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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science (JTAS) is the official journal of Universiti Putra Malaysia published by UPM Press. It is an open-access online scientific journal which is free of charge. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognized internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

JTAS is a **quarterly** (*February, May, August and November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open to authors around the world regardless of the nationality.

The Journal is available world-wide.

Aims and scope

Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include: agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

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Pertanika was founded in 1978. A decision was made in 1992 to streamline Pertanika into three journals as Journal of Tropical Agricultural Science, Journal of Science & Technology, and Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

After 37 years, as an interdisciplinary journal of Agriculture, the revamped Journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

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Pertanika is almost 40 years old; this accumulated knowledge has resulted in Pertanika JTAS being abstracted and indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge [BIOSIS & CAB Abstracts], EBSCO & EBSCOhost, DOAJ, Agricola, Cabell's Directories, Google Scholar, MyAIS, ISC & Rubriq (Journal Guide).

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International Standard Serial Number (ISSN)

An ISSN is an 8-digit code used to identify periodicals such as journals of all kinds and on all media–print and electronic. All Pertanika journals have ISSN as well as an e-ISSN.

Journal of Tropical Agricultural Science: ISSN 1511-3701 *(Print)*; ISSN 2231-8542 *(Online)*.

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Authors are not permitted to add or remove any names from the authorship provided at the time of initial submission without the consent of the Journal's Chief Executive Editor.

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Refer to Pertanika's **Instructions to Authors** at the back of this journal.

Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words **I**ntroduction, **M**aterials and Methods, **R**esults, **A**nd, **D**iscussion. IMRAD is simply a more 'defined' version of the "IBC" [Introduction, Body, Conclusion] format used for all academic writing. IMRAD indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: *Title, Authors, Keywords, Abstract, Conclusions*, and *References*. Additionally, some papers include Acknowledgments and Appendices.

The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's **Instructions to Authors**.

Editorial process

Authors are notified with an acknowledgement containing a *Manuscript ID* on receipt of a manuscript, and upon the editorial decision regarding publication.

Pertanika follows a **double-blind peer-review** process. Manuscripts deemed suitable for publication are usually sent to reviewers. Authors are encouraged to suggest names of at least three potential reviewers at the time of submission of their manuscript to Pertanika, but the editors will make the final choice. The editors are not, however, bound by these suggestions.

Notification of the editorial decision is usually provided within ten to fourteen weeks from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

As articles are double-blind reviewed, material that might identify authorship of the paper should be placed only on page 2 as described in the first-4 page format in Pertanika's **Instructions to Authors** given at the back of this journal.

The Journal's peer-review

In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts.

Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are seven steps to the editorial review process:

1. The Journal's chief executive editor and the editorial board examine the paper to determine whether it is appropriate for the journal and should be reviewed. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The chief executive editor sends the article-identifying information having been removed, to three reviewers. Typically, one of these is from the Journal's editorial board. Others are specialists in the subject matter represented by the article. The chief executive editor asks them to complete the review in three weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the literature.

3. The chief executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Edito-in-Chief, who reserves the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the chief executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The author(s) may also submit a rebuttal if there is a need especially when the author disagrees with certain comments provided by reviewer(s).

5. The chief executive editor sends the revised paper out for re-review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the chief executive editor in consultation with the editorial board and the editor-in-chief examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, an acceptance letter is sent to all the author(s), the paper is sent to the Press. The article should appear in print in approximately three months.

   The Publisher ensures that the paper adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the Publisher. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the article appears in the pages of the Journal and is posted on-line.

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EDITORIAL OFFICE

JOURNAL DIVISION
Office of the Deputy Vice Chancellor (R&I)
1st Floor, IDEA Tower II
UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 Serdang, Selangor Malaysia.
Gen Enq.: +603 8947 1622 | 1616
E-mail: executive_editor.pertanika@upm.my
URL: www.journals-jd.upm.edu.my

PUBLISHER

UPM PRESS
Universiti Putra Malaysia
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Tel: +603 8946 8855, 8946 8854
Fax: +603 8941 6172
E-mail: penerbit@upm.edu.my
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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

Pertanika is almost **40 years old**; this accumulated knowledge has resulted in the journals being abstracted and indexed in **SCOPUS** (Elsevier), Clarivate Analytics [*formerly known as Thomson (ISI)*] **Web of Science™ Core Collection-** Emerging Sources Citation Index (ESCI). Web of Knowledge [BIOSIS & CAB Abstracts], **EBSCO** and EBSCOhost, **DOAJ**, **ERA**, **Google Scholar**, **TIB**, **MyCite**, Islamic World Science Citation Center (ISC), ASEAN Citation Index (ACI), **Cabell's Directories** & Journal Guide.

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Contents

Foreword

Welcome to the **Third Issue 2018** of the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university and run on a non-profit basis for the benefit of the world-wide science community.

This issue contains 45 articles, out of which one is a review paper, two are short communications and the rest (42) are regular articles. The authors of these articles come from different countries, namely Malaysia, Indonesia, Thailand, Iran, Nigeria, India, Korea and Japan. Indonesia alone contributed 19 articles, the highest number of articles.

Articles submitted in this issue cover wide range of agricultural science fields including agricultural economics and management, agronomy, animal products, aquaculture, biotechnology, botany, ecology, fisheries sciences, food and nutrition development, forestry science, genetics and molecular biology, marine science, microbiology, nature products, organic chemistry, plant physiology, soil and water science, and zoology. An article is outlined from each of three favoured field in this issue: biotechnology; food and nutrition development; and plant physiology.

Selected from biotechnology field is a favourable article on bioactive potential of *Cosmos Caudatus* Kunth's leaves (locally known as '*ulam raja*') in scavenging free radicals and inhibiting α-glucosidase enzyme. The study was conducted by fellow researchers from Universiti Putra Malaysia (*Wan Nadilah Wan Ahmad, Khozirah Shaari, Alfi Khatib, Azizah Abdul Hamid* and *Muhajir Hamid*), Malaysia. The study shed some lights for future studies on plant phytochemicals and further development of the medicinal plant for health benefits. Details of the study is available on page 1367.

Selected from the field of food and nutrition development is an interesting article on contamination of pesticide and heavy metals in some vegetables and fruits, by Thailand scholars (*Sirikul Thummajitsakul, Rawitsara Subsinsungnern, Ngamrat Treerassapanich, Nutthida Kunsanprasit, Leeyaporn Puttirat, Patarapong Kroeksakul* and *Kun Silprasit*). The study samples were obtained from a local market and family farm in Ongkharak District of Nakhon Nayok Province, Thailand. They found high percentage of pesticides contamination and high level of heavy metals in the samples. This raises concern on health risk of the consumption of vegetables and fruits contaminated with pesticides and heavy metals. Details of the study is available on page 987.

Selected from the field of plant physiology is a pleasing article on effects of harmonic frequency and sound intensity levels on the opening of stomata, the growth and yield of soybeans, by fellow researchers from Indonesia (*Istirochah Pujiwati, Nurul Aini, Setyawan P. Sakti* and *Bambang Guritno*). They suggested the best combination of treatment to improve the productivity of soybean plants in Indonesia was exposure at a frequency of 4 kHz and sound intensity of 50 dB, followed by application of recommended dosage of leaf fertiliser. Details of the article is available on page 963.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This is to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers and editors, who have made this issue possible.

JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

Chief Executive Editor
Prof. Dato' Dr. Abu Bakar Salleh
executive_editor.pertanika@upm.my

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Review Article

Diversity of Nitrogen Fixing bacteria Associated with Various Termite Species

Sarannia Thanganathan and Kamariah Hasan*

School of Biological Sciences, Faculty of Sciences and Technology, Quest International University Perak, 30250 Ipoh, Perak, Malaysia

ABSTRACT

Nitrogen is one of the vital elements for the growth and survival of many organisms. Atmospheric N_2 can only be used by certain organisms like microbes that supply inorganic form of nitrogen to their host, insects, or plants via symbiotic or non-symbiotic interaction. Arthropods are diverse species on the earth, whose guts are inhabited by microbes that help in many physiological activities like N_2 fixation. As N_2 fixing bacteria ecologically play vital roles, many studies have demonstrated the presence of N_2 fixing bacteria in termite gut. Study on termite's gut omics has also supported a complex systemic understanding of gut digestome that is imperative in understanding the termite holobiome. This review gathers a variety of information from multifarious research which has been done on the isolation and diversity of N_2 fixing bacteria in various termite species.

Keywords: Acetylene reduction, *nifH*, nitrogen, nitrogen fixation, termite gut

INTRODUCTION

Nitrogen is an essential component of amino acids and proteins. N_2 is abundant in the atmosphere but atmospheric N_2 can only be used by certain organisms like microbes that supply inorganic form of N_2 to their host, insects, or plants via symbiotic or non-symbiotic interactions (Khan, Mohiuddin, & Rahman, 2008). Thus, N_2 fixation process is vital in providing fixed N_2 to organisms. Biological N_2 fixation (BNF) is the conversion of atmospheric N_2 into ammonia (Eskin, Vessey, & Tian, 2014). About 90 genera of microbes are able to fix N_2 by utilizing nitrogenase enzyme (Gaby & Buckley, 2012).

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E-mail addresses:
sf00948@qiup.edu.my (Sarannia Thanganathan)
kamariah@qiup.edu.my (Kamariah Hasan)
* Corresponding author

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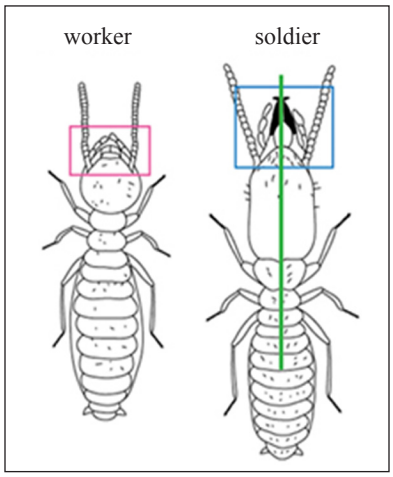
N_2 fixing bacteria are classified into two categories; symbiotic and non-symbiotic bacteria. They can be found in soils, gut of arthropods, or insects and root nodules. Arthropods are diverse species on the earth, whose guts are inhabited by microbes that help in many physiological activities like N_2 fixation and cellulose degradation (Bashir et al., 2013). Since N_2 is one of the limiting nutrients in insects' diet, most of the insects depend on mutualistic bacteria having N_2 metabolism in order to obtain sufficient amount of N_2 (Engel & Moran, 2013). N_2 fixing bacteria have symbiotic interaction in insects' guts, like in termites, cockroaches, and beetles. As N_2 fixing bacteria ecologically play vital roles, many studies have demonstrated the presence of N_2 fixing bacteria in termite gut that are capable of utilizing nitrogenous wastes excreted by termite and convert them into high-value N_2 (Ohkuma, Noda, & Kudo, 1999). This review gathers a variety of information from various researches, which have been done on the isolation and diversity of N_2 fixing bacteria in various termite species.

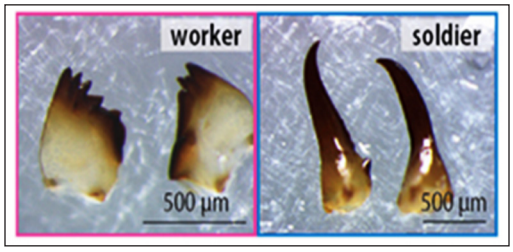
Termite Classification

Termites are classified into a few castes which comprise of queen, soldiers, workers, nymphs, and larvae (Kambhampati & Eggleton, 2000). Soldier class termites have the mandible, which is a jaw like structure on their heads that helps in protecting the colonies as shown in Figure 1 (Watanabe, Gotoh, Miura, & Maekawa, 2014; Watanabe & Maekawa, 2012).

According to the recent data, there are approximately 3106 species of termites. They are classified into 12 families which are further divided into 282 genera. From the 12 families, the lower temites' families are Cratomastotermitidae, Mastotermitidae, Termopsidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Archeorhinotermitidae, Stylotermitidae, Rhinotermitidae, and Serritermitidae whereas, the higher termites' family is Termitidae (Krishna, Grimaldi, Krishna, & Engle, 2013; Kambhampati & Eggleton, 2000).



(a)



(b)

Figure 1. The morphology of termites (worker and soldier): (a) The illustration of worker and soldier termite; (b) The mandible of soldier (right) and worker (left). Adapted from Watanabe et al. (2014)

Termite's Gut Structure

Termite gut is divided into three parts: foregut, midgut, and hindgut. The internal structure of a termite gut is shown in Figure 2. Termite's hindgut is colonized by diverse microbial symbionts from three different domains which are flagellate protists, bacteria, and archaea (Ohkuma & Brune, 2011). Lower termites' gut is colonized by a diverse species of flagellated protists and prokaryotes whereas the gut of higher termite is colonized only by prokaryotes (Brune, 2006; Peterson & Scharf, 2016).

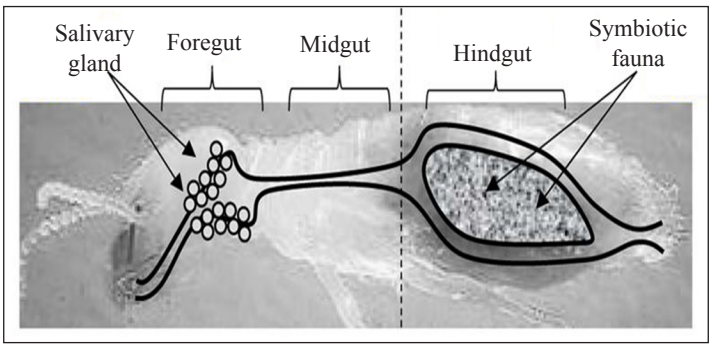


Figure 2. The internal structure of termite guts showing the foregut, midgut, and hindgut. Adapted and modified from Scharf, (2015)

Termite's Diet

Termites act as a decomposer where they feed on decaying and dead plant material with relatively high carbon to N_2 ratio. They also consume fungi and soil-rich organic matter (Engel & Moran, 2013). Such a feeding habit of termites contributes to the balanced ecosystem (Freymann, Buitenwerf, Desouza, & Olff, 2008).

According to Inward, Beccaloni and Eggleton (2007), and Donovan, Eggleton and Bignell (2001), gut contents and morphology were used to classify termites' feeding habits. Lower termites have simple gut and they feed on wood whereas, higher termites with complex gut feed on wood, grass, lichen, litter, epiphytes, and soil.

Microbiota of Termite's Gut

The complexity of termite gut leads to the presence of hundreds of phylotypes. Based on 16S rRNA studies, most of the species isolated from termite guts are novel. Those species are not environmental bacteria and, are specifically classified as termite gut specialists. Termites' gut microbes possess mutualistic relationship with their host in several ways. For instance, gut microbes aids in lignocellulose digestion which leads to the production of acetate, a main carbon source for the termites. Besides, the microbes also supply N_2 sources for the host (Breznak, Brill, Mertins, & Coppel, 1973; Odelson & Breznak, 1983).

About 90% of the termite's hindgut is colonized by flagellates from the phylum Parabasalia that occupy the gut of lower termites (Ohkuma & Brune, 2011). The flagellates found in termites' gut are unique and most of them cannot be found anywhere else (Brugerolle & Radek, 2006). Some of the flagellates such as *Trichonympha sphaerica* and *Trichonympha termosidis* remain uncultured. These two protists were isolated from the hindgut of *Zootermopsis angusticollis* (Tai, James, Perlman, & Keeling, 2013). There are also some protists associated with methanogens in termite gut. For instances, *Microjoenia* and *Dinenympha* are two different protists isolated from the termite *Hodotermopsis sjoestedti*. The flagellate *Spirotrichonympha leidyi* which was isolated from the gut of *Coptotermes formosanus* is associated with endosymbiotic methanogens (Hongoh & Ohkuma, 2011).

The gut of higher termites, especially soil-feeding termites of the family Termitidae, is colonized by a wide range of archaea. However, their presence is minute compared to bacteria (Brauman et al., 2001). At present, methanogens from the genus *Methanobrevibacter* and non-methanogens like *Thermoplasmoles* and *Crenarchaeota* have been isolated from termite gut (Friedrich, Schmitt-Wagner, Lueders, & Brune, 2001; Leadbetter, Crosby, & Breznak, 1998).

The most abundant bacteria found in the gut of wood-feeding termites are *Spirochaetes* whereas, *Bacteroideres* are abundant in the gut of fungus-cultivating termites (Dietrich, Kohler, & Brune, 2014; Hongoh, 2010). Several cellulolytic bacteria like *Cellulomanas, Citrobacter* and *Enterobacter* have also been isolated from the gut of termite (Upadhyaya et al., 2012). Cellulolytic bacteria secrete the enzymes cellulose and hemicellulose to allow them to degrade the cellulose and hemicellulose materials (Lima et al., 2014). *Dysgonomonas termitidis*, a lignocellulose degrading bacteria, was isolated from the gut of the termite *Reticulitermes speratus* (Pramono, Sakamoto, Limo, Hongoh, & Ohkuma, 2015). A study conducted on the termite *Odontotermes formosanus* had successfully isolated nine different isolates with cellulolytic property. All the nine isolates were closely related to *Bacillus cereus, Bacillus thuringiensis, Bacillus pumilus, Pseudomonas aeroginosa, Citrobacter freundii, Serratia marcescens, Salmonella enterica, Staphylococcus gallinaum*, and *Enterococcus casseliflavus* (Kavitha, Vijayarani, & Kumanan, 2014). There are also some acetogenic bacteria in the termite gut. Most of them are from the phylum *Firmicutes* and genera *Clostridium* and *Acetobacterium* (Drake, Gobner, & Daniel, 2008).

N_2 Fixing Bacteria in Termite Gut

Termites have a symbiotic relationship with microbes and play a vital role in N_2 fixation. Microbial fixation in termite gut is the main source of N_2 for the termite colony (Vecherskii, Kostina, Gorlenko, Dobrovol'skaya, & Umarov, 2008). Several studies have proved the presence of N_2 fixing

bacteria in the gut of various termite species. Several bacterial endosymbionts that have the ability to fix N_2 have also been found in the gut of lower termites (Peterson & Scharf, 2006). Since termites grow well in nitrogen-poor diet, there are many types of symbiotic N_2 fixing bacteria, living in the gut of termites (Masepohl et al., 2002).

In the study conducted by Doolittle, Raina, Lax and Boopathy (2008), *Klebsiella pneumoniae* was isolated from *Coptotermes formosanus* which was the Formosan subterranean termites (FST). N_2 fixation assay which was done using basic salt media supplemented with NaMo and $Fe_2(SO4)_3$ revealed that *K. pneumonia* was able to fix N_2 anaerobically which contributes to the N_2 source of FST. Apart from this, another study conducted by Potrikus and Breznak (1977), demonstrated the presence of *Enterobacter agglomerans* in the gut of *C. formosanus* and proved its ability to fix N_2 through acetylene reduction assay. Inhibition of acetylene reduction by oxygen proved that *E. agglomerans* can only fix N_2 anaerobically.

Few studies have demonstrated the presence of N_2 fixing bacteria in various Australian termite species. In order to confirm the presence of N_2 fixation in Australian termite, French, Turner and Bradbury (1976) used three different termite species including *Mastotermes darwiniensis*, *Nasititermes exitiosus*, and *Coptotermes zactues* which were obtained from mounds at Townsville, Seymour, and Canberra, respectively, to isolate and characterize N_2 fixing bacteria from the hindgut. Using acetylene reduction assay (ARA), N_2 fixation activity was discovered in all the isolates obtained from all three species. Based on $^{15}N_2$ incorporation test, isolate from *Mastotermes drawiniensis* showed highest $^{15}N_2$ incorporation. All the isolates obtained from this study were characterized as *Citrobacter freundii*. Another study was conducted by Eutick, O'Brien and Slaytor (1978), to isolate N_2 fixing bacteria from few species of Australian termites including *Coptotermes lacteus*, *Coptotermes acinaciformis*, *Cryptotennes primus* Hill, *Mastotennes darwiniensis*, *Nasutitermes exitiosus*, *Nasutitermes walker*, *Nasutitermes graveolus*, *Heterotermes ferox,* and *Schedorhinotermes intermedius intermedius*. Based on their morphological and biochemical characteristics, all the isolates were identified as *Enterobacter* spp.

Two facultative anaerobes, namely, *Clostridium* sp. and *Klebsiella* sp. with the ability to fix N_2 have been isolated from *Mactotermes* sp., the fungus cultivating termite. In this study, N_2 fixing bacteria were isolated from the gut of queen, soldier and worker termites. These bacteria were isolated using Hill's medium and were characterized using Hino and Wilson medium (Gomathi, Ramalakshmi, & Ramasamy, 2005). This research showed that, compared to others, the amount of bacteria enumerated was highest in worker termites. Previously, Breznak et al. (1973), also discovered similar findings whereby, in worker termites fed with wood, the N_2 fixing activity was higher, compared to the soldier termites. This was demonstrated using ARA.

N_2 Fixation Activity in Termite Guts

The distribution of sulfate reducing bacteria in termite gut was studied using various termite species that includes *Mastotermes darwiniensis*, *Neotermes jouteli*, *Neotermes castaneus*, *Nasutitermes nigriceps*, *Zootermopsis angusticollis*, *Zootermopsis nevadensis*, *Kalotermes flavicollis*, *Heterotermes indocola*, *Reticulitermes santonensis*, and *Odontotermes obesus*. A total of seven pure culture isolates were obtained in this study. Nitrogenase activity of those isolates was tested using ARA. All the isolates had the ability to fix N_2 which eventually provide N_2 source for the termites. Based on biochemical and physiological characteristics, the seven isolates were identified as *Desulfovibrio* sp. (Kuhnigk et al., 1996).

Several other researches were also conducted on the isolation of N_2 fixing bacteria from termites. The isolates with N_2 fixing ability which were isolated from various termite species have been tabulated in Table 1. These researches showed the variety of N_2 fixing bacteria that occupy termites gut and form symbiotic relationship, which benefits both hosts and microorganisms.

Culture independent molecular method was used to study the expression of N_2 fixation genes in the gut microbiota of *Neotermes koshunensis*. As a primary screening, the worker larvae were tested for N_2 fixation activity using ARA. The relationship between N_2 fixation activity and quantity of mRNA was tested by feeding the termite with two different diets; filter paper with added N_2 source and filter paper

Table 1
N_2 fixing bacteria isolated from various termite species

N_2 Fixing Bacteria	Host	Reference
Klebsiella pneumoniae	*Coptotermes formosanus*	Doolittle et al., 2008
Enterobacter agglomerans	*Coptotermes formosanus*	Potrikus & Breznak, 1977
Citrobacter freundii	*Mastotermes darwiniensis* *Nasititermes exitiosus* *Coptotermes zactues*	French et al., 1976
***Clostridium* sp.** ***Klebsiella* sp.**	*Mactotermes* sp	Gomathi et al., 2005
***Enterobacter* sp.**	*Coptotermes lacteus* *Coptotermes acinaciformis* *Cryptotennes primus* Hill *Mastotennes darwiniensis* *Nasutitermes graveolus* *Heterotermes ferox* *Schedorhinotermes intermedius intermedius*	Eutick et al., 1978
***Desulfovibrio* sp.**	*Mastotermes darwiniensis*	Kuhnigk et al., 1996
Desulfovibrio desulfuricans	*Reticulitermes santonensis*	
Desulfovibrio termitidis	*Heterotermes indicola*	
***Desulfovibrio* sp.**	*Odontotermes obesus*	

without N_2 source. The acetylene reduction activity was higher in termites fed with filter paper without any N_2 source. Nitrogenase activity was reduced in termites fed with filter paper containing N_2 source (Noda, Ohkuma, Usami, Horikoshi, & Kudo, 1999). This finding was similar to the experiment performed by another researcher using *Coptotermes formosanus* (Breznak et al., 1973).

The N_2 fixing activity was also studied by Desai and Brune (2012), to identify the presence of diazotrophs in four different termites, *Cryptotermes longicollis*, *Incisitermes marginipennis*, *Neotermes castaneus* and *Kalotermes flavicollis* which belong to genera *Kalotermitidae*. Termites were fed under two different diet conditions (wood and filter paper) followed by ARA to test nitrogenase activity. Although the termites were able to reduce acetylene in both conditions, the acetylene reduction activity was higher when termites were fed with filter paper compared to wood. PCR and RT-PCR were performed using universal primers IGK and YAA for hindgut homogenate of four termite species followed by creation of a clone library. Based on the clone library, it was revealed that the *nifH* homologous are diverse in hindgut of those termite species. Based on the phylogenetic analysis of *nifH* homologs, it was found that the gut of those termites is colonized by *Treponema* sp., *Bacteriodales*, and *Azoarcus* sp. All the *nifH* homologs obtained in this study were clustered into respective groups as referred to in the group nomenclature of Yamada, Inoue, Noda, Hongoh and Ohkuma (2007). The *nifH* homologs from hindgut of *I. marginipennis* and *N. castaneus* were clustered in group I and were closely related to *Azoarcus* strain BH72 and actively expressed. *NifH* homologs in group II were obtained from all three termites species except for *I. marginipennis* and had a close identity with *anfH* gene of *Clostridium pasteurianum*. Some homologs were related to *Spirochaetes* but not expressed. The *nifH* homologs in group III had a close relationship with *A. pseudotrichonymphae* and few homologs clustered together with *Treponema azotonutricium* where the *nifH* gene in both clusters was expressed.

In another study, lower termites of *Reticulitermes speratus* were used to investigate the diversity of *nifH* genes in their intestinal microbiota (Ohkuma et al., 1996). The DNA of mixed microbiota from termites' hindgut was extracted and *nifH* gene was amplified using four primer combinations (KAD-GEM, KAD-YAA, IGK-GEM and IGK-YAA). Four clones, TDG, TDY, TKG and TKY were isolated respectively. A total of 27 *nifH* amino acid sequences were obtained from *R. speratus* and all the sequences were not similar to any of the published sequences in database. Phylogenetic analysis was performed using neighbor joining tree constructed using 25 *nifH* sequences (Figure 3).

Based on the phylogenetic tree, although most of *nifH* amino acid sequences from the termite group are closely related to the sequences of *Clostridium pasterenium* and also *Desulfovibrio gigas* and *Chromatium buderi*, there are no similarities between

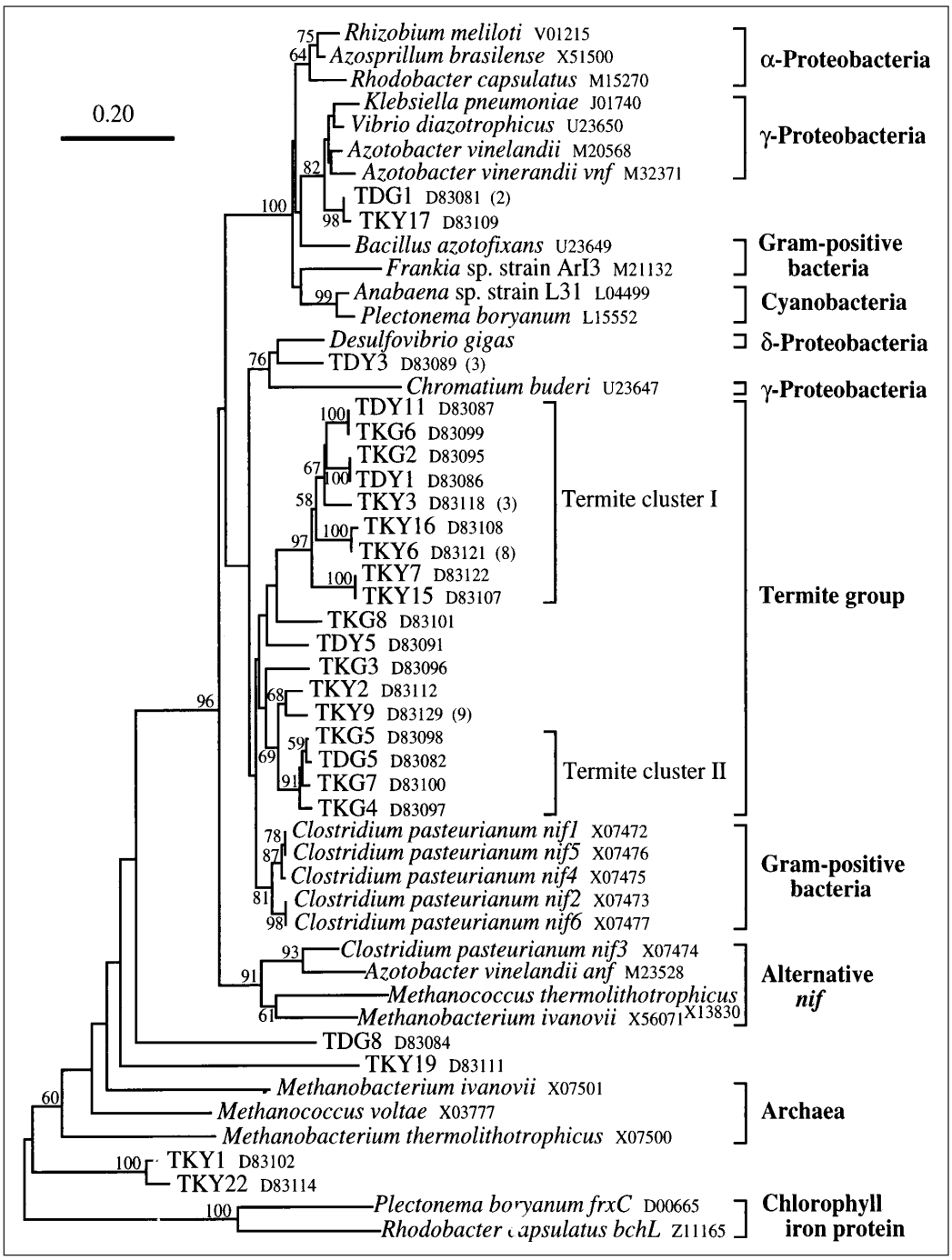


Figure 3. Neighbor-joining relationship constructed using *nifH* amino acid sequences of 24 sequences from database and 25 sequences from termites. Two chlorophyll sequences were used as outgroups. The *nifH* fragments used correspond to 45-153 amino acid residues of *K. pneumoniae* sequence. Scale bar 0.2 denotes substitutions of 20 nucleotides over 100 nucleotides. Numbers shown in the internal branches are the bootstrap values derived from 1000 replications when above 50% is shown at each node. Adapted from Ohkuma et al., 1996

the nucleotide sequences of clones and *C. pasterenium*. The *nifH* sequence of clone TDG1 and TKY17 are grouped together in gamma subclass of proteobacteria which includes *Azotobacter* sp., *Vibrio* sp. and *Klebsiella* sp. The sequence of TDY3 is clustered with *C. buderi* and *D. gigas*. The sequences of other clones are distantly related to the sequences obtained from database and they formed their own clusters (Ohkuma et al., 1996). There are also many copies of *nifH* sequence in one single organism due to the presence of many *nifH* genes like alternative nitrogenase genes. Although a few sequences were obtained from the same organism, there is a unique diversity among them.

Previous study proved that *Reticulitermes speratus* was contributed by N_2 source through N_2 fixation of microbes in its gut (Ohkuma et al., 1996). Likewise, the diversity of N_2 fixing microbes in intestinal microflora of *Reticulitermes chinensis* Synder were studied using the culture independent method. Primary screening was done using *nifH* gene amplification with few primers combinations (IGK–YAA, IGK–GEM, KAD–YAA, and KAD–GEM). From 63 clones, six chimeric sequences, and several similar sequences were eliminated. About 34 sequences were used for phylogenetic analysis. About 20 *nifH* sequences clustered together which were true functional *nifH* and 17 of them had a close relationship with *Clostridium* sp. with the similarity of 75-88%. One sequence had a higher relationship with a spirochetes, *Treponema primitia* ZAS-2, one sequence had 95% similarity with *Candidatus Azobacteroides pseudotrichonymphae genomovar,* CFP2 which is endosymbionts of *Coptotermes formasanus*. One more sequence is related to *D. vulgaris*. Another 14 *nifH* sequences are clustered together into alternative nitrogenases groups where six of them are related to proteobacteria, *Rhodospirillum rubrum*. Five sequences having higher similarity with spirochetes were obtained from *Z. angusticollis*. Besides, another three sequences are related to *Methanosarcina barkeri*. Although nitrogenase activity was not performed for all the isolates obtained from this study, there are diverse groups of N_2 fixer in the intestinal microbiota of *R. chinensis* (Du et al., 2012).

The microaerophilic nature of Termite Associated Verrucomicrobium 2 (TAV2) was studied by Isanapong et al. (2013). Through proteomic and transcriptome method, the genes and proteins expressed in different oxygen, O_2, concentration were identified. This study revealed the presence of peptides corresponding to *nifH* genes in the cells which were grown at 2% O_2 concentration. This indicates that TAV2 is involved in the BNF and contributes to the metabolism of microbial community in the gut of termites. In another study, the N_2 fixation activity of TAV2 was confirmed through genomic analysis. The TIGRFAM and Pfam protein family databases were used to perform protein model comparisons. In addition, Kyoto Encyclopedia (KEGG) and Clusters of Orthologous Groups (COG) databases were also used to justify the

presence of N_2 fixing activity. Based on the results obtained, the presence of *nifHDK* and *anfHDGK* operons in TAV2 was confirmed (Wertz, Kim, Breznak, Schmidt, & Rodrigues, 2012).

Other than that, the ability of symbiotic N_2 fixation in fungus growing termites was tested using ARA. Two termite species, *Mactotermes natalensis* and *Odontotermes badius* were used in this study. The study revealed that there was a positive acetylene reduction activity in live termites of those two termite species and not in the fungus comb. This indicated the presence of N_2 fixer in termite guts. The fixation was higher in worker than in soldier in both the species (Sapountzis et al., 2016).

N_2 Fixing Bacteria and *NifH* Gene

NifH gene is responsible for N_2 fixation and used as identification marker to detect the presence of N_2 fixing microbes (Zehr, Jenkins, Short, & Steward, 2003). The genome of spirochetes strains ZAS-1, ZAS-2, and ZAS-9 were examined in the previous study to check for the presence of *nifH* gene and the ability to fix N_2 (Noda, Ohkuma, & Kudo, 2002). The study showed the presence of two *nifH* homologs in each strain.

Study conducted on *Spirochaeta aurantia, Spirochaeta zuelzerae,* and treponeme ZAS-9 showed their ability to fix N_2 due to the presence of *nifH* gene (Hongoh, Ohkuma, & Kudo, 2003; Lilburn et al., 2001). The ZAS-strain which belongs to *Treponema* sp. isolated from the gut of *Zootermopsis angusticollis* has two homologous *nifH* genes with nitrogenase activity (Hongoh et al., 2003). Apart from this, the *nifH* gene was also found in the bacteria isolated from *Hodotermopsis sjostedti* and *Zootermopsis nevadensis*.

In another study, *Endobacterium proavitum* strain Rsa215 was isolated from *Reticultitermes santonensis* (Rsa) and *Zootermopsis nevadensis* and was tested for the ability to fix N_2. This strain contains a single gene cluster encode for nitrogenase. The phylogenetic analysis of *nifD, nifK,* and *nifH* demonstrated that Rsa215 has group IV nitrogenase. The group IV nitrogenase gene is known as *nif*-like gene with some of these genes involved in N_2 fixation. *Endobacterium proavitum* has a single set of *nifHDK* genes which are functional in N_2 fixation (Zheng, Dietrich, Radek, & Brune, 2016).

According to Noda et al. (2002), a strong N_2 fixation activity had been shown by *C. formosanus* in Japan. The culture independent studies of *nifH* gene show that there are diverse species of microbes such as spirochetes, clostridia, archaea, and proteobacteria which are able to fix N_2 available in the gut of *C. formosanus*.

Besides, the termite *Neotermes koshunensis* has abundant *nifH* genes in its gut and it possesses high N_2 fixation activity (Noda et al., 1999). There is also potential N_2 fixer which has symbiotic interaction in the gut of *Reticulitermes chinensis*. This was demonstrated from the phylogenetic analysis of the clones of *nifH* gene isolated from *R. chinensis* (Du et al., 2012).

Role of Metagenomic Technologies in Studying the N_2 Fixing Bacteria

In termites, gut consortium play direct roles in N_2 fixation, amino acid biosynthesis and lignocellulose digestion. However, a majority of gut microbes are unculturable thus molecular methods such as single-species-targeting metagenomics analysis and other omics approaches are crucial in capturing and revealing the diversity of the termite's gut microbe. Metagenomics studies employing bacterial 16S rRNA sequences have been used to catalog bacteria and archaea in termites (Do et al., 2014; He et al., 2013; Peter & Scharf, 2016; Rajarapu, Shreve, Bhide, Thimmapuram, & Scharf, 2015; Tartar et al., 2009). Large metagenomic dataset will allow in-depth analysis of microbial functions and could offer resources for advancing integrative sociogeomic, digestomic, and termitosphere in order to better understand the intricate symbiotic relationships between termites and their gut microbes. Overall, six main bacterial phyla are represented across higher and lower termites, namely Bacteroides, Firmicutes, Sphirochetes Proteobacteria, Fibrobacteres, and Elusimicrobia (Brune, 2014). Metagenomic study on wood and soil-feeding higher termites revealed that community structure and functional potential of microbes in gut compartments are determined by digestive approach of the host (Rossmaler et al., 2015). Omics science collectively catalog, enumerate, and illustrate biological molecules that transform into organization, function, and life processes of an organism. Omics research has also provided advances in understanding symbiotic roles of individual microbial species. Currently, 82 termite species have been studied using several omics methods, with bigger representation by lower (72%) compared to higher termites (28%).

Recently, studies on metatranscriptome using ribodepletive strategy on lower termite *Reticulitermes flavipes* substantiate earlier discoveries of physiological contribution of bacteria with regard to biosynthesis, catabolism, and transport of major organic molecules and ions (Peterson & Scharf, 2016). The existence of N_2 metabolism genes like nitrogenase, nitroreductases, and ureases for N_2 recycling and fixation in lower termites gut bacteria were also identified through genome sequencing (Hongoh et al., 2008; Inoue et al., 2015).

Metagenomic approach is an important tool to uncover the diversity of uncultured N_2 fixing microbes associated with termites' gut. Currently, metagenomic studies focusing on N_2 fixing ability of guts microbiome utilizing gene encoding nitrogenase enzyme, *nifH* are still lacking as many studies focus more on lignocellulase and xylanase screening from genome sequence compared to N_2 metabolism (Bastien et al., 2013; Liu et al., 2013). Although the nature of N_2 fixation in termites is still in the dark, they deserve attention because of their potential influence on N_2 metabolism in tropical soil. By uncovering the diversity of N_2 fixing bacteria in termite gut through metagenomic

and other omics study, we might uncover a unique and exceptional N_2 fixer that could be incorporated into soil and plant to promote plant growth.

CONCLUSION

The main source of N_2 for termites is from the microbial fixation of N_2. There is an abundance of microbes in the gut of various termite species which help in N_2 fixation. They are detected by looking at the presence of N_2 fixing genes, most commonly nitrogenase encoded by *nifH*. The importance of N_2 fixation in termite should be analyzed further using other housekeeping genes, for instance, other *nif* genes and *fix* genes that are involved in N_2 fixation. The importance of N_2 fixing genes should be emphasized because those genes can be manipulated to provide significant impact on agriculture since N_2 fixing bacteria have the potential to increase plant productivity. The bacteria can be released into plants as free living bacteria. If there is significant improvement in the plants' nutrients, the *nifH* gene from these bacteria can be manipulated and incorporated into non-leguminous crops for better conversion of N_2 gas. At the same time it may reduce contamination caused by chemical fertilizers.

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Evaluation of Agronomic Traits of Wheat Genotypes under Different Irrigation Regimes

Babak Hooshmandi[1]* and Ebrahim Khalilvand Behrouzyar[2]

[1]*Young Researchers and Elite club, Tabriz Branch, Islamic Azad University, Tabriz, Iran*
[2]*Department of Agronomy and Plant Breeding, Tabriz Branch, Islamic Azad University, Tabriz, Iran*

ABSTRACT

Wheat is one of the important cereal crops in the world and is the main staple food for many. Among the different environmental stresses, drought is the most critical threatening wheat productivity worldwide. This study evaluated and classified morphological and physiological characteristics of wheat genotypes in two non-stress and drought-stress conditions. A field study was conducted at the Research Station of Agricultural Faculty of Islamic Azad University of Tabriz, Iran between 2012 and 2013. Thirty wheat genotypes with six replications were sown in a randomised complete block design. As indicated in the results analysis of variance, the studied genotypes were genetically different in all characteristics. The grain yield had positive correlation with straw yield, harvest index, and biological yield Based on factor analysis, in the non-stressed condition, the first factor was referred to as yield, and in the stressed condition, the first factor was called yield components. To classify genotypes, cluster analysis was performed on the Ward method. The results of the analysis were divided into three groups in non-stressed experiment genotypes while in stress experiment genotypes, they were divided into four groups. Considering the cluster analysis, the first group was presented as the optimal one in the non-stress condition. The results indicated that in order to obtain the desirable grain yield, we can increase most of the traits with positive and significant correlations with the yield.

Keywords: Cluster analysis, water regimes, wheat genotypes, yield

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E-mail addresses:
hooshmandi.babak@yahoo.com
(Babak Hooshmandi)
e.khalilvand@iaut.ac.ir (Ebrahim Khalilvand Behrouzyar)
* Corresponding author

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INTRODUCTION

Cereals are considered the most significant source of calories for human beings (Daryanto, 2016). Although cereals are considered a staple food for many, drought reduces more than 50% of the crop (Amiri et al., 2014). It is also used as a main source of straw for animal nourishment (Salwa & Osama, 2014). The global wheat production was about 735.23 million tonnes in 2016 (FAO, 2016). In many semi-arid environments, the relative humidity at the beginning of growing season is at the highest level and with increasing temperature, the amount of rainfall decreases. In these regions, wheat grain filling duration is simultaneous with water shortage and increased evaporation of soil surface; Thus, the yield is reduced (Heyne, 1987). Iran, with an annual average precipitation of 240 mm, is located in the semi-arid and arid areas of the world. In Iran, most farmers do not get a desirable result from planting water-expecting cultivars due to inadequate water in terms of assigning late-season irrigation to summer farming. Consequently, wheat farming results in the late-season drought stress. Therefore, to obtain and introduce cultivars capable of producing greater and reliable yields, managing normal and late-season drought stress is important (Koocheki et al., 2014). In arid and semi-arid regions, drought stress is one of the most important factors limiting agricultural production (Mollasadeghi et al., 2011).

Drought stress in different stages of wheat growth reduces grain yield, harvest index, biological yield, and grain yield components (Araus et al., 2003). A marked effect of humidity stress is shorter plants, which occurs as the result of a decrease in distances between internodes. Decrease in the height of plants and internodes occur due to drought stress usually before the emergence of spikes but would rarely be affected after that (Annicchiarico et al., 2000).

Zaefyzadeh et al. (2009) classified 13 wheat genotypes into three clusters using the Ward method in drought stress. Poudel et al. (2017) were categorised the clusters in into two groups in stress environment: Group A and Group B. Cluster 1, Cluster 2, Cluster 3 and Cluster 4 are included in Group A, while Group B consists of only one cluster, Cluster 5. Grain yield is a complex multi-component property which undergoes different environmental conditions. Different morphological and physiological characters contribute to grain yield (Naghavi & Khalili, 2017). Zi-Zhenali et al. (2004) indicated the number of spikes per unit area along with the number of grains per spike was considered as the main determining factor. However, the number of spikes per unit area has a negative influence on the number of grains per spike. Samarah (2005) had reported decreased grain yield under the drought stress condition as a result of decreased 1000-grain weight, the number of tillers, and the number of spikes and grains in the plant. He reported drought stress reduces grain yield by decreasing the number of grains per spike. Intense drought in pre-pollination stages decreases the number of spikes and spikelets, making

the remaining spikelets fertile. In addition, the duration of this stage and acceleration of aging, as well as the period of grain filling in the later development stages are reduced by the drought stress in the flowering stage (Shepherd et al., 2002).

Slafer and Araus (1998) showed that if the late-season drought threatened crop growth, cultivars and lines capable of turning the vegetative to the generative stage—when more usable moisture is available in soil—led to a higher harvest index and grain yield, because they had more opportunity to use moisture stored in the soil prior to the late-season drought. By analysing wheat resistance to drought, it was found under adequate moisture conditions in the soil, the number of grains in spike and 1000-grain weight were among the influential factors. It was suggested that these two traits should be underlined in selecting cultivars for cropping in regions with limited water. In a study on wheat, Sio-Se Mardeh et al. (2006) indicated that grain yield under non-stress conditions was inversely correlated with that under stress conditions. On that basis, it was concluded that high yield in desirable irrigation conditions did not necessarily result in improved yield under stress conditions. They also expressed that under stress conditions, the potential gene expression is decreased. Thus, the genetic progress rate becomes higher in the non-stress than the stress condition. Therefore, that selection based on genotype yields in the stress condition is only suitable for that, but selection based on genotype yields in the non-stressed condition may be adopted to either condition. This study aims to investigate the relationships between different traits in non-stressed and drought-stressed conditions and to identify the effective factors in the genetic improvement of yield.

MATERIALS AND METHODS

This research was conducted between 2012 and 2013 at the Research Station of Agricultural Faculty of Islamic Azad University of Tabriz, Iran (longitude 46°17′ east, latitude 38°5′ north, and altitude 1364 m above sea level). In this study, 30 wheat genotypes were tested. The cultivars were provided by the Corn Research Department, Centre for Agricultural and Natural Resources, East Azerbaijan Province (Table 1).

The experiment was conducted in a randomised complete block with six replications, three for non-stressed and three for drought-stressed conditions, separately and simultaneously. Surface irrigation was performed. That is, the usual irrigation treatment was performed until the late-season growing based on the water needs of plants. Irrigational stress treatment was performed until the early heading stage as necessary. Then, stress irrigation was performed in three stages. The first irrigation of the stress treatment was performed after the stem elongation stage and the plants were under stress in the heading stage. Twenty days after the first irrigation, the second irrigation of the stress treatment was conducted while the third irrigation was performed 34 days after the second

Table 1
Wheat genotypes used in this study

Number	Pedigree	Origin
1	Seri/Avd/3/Rsh/Afn/4/jup/Bjy/Kauz	Iran
2	Yan 7578. 128//Chill/2*Star	Iran
3	Shi# 4414/Crow"s"//Kvz	Iran
4	Merual/4/Bloudan/3/Bb/7c*2/Y50E/Kal*3/5/shiroodi	Iran
5	Bloudan/3/Bb/7C*2//Y50e/3*Kal/4/MV 17	Iran
6	Gaspard/Attila	Iran
7	Tbs/Flt/3/Evwy2/Azd/Rsh*2/10120/4/M-75-7	Iran
8	1-66-22/5/1-66-31/4/Anza/3/Pi/Nar/Hyz/6/M-75-7	Iran
9	Alvand//Aldan/las58	Iran
10	Attila (CM85836-50Y-0M-0Y-3M-0Y)	Iran
11	Sha/Chil	Iran
12	Hereward/Siren/5/Gov/Az/Mus/3/DoDo/4/Bow	Iran
13	Owl*2/Shiroodi	Iran
14	Alondra"s"	Iran
15	Bilinmiyan96.40	Iran
16	Fr3*/MM/Mt-Y50//Rsh	Iran
17	200H/Vfn//Rsh	Iran
18	Kal/Bb//Cj"s"/3/Hork"s"/4/Gascogne	Iran
19	Bhr*5/Aga//Sni/3/Trk13/4/Drc	Iran
20	Gascogne/3/Nai60/Hn7//sy	Iran
21	Emu"s"/Tjb84-1543//1-27-7876/Cndr/3/ Azd//Tob/Chb	Iran
22	Dove"s"/Buc"s"//2*Darab	Iran
23	Maya"s"/Nac	Iran
24	Ghk"s"/Bow"s"//90Zhong87/3/Shiroodi	Iran
25	Choti/Lerma	Iran
26	Alvd/Aldan"s"/las58/4/Kal/Bb/Cj"s"/3/Hork"s"	Iran
27	Mv22-77Stepphon/3/mon"s"/Imu"s"//Falka/4/Zarrin	Iran
28	Appolo/4/Seri/Avd/3/Rsh//Ska/Afn/5/Pyn/Bau	Iran
29	4820/1-32-15409//Mexp	Iran
30	Omid/H7/4/839/3/Omid/Tdo/5/Kal/Bb/Cj"s"/Hork"s"	Iran

irrigation. Starting from mid-April 2013, drought stress was applied to encourage natal growth. The land preparation operation included single ploughing with mouldboard ploughshare and a single use of a disk and a trowel. Seeds of each genotype were sown in two-metre lines with 20 cm line distances. Planting and weeding were done mechanically. Since the studied genotypes were taken from wheat planted during winter, mid-October was considered as the planting date. Information related to temperature and rainfall was obtained from the Iran Meteorology Organization, Tabriz (Table 2).

Table 2
Meteorological statistics during 2010-2013 in Tabriz, Iran

	Total monthly rainfall				Mean monthly temperature			
	2010	2011	2012	2013	2010	2011	2012	2013
Jan	11.9	8.5	25.1	36.7	3.7	-1.2	-0.5	0.4
Feb	35.1	19.3	6.4	43.8	4.5	1.1	-0.3	3.8
Mar	20.4	41.9	20	9.6	8.8	5.9	2.8	8
Arp	51.2	83.2	35.6	47.3	12.2	12.3	13.6	13.3
May	38.5	50.3	22.2	39.5	17	17.2	19.1	16.6
Jun	6.9	0.7	15.8	7.8	25.3	23.8	23.8	23
Jul	0.4	11.4	14.9	4.5	28.1	28	25.8	26.4
Aug	10	4.5	0	0	26.7	25.6	28.3	25.3
Sep	2.4	16.1	5.1	0.4	23.8	21.3	21.7	21.8
Oct	6.9	15	9.2	7.6	16.6	13.2	16	13.2
Nov	0	23.8	20.2	47.4	8.2	1.8	8.8	8.3
Dec	0.3	7.6	42.8	18	3.7	-1.3	2	-5.8

In each row, 10 plants were randomly labelled and each studied for the number of grains per spike, straw yield, grain-filling duration, harvest index, 1000-grain weight, peduncle length, and days to physiological maturity, biological yield, and the number of fertile spikelets per spike, spike length, plant height, and grain yield were noted. In order to understand the relationship between the traits better, the correlation coefficients between all traits were measured and their significance at 5 and 1 percent probability levels was studied in each test. Factor analysis was done based on the analysis method for main components and varimax rotation on the data in either condition. The analysis method for the main components was used to extract load factor matrices, as well as estimate the number of factors. On that basis, the factors with characteristic root>1 were selected, and factor coefficients were employed for matrix formation. In this study, the cluster analysis with the Ward method was also used to classify genotypes. Data was subjected to statistical analysis using SAS and SPSS software. Analysis of variance and correlation coefficient were performed using SAS software and factor analysis and cluster analysis using SPSS software.

RESULTS AND DISCUSSION

Based on the results shown in Table 3, there was a significant difference between two non-stressed and drought stressed experimental conditions in terms of all the measured traits at 1% probability level, indicating the changed value of the studied traits and the effect they have received from the environmental test results.

Table 3
Analysis of variance of measured traits

S.O.V	df	GS	SY	GFD	HI	TKW	PL	PM	BY	FSS	SL	PH	GY
Stress	1	33121**	437**	14622**	1510**	13364**	396.06**	359**	1600**	67**	85**	54**	11939**
Error1	4	1.69	2.5	1.1	0.06	0.82	0.048	0.032	2.72	0.063	1.05	0.38	9.87
Genotype	29	60.9**	105.2**	130.6**	20.6**	33**	10.672**	2.86**	180.1**	5.42**	9.881**	8.1**	56.8**
Genotype × Stress	29	50.7**	4.035**	50.7**	12.2**	21**	5.788**	2.74**	23.58**	0.47**	7.14**	0.91 **	19.8**
Error2	116	0.585	0.965	0.924	0.039	0.696	0.027	0.011	2.720	0.053	0.923	0.172	0.435
CV (%)		2.38	2.69	3.25	1.75	2.69	2.18	2.86	4.58	2.80	8.22	2.84	0.25

GS: Grain per spike, SY: Straw yield, GFD: Grain filling duration, HI: Harvest index, TKW:1000 grain weight, PL: Peduncle length, PM: Physiological maturity, BY: Biological yield, FSS: Fertile spikelets per spike, SL: Spike length, PH: Plant height, GY: Grain yield. * and **significant at 5% &1% respectively

Table 4
Correlation coefficients between different agronomic characteristics of wheat genotypes drought under stressed condition

	GS	SY	GFD	HI	TKW	PL	PM	BY	FSS	SL	PH
SY	0.29										
GFD	0.16	-0.09									
HI	-0.3	0.58*	0.24								
TKW	-0.55*	-0.11	0.31	0.3							
PL	0.08	0.61*	-0.17	-0.67**	0.19						
PM	0.016	-0.1	0.67**	-0.08	-0.14	-0.02					
BY	0.21	0.96**	-0.01	-0.16	-0.01	0.49*	-0.14				
FSS	0.83**	0.04	-0.26	-0.49*	0.56*	0.1	0.17	-0.01			
SL	0.80**	0.29	0.02	-0.51*	-0.12	0.18	0.14	0.2	0.74**		
PH	0.51*	0.69**	-0.17	-0.80**	-0.49*	0.85**	0.11	0.54*	0.53*	0.60*	
GY	-0.21	0.69**	0.16	0.62**	0.19	-0.07	0.18	0.65**	-0.25	0.03	-0.05

GS: Grain per spike, SY: Straw yield, GFD: Grain filling duration, HI: Harvest index, TKW: 1000 grain weight, PL: Peduncle length, PM: Physiological maturity, BY: Biological yield, FSS: Fertile spikelets per spike, SL: Spike length, PH: Plant height, GY: Grain yield. *and **: Significant at 5% and 1% probability levels respectively.

There was a positive and significant correlation between the number of fertile spikelets per spike and grains per spike (Table 4).

The correlation between the number of fertile spikelets per spike and 1000-grain weight was positive and significant. There is a positive and significant correlation between plant height and biological yield. In this study, it was observed that, there was a positive and significant correlation between grain yield and the harvest index. Ali et al. (2008) reported similar results for the correlation between the number of fertile spikelets per spike and grains per spike. The flowering stage is one of the most drought-stress-sensitive stages of wheat life. At this time, water deficit causes a lack of insemination and infertility of flower spikes. Also, some of the vaccinated ovules are stillborn as a result of drought stress, and consequently, the number of grains per spike decreases. In the pollination stage, stress causes infertile pollen grains, disrupted current photosynthesis, and transmission of stored materials to grains, which is a reason for a reduced number of grains per spike (Wang et al., 2001). While studying 25 local wheat varieties, Nawaz et al. (2013) observed a positive correlation between the number of fertile spikelets per spike and 1000-grain weight. Marc et al. (1985) reported that drought stress after the flowering stage reduces the number of grain endosperm cells in the base, and finally reduced grain weight. Drought stress after flowering reduces grain weight, which shortens the duration of grain filling. In order to deal with drought stress and to prevent excessive wastage of water, the plant closes the stomata to reduce photosynthesis and assimilates for grain-filling. This, in turn, reduces the mean weight per grain. Mursalova et al. (2015) indicated a positive significant correlation between plant height and biological yield. In cultivars with greater plant height, the amount of production, especially in the later growing stages, depended on the transmission power of assimilates. Genotypes with greater plant height show increased biological yield (Nasri et al., 2014). Bisht et al. (2017) reported a non-correlation between grain yield and harvest index. Increasing the harvest index in case of sufficient photosynthetic organs led to increased grain yield, because at the end of the plant growth period, a large amount of photosynthetic material produced during the growth period entered the seeds. Duggan and Fowler (2006) observed in a study that in a drought-stress condition, two factors—the number of grains per spike and grain weight—played a significant role in the formation of grain yield. But in a favourable moisture condition, grain weight did not significantly influence grain yield. When 50% of the spikelets in a spike were removed artificially, the grain weight increased under humid stress, finally resulting in increased grain yield. But under non-stress conditions, these were not observed. That is, in stress and complete irrigation conditions, there were source and sink limitations respectively.

In factor analysis, considering that Eigen values were higher than 1, and factor coefficients were higher than 67%, four factors were identified for non-stressed and drought-stressed conditions (Table 5).

Table 5
Factor analysis for different agronomic characteristics of wheat genotypes in non-stressed and drought stressed conditions

	Non-stress				Drought stress			
	Factor 1	Factor 2	Factor 3	Factor 4	Factor 1	Factor 2	Factor 3	Factor 4
Grain per spike	0.20	0.87	0.13	0.10	0.91	0.07	0.16	-0.01
Straw yield	0.92	0.06	-0.28	0.22	0.17	0.38	0.83	-0.07
Grain filling duration	0.04	-0.01	0.97	-0.13	-0.24	-0.19	0.11	0.89
Harvest index	-0.18	0.40	0.86	0.01	-0.30	-0.34	0.12	0.04
1000 grain weight	-0.17	-0.33	0.77	0.14	-0.31	-0.30	0.15	0.21
Peduncle length	0.15	0.18	0.03	0.94	-0.01	0.85	0.19	-0.08
Physiological maturity	0.35	0.24	0.40	-0.02	0.24	0.10	-0.23	0.83
Biological yield	0.93	0.21	-0.01	0.26	0.08	0.27	0.94	-0.06
Fertile spikelets per spike	0.20	0.86	-0.2	0.08	0.88	0.13	0.15	-0.05
Spike length	0.07	0.72	0.22	0.31	0.74	0.21	0.19	0.25
Plant height	0.35	0.16	-0.10	0.87	0.34	0.82	0.20	0.04
Grain yield	0.88	0.31	0.43	0.24	-0.15	-0.30	0.91	0.01
Eigen value	2.70	2.60	2.50	1.90	2.80	2.70	2.70	1.60
Variance	22.11	21.65	21	16.24	23.31	22.47	22.20	13.39
Component variance	22.11	43.76	64.76	81	23.31	45.78	67.97	81.36

Felenji et al. (2011) identified three factors through factorial analysis based on the main components, which explained that 80.01% of the total data variants of the whole, at the first factor (yield), had contributed most to this explanation (33.3%). In non-stressed conditions, four factors explained 81% of the total data variants. The first factor, showing the greatest contribution (22.1%) of data variants, has significant positive coefficients for biological yield, straw yield, and grain yield. With respect to the existing traits in this group, this factor can be called the yield factor. Since this factor involves yield, it can be considered as the most important and valuable factor. The second factor explains 21.7% of data variants, which have higher coefficients for factors such as spike length, the number of fertile spikelets per spike, and the number of grains per spike, which can be called the grain yield component factor. The third factor, with an explanation for 21% of the total variance, has a positive significant factor coefficient for traits, such as grain-filling duration, 1000-grain weight,

and harvest index. It was named as a factor affecting grain weight and harvest index. In the fourth factor which involves 16.2% of the variants, the coefficients for plant height and peduncle length is high. Thus, it can be called an effective factor for height. The choice of every factor would lead to the selection of studied lines and cultivars on the basis of critical sub-traits in every trait. Under limited irrigation conditions, four main factors extracted from factor analysis explained 81.4% of all variants (Table 4). In the drought-stress experiment, the first factor that involves the biggest part (23.3%) of data variants has positive and greater coefficients for the following traits: spike length, the number of fertile spikelets per spike, and the number of grains per spike. Therefore, this factor can be represented as the components of grain yield. The second factor, involving 22.5% of variations, had great coefficients for traits, such as plant height and peduncle length. So, it can be called the influential factor for height. Thus, selecting and breeding based on these factors increase plant height, and as a result, leads to resistance to drought and earliness. By explaining 22.2% of the total variance, the third factor was affected by positive biological yield, straw yield, and grain yield, and it was introduced as an effective factor on yield. Traits such as the days to physiological maturity and grain-filling duration also had positive and significant load factors in the fourth factor, which explained 13.4% of the total variations. The fourth factor was related to plant phenology. Hence, these factors can be a good means of classification between lines and cultivars. With respect to the results from factor analysis under both non-stressed and stressed conditions, it can be seen the arrangement manner of traits in any factor is highly similar to each other. For example, in either conditions, traits such as plant height, peduncle height, biological yield, straw yield, and grain yield were located in the same factor, implying a significant correlation between these traits. This was also true for most traits within the same factor.

The results obtained from cluster analysis were used as a criterion for similarity through the Ward method using the Euclidean distance. They were divided into three groups in non-stressed drought experiments of genotypes (Figure 1).

As shown, peduncle length and plant height in the first group were greater than other groups (Table 6).

Slafer and Savin (1994) reported the influential role of peduncle length in improving yield, as cultivars with a taller peduncle length had greater stored and grain-transferable carbohydrates than those with a shorter length. The genotypes of this group had a good yield due to the maximal value for traits, such as straw, biological, and grain yields. The second group showed the earliest maturing genotypes due to the least number of days for physiological maturity, which was very important in breeding investigations. The results of cluster analysis in drought-stressed experiments divided the genotypes into four groups (Figure 2).

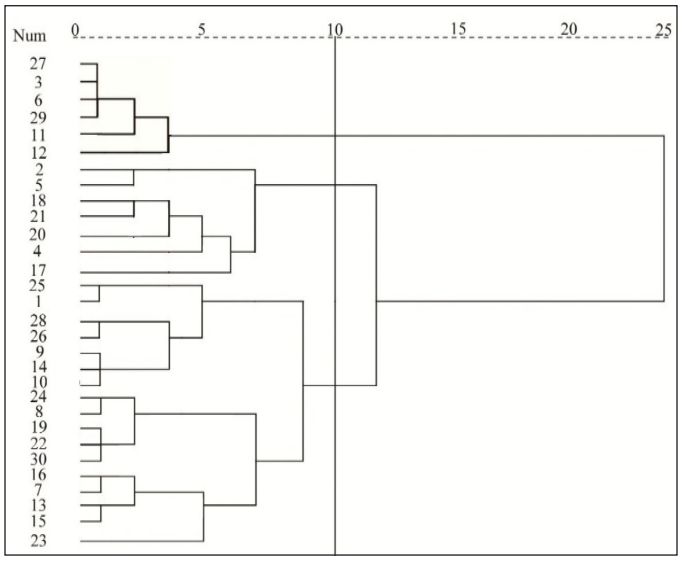

Figure 1. Grouping wheat genotypes based on all characteristics obtained from cluster analysis in non-stressed condition

Table 6
Comparison of groups between cluster analysis of wheat genotypes in non-stressed condition

	Cluster 1	Cluster 2	Cluster 3	Total average
Grain per spike	50.47	38.97	38.79	42.74
Straw yield	12.33	11.31	9.17	10.94
Grain filling duration	44.23	38.3	42.75	41.76
Harvest index	42.84	33.72	39.26	38.61
1000 grain weight	40.95	35.58	43.47	36.99
Peduncle length	44.25	41.79	38.95	41.66
Physiological maturity	275.71	268.81	272.79	272.44
Biological yield	18.23	15.95	13.9	16.03
Fertile spikelets per spike	17.37	16.99	15.7	16.69
Spike length	12.02	9.97	9.66	10.55
Plant height	110.03	106.27	96.68	104.33
Grain yield	8.02	5.72	5.82	6.52

Considering the genotypes in the first group, the values of these cultivars are the highest in terms of traits, such as peduncle length and plant height (Table 7).

In this group, straw yield, biological yield, and grain yield have the maximum values. This group is superior to other groups in terms of yield. On the other hand, the first group showed the lowest value among all groups in terms of the number of days to physiological maturity. Hence, the genotypes in this group are among the earliest maturing ones. Therefore, this group is introduced as the best of all groups

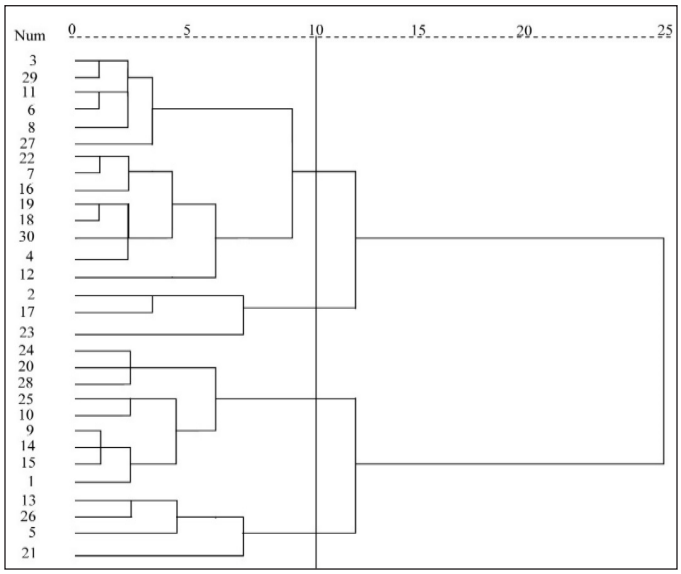

Figure 2. Grouping wheat genotypes based on characteristics obtained from cluster analysis in a drought-stressed condition

Table 7
Comparison of groups between cluster analysis of wheat genotypes in drought stressed condition

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Total average
Grain per spike	37.81	34.21	31.61	34.93	34.64
Straw yield	8.59	7.62	6.68	5.16	7.01
Grain filling duration	23.28	25.73	27.29	23.65	24.99
Harvest index	28.26	27.01	33.26	31.8	30.08
1000 grain weight	21.56	20.22	26.51	22.96	22.81
Peduncle length	42.71	39.25	35.24	32.92	37.53
Physiological maturity	248.55	259.54	256.8	257.52	255.6
Biological yield	10.28	9.81	9.19	6.85	9.03
Fertile spikelets per spike	15.69	15.63	14.02	16.11	15.36
Spike length	9.23	8.81	8.47	8.7	8.8
Plant height	104.34	94.05	85.24	84.97	92.15
Grain yield	4.49	3.28	3.61	2.79	3.54

because of the genotypes' greater peduncle length, plant height, and higher yield as well as their earliness, and not facing late-season drought stress, which Mitra (2001) termed 'drought escape'. Since early-maturity cultivars enter the generative stage in more favourable conditions, they could avoid warming and late-season moisture tensions, and thus, had more yield resistance, while late-maturing cultivars suffered severe damages during water shortage due to late-season warming with much more need for water consumption.

CONCLUSION

The results of analysis of variance for the evaluated genotypes was highly varied in terms of their significance. The analysis of correlation coefficients proved that increased biological yield, straw yield, and harvest index culminated in genetic improvement of plant grain. Regarding factor analysis under two irrigation regimes, it can be concluded four factors are responsible for most variations in this study. In cluster analysis through the Ward method, genotypes were divided into four and three groups in and drought-stress and non-stress conditions, respectively. In non-stress conditions, the current superior traits could be used in group 1 and 2, and the first group can be introduced as the superior group in stress conditions because of its high yield and grain earliness.

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Chemical Constituents of Malaysian *Geniotrigona thoracica* Propolis

Harshana Nazir, Wan Nazatul Shima Shahidan, Hanim Afzan Ibrahim and Tuan Nadrah Naim Tuan Ismail*

School of Dental Sciences, Universiti Sains Malaysia (USM), 16150 Kubang Kerian, Kelantan, Malaysia

ABSTRACT

Propolis produced by a stingless bee (*Geniotrigona* spp.), commonly known as "Kelulut" in Malaysia, is known to possess various medicinal values. It is a resinous product that is used to build the beehives. Chemical constituents of propolis differ and depend on many factors such as regions and species. To date, the study of the chemical constituents of *Geniotrigona thoracica* propolis from Malaysia is still lacking. Hence, this study report the chemical constituents from Malaysian *G. thoracica* propolis collected from Kota Bharu, Kelantan, Malaysia. The ethanolic extract of propolis (EEP) was derivatized and analyzed by gas chromatography–mass spectrometry (GCMS). The compounds were later identified by library searching Wiley 275 and NIST 02 mass spectral databases. Out of the 48 individual compounds identified, 30 compounds were identified for the first time from propolis. The main class group compounds were phenolic compounds and terpenoids. 1H-Pyrrole-2-carboxylic acid, 1-(2-hydroxy-2-phenylethyl) and fren-9(11)-en-2.alpha.-ol were the main identified phenolic compound and terpenoid, respectively.

Keywords: GCMS, *Geniotrigona thoracica*, Malaysian propolis

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E-mail addresses:
shana8088@hotmail.com (Harshana Nazir)
shima@usm.my (Wan Nazatul Shima Shahidan)
afzankk@usm.my (Hanim Afzan Ibrahim)
tnadrah@usm.my (Tuan Nadrah Naim Tuan Ismail)
* Corresponding author

INTRODUCTION

Stingless bees belong to the former genus *Trigona* with many subgenera that have been elevated to generic status such as *Geniotrigona, Heterotrigona, Lepidotrigona, Lisotrigona*, and *Tetragonula* (Michener, 2000). They are commonly known as "Kelulut" in Malaysia. These bees can be commercially reared and stingless bee keeping industry in Malaysia

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has increased drastically in the last 6 years. Currently there are more than 1000 registered farmers nationwide (Harun et al., 2015). In Malaysia, five species of *stingless bees* have been recorded so far (Kelly, Farisya, Kumara, & Marcela, 2014). They are *Geniotrigona thoracica, Heterotrigona itama, Lepidotrigona terminata, Lisotrigona scintillans,* and *Tetragonula laeviceps*. However, stingless bee keeping in Malaysia is limited to *G. thoracica* and *H. itama*.

Stingless bees can provide a lot of propolis per hive compared to honeybees (*Apis* spp.). Propolis is one of the most fascinating bee product, both for hive-building material and defensive substance. It is well known to have various biological activities such as antimicrobial (Shehu et al., 2016; Choudhari, Punekar, Ranade, & Paknikar, 2012), antihyperglycemic (Mahani, Jannah, Harahap, Salman, & Habib, 2013), anticancer (Choudhari, Haghniaz, Rajwade, & Paknikar, 2013), and anti-inflammatory activities (Campos et al., 2015). These activities were attributed to the presence of biologically active compounds such as phenolic compounds (flavonoids, phenolic acids, and their esters), terpenoids, and steroids.

Generally, propolis contains resins that comprised flavonoids, phenolic acids, fatty acids, terpenoids, aromatic acids, pollen, and minerals (Krell, 1996). The chemical composition of propolis depends on a few factors such as bee species, source of plant resin, season, and region. To date, the study of chemical composition in Malaysian stingless bee propolis is still lacking. Previous study of Malaysian propolis (*H. itama*) had analyzed the nonpolar or volatile compounds only (Usman & Mohamed, 2015). Hence, this study was aimed to identify both volatile and nonvolatile compounds of propolis from *G. thoracica* by using gas chromatography–mass spectrometry (GCMS). The propolis extract was derivatised to increase the volatility and thermal stability of the compounds, thus making compounds amenable to GCMS analysis.

MATERIALS AND METHODS

Propolis Sample and Preparation of Ethanolic Extract of Propolis (EEP)

Raw propolis sample from *G. thoracica* was obtained from the local stingless bee keeper (Razip International Trade, Kota Bharu, Kelantan). The sample was collected from Kota Bharu, Kelantan, Malaysia and transported in sealed bottles at −20°C. The extract was prepared based on the methods described by Krell (1996) with some modifications. Propolis sample was cooled in a freezer (−20°C) for a day and was ground into a fine powder. A 50 g of propolis sample was then mixed with 167 mL of 70% ethanol to obtain 30% (w/v) propolis extract. The mixture was shaken moderately by hand twice a day for a week at room temperature. The mixture was then filtered twice through Whatman filter paper No. 1. Prior to the second filtration, the extract was kept in refrigerator (2-8°C) for a day to remove the wax. The ethanol was evaporated by using a rotary evaporator under vacuum at 35°C. The remaining water in the extract was dried

by using freeze dryer and the dry extract was stored in the amber glass with a screw cap and put in the freezer (−20°C) until used.

Gas Chromatography–Mass Spectrometry (GCMS) Analysis

Sample Preparation for GCMS. The dried extract (0.5 mg) was derivatised with 50 µl *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) in a sealed glass tube for 30 min at 60°C. The trimethylsilylated extract was cooled to room temperature before diluting with 50 µl of dodecane. The extract was transferred into crimped cap vial before GCMS analysis. Blank 70% of ethanol (negative control) was dried and treated similarly as the sample.

GCMS Conditions and Parameters. GCMS was carried out using Hewlett Packard 6890 Gas Chromatograph fitted with 5973N Mass Selective Detector. The column used was fused silica capillary, HP-5 column (30 m × 0.25 mm i.d × 0.25 µm film thickness; Agilent Technologies, USA). The carrier gas was helium with flow rate at 1.0 mL/min with the oven temperature was programmed from 50°C (held for 2 min) to 280°C (held for 10 min) at a rate of 10°C/min. The injection and interface temperatures were set at 250°C and 280°C, respectively.

One microliter sample was injected in splitless mode and analyzed in MS full-scan mode (*m/z* 40–650). The electron ionization was set at 70 eV. Acquisition of data was performed using Chemsation software.

Identification of the Chemical Constituents. The National Institute of Standards and Technology (NIST) library of mass spectra was used to match and to identify an unknown chemical in the sample mixture. The mass spectrum produced by a certain chemical compound is basically the same every time. Therefore, the mass spectrum is essentially a fingerprint for the molecule. This fingerprint can be used to identify the compound. The propolis compounds were identified by library searching Wiley 275 and NIST 02 mass spectral databases. The percentage compound was calculated from the summation of the peak areas of the propolis compounds.

RESULTS AND DISCUSSION

Forty-eight individual compounds were identified. From this, 30 compounds are identified for the first time from propolis, that are hydroginkgol, resorcinol, pentadecyl-,1H-Pyrrole-2-carboxylic acid, 1-(2-hydroxy-2-phenylethyl), ethyl (3-phenylcyclobutylidene)acetate, 1-(2-Methoxyphenyl)-2,5-dihydro-1H-pyrrole-2,5-dione, nootkatone, fren-9(11)-en-2.alpha.-ol, beta-amyrenol, friedelan-y-al, 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-, cycloeucalenol, 3-(Dimethylphenylsilyl)-2-methylpropionamide, 3,7-Dioxa-2,8-disilanonan-5-one, 2,2,8,8-tetramethyl-, cyclopentane-1-carbonitrile,1-(4-hydroxy-3-methoxyphenyl)-, vinyl palmitate, methyl 9,10-methylene-octadec-9-enoate, oleyl alcohol, 2-(2',4',6'-Trichlorophenoxy)-4,5,6-trichlorophenol, 8-methyl(6)

(2,4) thiophenophane, curan-17-oic acid, 2,16-didehydro-20-hydroxy-19-oxo-, methyl ester, 1,8-Dimethyl-3,6-diazahomoadamantan-9-ol, 2-Amino-3-methylpyridine-N-oxide, 29,30-Dinorgammaceran-3-one, 22-hydroxy-21,21-dimethyl-, (8.alpha.,9.beta.,1, 12-Oleanen-3-yl acetate, (3.alpha.)-, 8-Amino-2,6-dimethoxylepidine, octahydroisocolumbinic acid methyl ester, 3-hydroxy-4-dimethyloxetanecholest-4-ene, 9,19-Cyclolanost-24-en-3-ol, acetate, (1RS,2SR,4SR,9SR)-1,6,6-trimethyl-9-isopropenyl-10-oxatricyclo[5.3.0.0(2,4)], and cycloisolongifolene, 7-bromo-.

The chemical constituents of Malaysian *G. thoracica* propolis as percentage of total ion chromatogram (TIC) are presented in Table 1. The classes of compounds that were identified are phenolic compounds, terpenoids, steroids, fatty acids, and sugar alcohol, which were similar with propolis from other stingless bees (Choudhari et al., 2012).

Table 1
Chemical compounds of Malaysian Geniotrigona thoracica *propolis*

Retention time	Compound names	% of total ion chromatogram
	Phenolic compounds	
7.32	Phenol	0.11%
9.12	Benzoic acid trimethylsilyl ester	0.13%
14.45	Hydroginkgol	0.46%
15.39	Resorcinol, pentadecyl-	0.24%
19.47	1H-Pyrrole-2-carboxylic acid, 1-(2-hydroxy-2-phenylethyl)-	1.26%
20.11	Ethyl (3-phenylcyclobutylidene)acetate	0.45%
21.46	1-(2-Methoxyphenyl)-2,5-dihydro-1H-pyrrole-2,5-dione	0.31%
	Terpenoids	
10.62	Delta-cadinene	0.14%
12.46	Nootkatone	0.26%
16.56	Lup-20(29)-ene-3,21-dione, 28-hydroxy-	0.51%
17.98	Fren-9(11)-en-2.alpha.-ol	0.60%
19.42	Beta-amyrenol	0.36%
23.54	Friedelan-y-al	0.17%
	Steroids	
19.58	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	0.48%
19.73	Cycloeucalenol	0.37%
	Sugar alcohol	
10.59	Erythritol, 1,2,3,4-tetrakis-O-(trimethylsily	0.10%
	Fatty acids	
12.06	Myristic acid, trimethylsilyl ester	0.27%
12.79	Palmitic acid, trimethylsilyl ester	0.21%

Table 1 *(continue)*

Retention time	Compound names	% of total ion chromatogram
12.98	Linoleic acid	0.22%
13.55	Octadecanoic acid, trimethylsilyl ester	0.14%
	Others	
8.19	3-(Dimethylphenylsilyl)-2-methylpropionamide	0.53%
8.90	3,7-Dioxa-2,8-disilanonan-5-one, 2,2,8,8-tetramethyl-	0.42%
9.15	Glycerol tms	0.61%
10.95	Diethyl Phthalate	0.27%
11.01	Caryophyllene oxide	0.21%
12.04	Cyclopentane-1-carbonitrile,1-(4-hydroxy-3-methoxyphenyl)-	0.20%
12.35	Hexadecanoic acid, methyl ester	0.29%
12.53	Phthalic acid, butyl isobutyl ester	0.17%
12.60	Vinyl palmitate	0.69%
13.07	Linolenic acid, methyl ester	0.38%
13.16	Ethyl linoleate	0.36%
13.34	Stearic acid, ethyl ester	1.25%
13.50	Methyl 9,10-methylene-octadec-9-enoate	0.21%
13.60	Oleyl Alcohol	0.75%
15.46	Squalene	0.93%
15.61	2-(2',4',6'-Trichlorophenoxy)-4,5,6-trichlorophenol	0.60%
15.66	8-methyl(6)(2,4) thiophenophane	0.68%
16.86	Curan-17-oic acid, 2,16-didehydro-20-hydroxy-19-oxo-, methyl ester	0.46%
17.04	1,8-Dimethyl-3,6-diazahomoadamantan-9-ol	0.28%
17.48	2-Amino-3-methylpyridine-N-oxide	0.36%
18.39	29,30-Dinorgammaceran-3-one, 22-hydroxy-21,21-dimethyl-, (8.alpha.,9.beta.,1	1.05%
18.66	12-Oleanen-3-yl acetate, (3.alpha.)-	1.94%
19.00	8-Amino-2,6-dimethoxylepidine	1.33%
19.67	Octahydroisocolumbinic acid methyl ester	0.58%
20.01	3-hydroxy-4-dimethyloxetanecholest-4-ene	1.18%
21.06	9,19-Cyclolanost-24-en-3-ol, acetate	0.43%
21.18	(1RS,2SR,4SR,9SR)-1,6,6-trimethyl-9-isopropenyl-10-oxatricyclo[5.3.0.0(2,4)]...	0.19%
21.31	Cycloisolongifolene, 7-bromo-	0.29%

The major compounds identified were phenolic compounds that make up 2.96% of TIC. The main identified phenolic compounds were 1H-Pyrrole-2-carboxylic acid, 1-(2-hydroxy-2-phenylethyl) (1.26%), hydroginkgol (0.46%), and ethyl (3-phenylcyclobutylidene) acetate (0.45%). Interestingly, all these compounds are

identified for the first time in propolis. In contrast, phenolic compounds were the minor compounds that were found in other species (*H. itama*) of stingless bee reared in Kelantan (Usman & Mohamed, 2015). Phenolic compound has relatively low volatility and is not suitable for direct GC analysis and this factor could be the contribution to the discrepancies. In the present study, the propolis extract was derivatised to increase their volatility and thermal stability. Compounds that are adequately volatile and stable in high temperature in GC conditions can be analyzed appropriately by GCMS.

After phenolic compounds, terpenoids were clearly the dominant compounds in the propolis (2.04%). Terpenoids are one of the important classes in the propolis compounds due to their valuable biological activities. The main terpenoids identified in this study were fren-9(11)-en-2.alpha.-ol (0.60%), lup-20(29)-ene-3,21-dione, 28-hydroxy- (0.51%), and beta-amyrenol (0.36%). Fren-9(11)-en-2.alpha.-ol and beta-amyrenol are identified for the first time in propolis. Other terpenoids that has been identified from this propolis was nootkatone. Recent study of this compound showed that it has an anti-inflammatory effect (Choi, Lee, & Jung, 2014).

Steroids are the other main compounds identified from propolis that make up 0.85% of TIC. The steroids that were discovered are 9,19-cyclolanostan-3-ol, 24-methylene-, (3.beta.)- and cycloeucalenol. Both of these steroids are identified for the first time in propolis. Study of cycloeucanol revealed that it has a hypoglycemic effect (Ragasa, Lim, Shen, & Raga, 2015). In contrast, steroids were not detected from other Malaysian *G. thoracica* propolis (Ibrahim et al., 2016). Although the bee species are similar, the main plant source, shrubs and fruit orchards surrounding the two apiaries may differ and contribute to the discrepancies.

Similar with propolis from other stingless bee species, erythritol was also discovered in this study that made up 0.10% of TIC. Erythritol is a sugar alcohol that tastes like sugar but has no calories. It has been approved for use as a food additive in the United States and throughout the world. The other compounds that were identified were fatty acids. The amount of fatty acid identified was 0.84% of TIC. The compounds identified were myristic acid, trimethylsilyl ester, palmitic acid, trimethylsilyl ester, linoleic acid, and octadecanoic acid, trimethylsilyl ester. Similarly, these fatty acid compounds are also identified in propolis from other stingless bee.

Diethyl phthalate was identified in this propolis sample most probably due to the plastic container that was used to transport this sample. It is a synthetic substance that is commonly used to make plastics.

The various polarities of the chemical compounds of propolis thus affect their method of extraction. As ethanol has a polar end, the hydroxyl group (OH) and a nonpolar end, the ethyl group (C_2H_5), it can dissolve both polar and nonpolar compounds. Therefore, it is important that

solvents for the identification of propolis chemical constituents should be able to extract both polar and nonpolar compounds. In this study, 70% of ethanol has been used to extract the propolis.

CONCLUSION

In the present study, 48 chemical compounds were identified from Malaysian *G. thoracica* propolis. From this, 30 compounds were identified for the first time from propolis by using GCMS analysis. Most of the identified compounds are known to have biological properties. Furthermore, stingless bee keeping industry in Malaysia has been increasing in trend. Hence, it is needed to further identify the biological properties of this Malaysian propolis that may be beneficial for the human nutrition and health.

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CONFLICT OF INTEREST

None.

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The Effect of Harmonic Frequency and Sound Intensity on the Opening of Stomata, Growth and Yield of Soybean (*Glycine max* (L.) Merrill)

Istirochah Pujiwati[1]*, Nurul Aini[2], Setyawan P. Sakti[3] and Bambang Guritno[2]

[1]Department of Agrotechnology, Faculty of Agriculture, University of Islam Malang, 65144 East Java, Indonesia
[2]Department of Agricultural Cultivation, Faculty of Agriculture, Brawijaya University Malang, 65145 East Java, Indonesia
[3]Physics Department, Faculty of Mathematics and Natural Sciences, Brawijaya University Malang, 65145 East Java, Indonesia

ABSTRACT

Seventy percent of Indonesia's soybean demands and consumption are met from imports, and therefore, it is necessary to increase its local production. This study assessed the effects of harmonic frequency and sound intensity levels on the opening of stomata, the growth and yield of soybean. Experiments were conducted in a split plot design, with frequency harmony (F_4: 4 kHz, F_8: 8 kHz, F_{12}: 12 kHz) being the main plots and sound intensity (A_{50}: 50 decibels (dB), A_{80}: 80 dB and A_{110}: 110 dB) used as sub plots. The results showed there was no significant effect of frequency and intensity on the measured response (stomata opening). However, if they were compared with those at 0 frequency and 0 intensity, the stomata openings were significantly different based on t-test at p = 0.05. This means the opening of stomata was affected by resonance. In general, the sound level pressure attempted in the range of 50-110 dB had no effect on the width of stomata opening, but it affected to the growth and yield of soybean. The best growth of the leaf area and relative growth rate were in the presence of sound waves at a frequency of 4 kHz. Likewise, the best result of the average fresh weight of seed, dry weight of seed and harvest index were at a frequency of 4 kHz sound waves. The leaf area, seeds fresh and dry weight, and harvest index were also significantly highest at sound intensity of 50 dB. Therefore, to improve the productivity

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E-mail addresses:
isti.wati@yahoo.co.id (Istirochah Pujiwati)
rully_aini@yahoo.com (Nurul Aini)
sakti@ub.ac.id (Setyawan P. Sakti)
bbguritfp@yahoo.com (Bambang Guritno)
* Corresponding author

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of soybean plants, exposure at a frequency of 4 kHz and sound intensity of 50 dB, followed by an application of leaf fertiliser according to recommended dosage is the best combination of treatment in growing soybean in Indonesia.

Keywords: Harmonic frequency, resonance, sound intensity, soybean

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is the most important agricultural commodity after rice and maize in Indonesia. Soybean is a legume that contains vegetable protein and oil. The Central Statistics Agency data shows that soybean production fell by 780.16 thousand tons between 2012 and 2013, which is a decrease of 63 thousand tons (7.47%) compared to 2012. The decline in soybean production was due to decreased productivity by 0.07 t/hectare (4.65%) and a decrease in the harvested area of 16.83 thousand hectares (2.96%). Thus, strategic effort is needed to increase domestic soybean production. One of the strategies is through the application of sonic bloom technology.

Sonic bloom is a stimulation technology targeting wider stomata opening by using high frequency of sound waves. This is followed by an application of liquid fertiliser through leaves (Carlson, 2013). When the plants are at the stage of fruit bearing, and with the exposure of sound wave of 3.5 - 5 kHz, they are sprayed with foliar fertilisers. By a wider opening of the stomata, the absorption of fertiliser increases by the leaves (Carlson, 2013). The opening of stomata is affected by light, temperature, and other factors. However, by using scattered sound waves, the stomata can be maximally opened despite the above factors (Dalimunthe, 2004).

According to Weinberger and Graefe (1973), the stimulation of stomata opening by sound waves may be due to one or more of the following mechanisms: (1) certain sound frequencies can turn on certain genes in cells that affect growth and cell expression, (2) the frequency of resonating sound improves the movement of cytoplasm in the cell, (3) the cavitation phenomenon, a phenomenon caused by noise in the liquid. Sound waves coming from a cell's wall hits the cytoplasm. Certain frequencies affecting the cytoplasm cause micro-bubbles. The micro-bubbles resonate with the sound and spur the rise of guard cells turgidity, which causes maximal stomata opening. Additionally, the fourth (4) mechanism is the propagating sound through the liquid stimulates the movement of molecules such as in the diffusion process. However, until now, the proper mechanism of sound effect on plant is not fully understood. Better understanding is necessary to establish a mechanism and to develop models for application of this potential technology in addition to experiments to find the best sound frequencies and intensities for growth and yield of soybean.

Frequency is the number of vibrations in one second. Harmonic frequency is the integer multiples frequency of basic frequency (Satira, 2013). The basic frequency used in this study was 4 kHz, so

that the harmonic frequencies used were at 8 kHz and 12 kHz. Pujiwati and Djuhari (2011) suggested exposure of sound wave frequency of 4-5 kHz causes an increase in the width of stomata opening. Furthermore, Pujiwati and Djuhari (2014) found that the 15-day old Anjasmoro soybean variety when exposed to sound waves with a duration of 40 minutes produced the best results with seed production reaching 3.93 t/ha, or increased by 71%, compared with the average production of 2.25 to 2.30 t/ha.

Exposure to high frequency sound waves is proven to optimise the stomatal opening of plant leaves (Collins & Foreman, 2001; Haryanti & Meirina, 2009). Rohmah (2012) reported that the stomata of soybean plant leaves opened wider when exposed to noise. The sound waves affect several growth processes, such as seed germination, growth of callus in tissue culture techniques, growth and development of vegetables, fruits and plantation crops (Hassanien et al., 2014). There is a biological effect of sound waves on the growth of rice seeds when exposed to sound frequency and intensity of 0.4 kHz and 106 dB respectively (Baldocchi, 1997). Their germination index, plant height, and root development are significantly increased by sound.

A tissue culture of *Actinidia chinensis* exposed to sound wave at frequency and intensity of 1 kHz and 100 dB respectively at a distance of 0.2 meters had resulted in an increase of ATP (soluble protein) and the activity of superoxide dismutase (SOD) enzyme of the plant. The SOD enzyme catalyses superoxide into oxygen and hydrogen peroxide (Yang et al., 2002), and it acts as an antioxidant and protects cell components from oxidation by reactive oxygen species (ROS) due to drought, deficiency of nutrients, heavy metals, reaction of ultra-violet or gamma rays and the metabolic activities of some plants (Alscher et al., 2002; Raychaudhuri & Deng, 2000).

At an Agricultural Engineering Research Centre in China, research was conducted on several kinds of vegetables using Plant Acoustic Frequency Technology (PAFT) with a frequency and a sound pressure level of 0.06-2 kHz and 50-120 dB respectively at a distance of 50-100 meters (Jun-ru et al., 2011; Meng et al., 2011). The results obtained in cucumber, tomatoes, watermelon, cowpea and eggplant showed significant increases in chlorophyll content, net photosynthetic rate, and the number of flowers and fruit. It was found that PAFT stimulates endogenous production of some hormones, such as IAA and GA. Sound wave technology is also effective in increasing the plant's immune system against disease and insect pests (Hou et al., 2010; Yu et al., 2013). In the case of cotton plants that were grown in an open area, a minimum yield increase of 5.2% was noted when crop received sound waves of 75-110 dB at a distance of 30 meters while the highest of 18.6% was recorded at the sound pressure of 70-75 dB (Hou et al., 2010).

This study was aimed at finding the initial proof that resonance events that might have caused the stimulation of stomata opening related to selected growth and yield

parameters of soybean with the sound wave frequency and intensity, especially under the Indonesia's growing condition.

MATERIALS AND METHODS

Study Site

The experiment was conducted from June to September 2016 at the experimental station of Brawijaya University located at the Faculty of Agriculture, Jatimulyo village, Lowokwaru District Malang, East Java Province, Indonesia at the height of 540 meters above sea level. It lies at 07°59'S and 112°36'E. Its average temperature was 24.1°C with air humidity of 72%. The average rainfall was 1883 mm per year. The intensity of solar radiation ranged from 327.0 x 10^5 to 603.4 x 10^5 lux.

Experimental Design and Treatments

The experiments were conducted in a split plot design. The main plot was the frequency of sound waves with three levels (F_4, F_8, and F_{12} for 4, 8, and 12 kHz respectively). The sub plot was the sound level pressure (sound intensity) consisting of three levels (A_{50}, A_{80}, and A_{110} representing 50, 80, and 110 decibels [dB], respectively). The main plot and sub plot were repeated three times. The control was the samples that were not exposed to frequency and sound intensity.

Soybean plants were exposed to sound waves at the above levels of frequency and sound intensity from age 20 to 70 days after planting (DAP) with 10-day intervals. Each exposure lasted for 20 minutes. Subsequently, soybean plants were sprayed with Growmore liquid fertiliser at a dose of 2 g/litre. The observation of stomata width opening was made by using the replica method with a clear nail polish fixation. The recorded and computed growth parameters were plant height, leaf area, and relative growth rate ($RGR = (\ln W_2 - \ln W_1)/(t_2-t_1)$) (Sitompul and Guritno, 1995). The fresh weight of seed, dry weight of seed, and harvest index (HI) were also observed and computed (HI = (dry seed weight/total dry weight of the plant) x 100%)) (Sitompul and Guritno, 1995).

Statistical Analysis

Statistical analysis was conducted by using analysis of variance (ANOVA test) and means separation were tested by using the Least Significant Difference test at p = 5%. The analysis of comparison between control and each treatment was performed by a t-test at p = 5% and p = 1%.

RESULTS AND DISCUSSION

The Effect of Harmonic Frequency and Sound Intensity on the Width of Stomata Opening

The ANOVA was performed at frequencies of 4, 8, and 12 kHz, and sound intensities of 50, 80, and 110 dB which affect the stomata opening at each DAP. The results also indicated there was no significant effect of sound frequency and intensity on the measured responses. However, there was highly significant effect between the plants exposed to sound waves at a frequency of 4-12 kHz and at intensity of 50-110 dB with controlled plants as shown in Table 1.

Table 1
The average width of stomata opening (µm) on harmonic frequency and sound intensity at different age observations

Treatment	Mean of stomata opening width (µm) at different plant age					
	20	30	40	50	60	70
Frequency (kHz)						
4	10.38a	11.32a**	11.39a**	9.27a	9.47a	11.70a**
8	10.45a*	11.35a**	10.45a*	8.85a	9.29a	11.32a**
12	11.46a*	10.66a	11.42a**	9.38a	9.28a	10.31a*
Control	9.07	8.65	7.50	7.50	7.59	6.88
LSD 5%	2.56	2.41	1.69	1.76	2.29	2.25
Sound Intensity (dB)						
50	10.94a	11.49a*	10.21a*	8.85a	9.64a	11.56a*
80	10.83a	11.63a	11.77a**	9.58a	9.04a	11.88a**
110	10.83a	10.21a	12.19a*	9.38a	9.73a	11.67a
Control	9.07	8.65	7.50	7.50	7.59	6.88
LSD 5%	2.56	2.41	1.69	1.76	2.29	2.25

Notes: Means followed with same letter within a column of each main effect are not significantly different at p=0.05 by LSD test
* and ** are different at p=5% and 1%, respectively by t-test for comparison between control and each level of frequency and sound intensity
DAP: days after planting

Previous works (Pujiwati & Djuhari, 2011) showed that the width of stomata opening was different when the plant's stomata opening occurred at 4 to 5 kHz. The result of this experiment (Table 1) showed the width of stomata opening was not different when exposed by sound wave at 4, 8 and 12 kHz, but it was different to that of the control.

The width of stomata openings were similar among those frequencies possibly due to resonance effect to the stomata opening caused by sound waves. In a physical system, when a system affected by a specific frequency from external stimulus, for example sound wave, the system will have a similar response to the harmonic frequency of the stimulus signal. The same mechanism can also exist in the stomata opening affected by sound wave.

The Effect of Harmonic Frequency and Sound Intensity on Growth

The results showed the frequency affected plant height at 41 and 48 days after planting (DAP) (Figure 1), leaf area at 48 and 62 DAP (Figure 3), and relative growth rate at an interval of 49 to 56 DAP (Figure 5). Additionally, the intensity of the sound affected plant height at 41 DAP (Figure 2) and leaf areas at 48 DAP (Figure 4).

At 41 DAP control plants without frequency produced the highest plant height while at 48 DAP plants exposed to the

frequency of 8 kHz had similar height as the control plants (Figure 1). Meanwhile, at 41 days, the highest plant height was at 80 dB and the shortest plant height was at 110 dB among all intensity treatments excluding control (Figure 2).

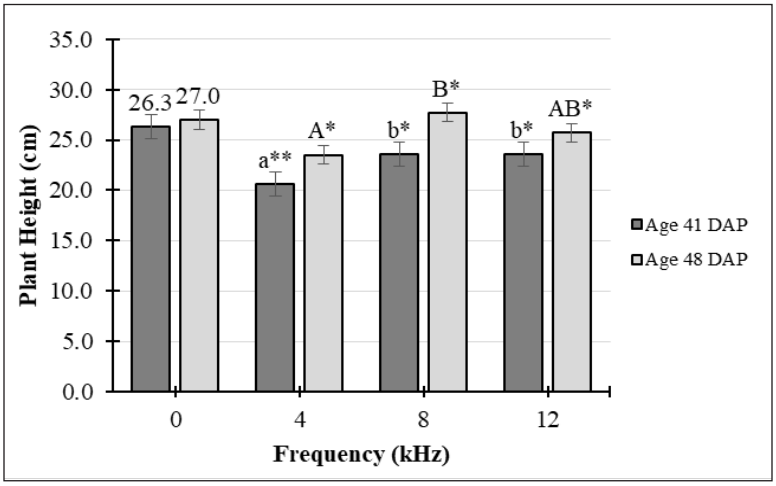

Figure 1. The effect of the frequency on plant height at 41 and 48 days after planting. Means followed by same letter among 4, 8, and 12 kHz are not different based on LSD at p=5% (lower and upper cases are for 41 and 48 DAP plants, respectively). The * and ** indicate significant difference between control (0 kHz) and each level of frequency by t-test at p=5% and 1% respectively.

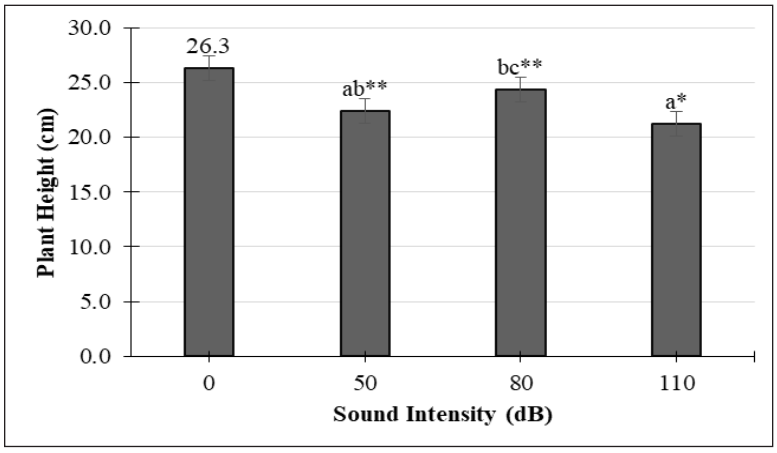

Figure 2. The effect of the sound intensity on plant height at 41 days after planting. Means followed by same letter among 50, 80, and 110 dB are not different based on LSD at p=5%. The *and ** indicate significant difference between control (0 dB) and each level of sound intensity by t-test at p=5% and 1% respectively.

The frequency of 8 kHz and 12 kHz produced a better leaf area than the frequency of 4 kHz on the growth of leaf area at 48 DAP entering pod phase formation, whereas at 62 DAP (charging phase and ripening seeds), many leaves turned yellow and fell off the plant (Figure 3).

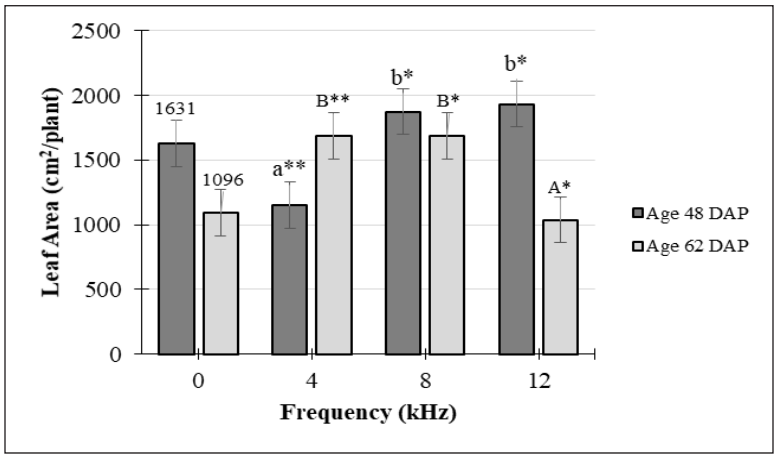

Figure 3. The effect of the frequency on leaf area at 48 and 62 days after planting. Means followed by same letter among 4, 8, and 12 kHz are not different based on LSD at p=5% (lower and upper cases are for 48 and 62 DAP plants, respectively). The * and ** indicate significant difference between control (0 kHz) and each level of frequency by t-test at p=5% and 1% respectively.

At 48 DAP, the leaf area decreased with the increasing of sound intensity and the best leaf area was produced at the 50 dB intensity (Figure 4).

Figure 5 shows that at the interval age of 49 to 56 DAP with the exposure of soybean crop at 4 and 12 kHz, produced a relatively higher relative growth rate than that of the 8 kHz level.

The soybean crop that was exposed to harmonic frequency and sound intensity of 50 to 110 dB showed different plant height (at 41 and 48 DAP), leaf area development (at 48 and 62 DAP), and relative growth rate (at 49 to 56 DAP). This finding is in line with that of Hassanien et al. (2014) as every plant has different responses toward sound frequency and sound intensity. Furthermore,

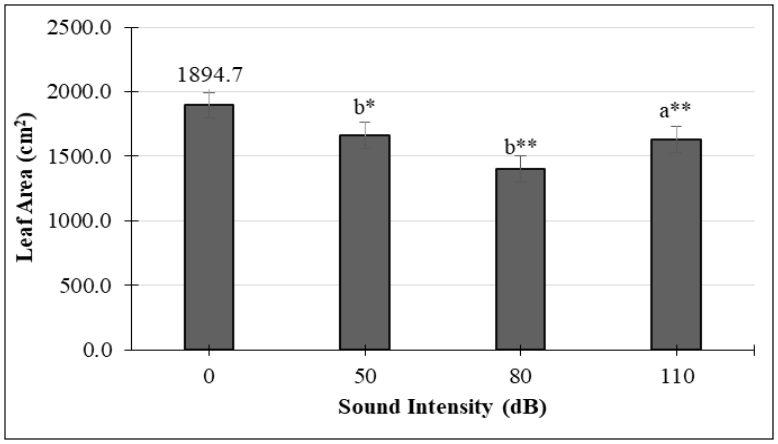

Figure 4. The effect of the sound intensity on leaf area at 48 days after planting. Means followed by same letter among 50, 80, and 110 dB are not different based on LSD at p=5%. The *and ** indicate significant difference between control (0 dB) and each level of sound intensity by t-test at p=5% and 1% respectively.

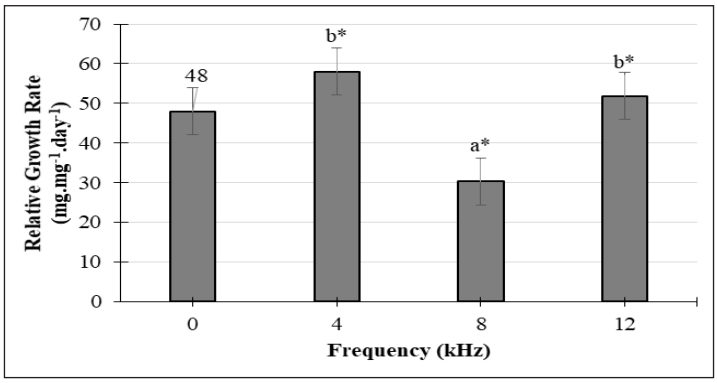

Figure 5. The effect of frequency on the relative growth rate at 49-56 days after planting. Means followed by same letter at 4, 8, and 12 kHz are not different based on LSD at p=5%. The * indicate significant difference between control (0 kHz) and each level of frequency by t-test at p=5%.

this finding was supported by Chowdhury et al. (2016) who reported that sound was an external factor that had a great impact on the biological index of plant and could either promote or suppress growth.

The Effect of Harmonic Frequency and Sound Intensity on the Yield

The harmonic frequency and the sound intensity showed a significant result on the fresh and dry weight of seed, and the harvest index.

The frequency of 4 kHz produced the highest fresh weight of seed that was 2.84 $t.ha^{-1}$, an increase of 26.2% compared with the potential production by 2.25 - 2.30 $t.ha^{-1}$. Similarly, for dry weight of seed, 4 kHz frequency also gave the highest yield (Figure 6).

The sound intensity of 50 dB produced the highest fresh weight and dry weight of the seed (Figure 7).

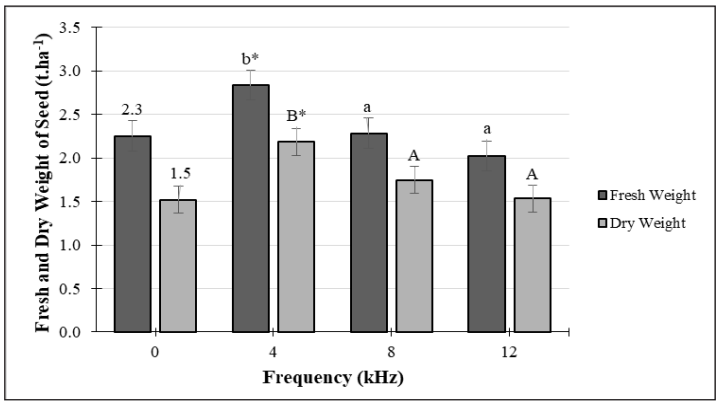

Figure 6. The effect of the frequency on fresh weight and dry weight of seed. Means followed by same letter among 4, 8, and 12 kHz are not different based on LSD at p=5% (lower and upper cases are for fresh weight and dry weight, respectively). The * indicate significant difference between control (0 kHz) and each level of frequency by t-test at p=5%.

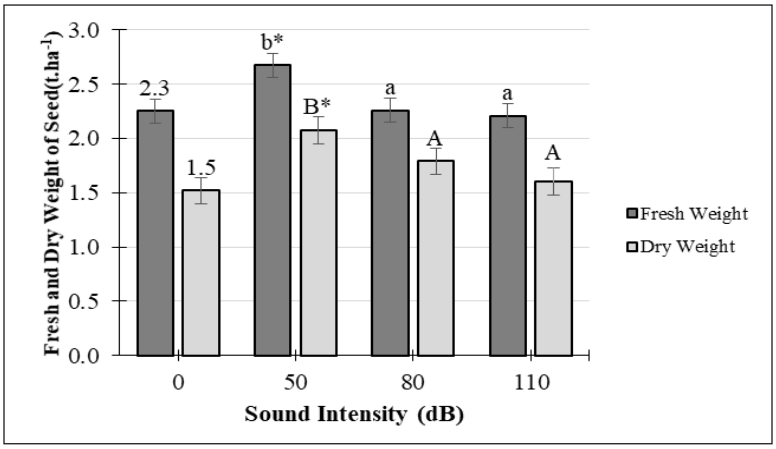

Figure 7. The effect of the sound intensity on fresh weight and dry weight of seed. Means followed by same letter among 50, 80, and 110 dB are not different based on LSD at p=5% (lower and upper cases are for fresh weight and dry weight, respectively). The * indicate significant difference between control (0 dB) and each level of sound intensity by t-test at p=5%.

The average of harvest index at the frequency of 4 kHz produced the highest result, 42.65%. The harvest indexes among frequencies of 8 kHz and 12 kHz, and without frequency were not significantly different (Figure 8). Additionally, the highest harvest index was generated at a sound intensity of 50 dB with 41.22%. Meanwhile the harvest indexes were not different without sound intensity and intensities of 80 dB and 110 dB (Figure 9).

The best results of the average of fresh and dry seed weight per hectare and harvest index were obtained when the plants were exposed to the sound wave frequency of 4 kHz. An increase in grain yield amounted

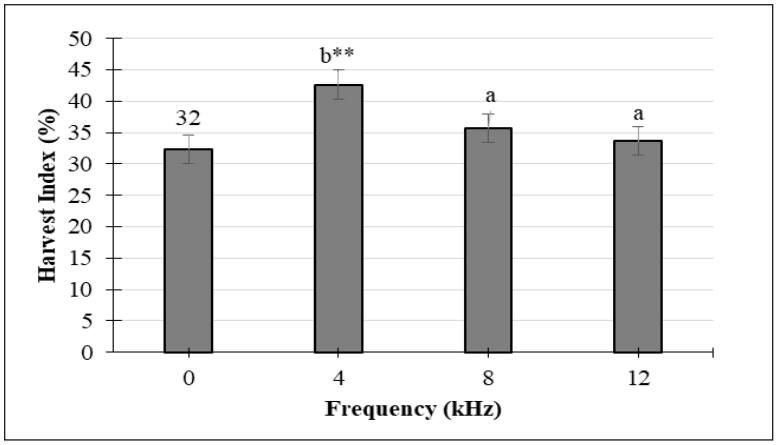

Figure 8. The effect of the frequency on harvest index. Means followed by same letter among 4, 8, and 12 kHz are not different based on LSD at p=5%. The ** indicate significant difference between control (0 kHz) and each level of frequency by t-test at p= 1%.

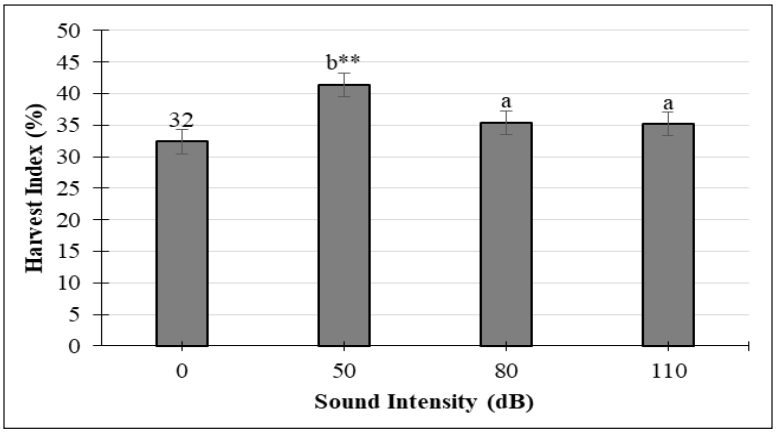

Figure 9. The effect of the sound intensity on harvest index. Means followed by same letter among 50, 80, and 110 dB are not different based on LSD at p=5%. The ** indicate significant difference between control (0 dB) and each level of sound intensity by t-test at p=1%.

to 26.2% was achieved when compared to the potential production of the Anjasmoro soybean variety (2.25 to 2.30 $t.ha^{-1}$). The result was supported by previous work (Pujiwati & Djuhari, 2011) which showed that the stomata opening increased at sound frequency from 4-5 kHz. However, if the frequency reached 6-7 kHz, the opening of the stomata decreased. This finding was in line with the experimental result of Martens et al. (1982) which found that only the optimal frequency range could stimulate growth and yield. Furthermore, Chowdhury et al. (2014) reported that sound waves with specific frequencies and intensities could have positive effects on various plant biological indices, including seed germination, root elongation, plant height, callus growth, cell cycling, signalling transduction systems, enzymatic and hormonal activities and gene expression.

The fresh weight and dry weight of the seed, harvest index decreased as the sound intensity increased. The high sound intensity, could be in a level of intensity with energy that affected the biomass by photosynthesis mechanism (principally through affecting stomata opening). It means that the sound level intensity should be optimised to achieve the best biomass. Other studies on microorganism (*E. coli*) showed a negative effect of high sound intensity to bacteria growth (Gu et al., 2016).

CONCLUSION

This study concluded the stomata opening mechanism was due to the resonance of events indicated by their higher stomata opening than the value at the control treatment (0 kHz and 0 dB). The phase of vegetative growth of soybean plants had different patterns of response to the

frequency of sound waves. The yield of soybean crop exposed to the sound wave frequency of 4 kHz showed an increase of 26.2%. The highest result of the leaf area, fresh weight, dry weight of seeds and harvest index was also found at 50 dB sound intensity.

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Growth and Yield Performance of Five Purple Sweet Potato (*Ipomoea batatas*) Accessions on Colluvium Soil

Martini Mohammad Yusoff[1]*, Siti Nurjiah Abdullah[2], Mohd Ridzwan Abd Halim[1], Erwan Shah Shari[3], Nur Arina Ismail[1] and Masnira Mohammad Yusoff[4]

[1]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[2]Tambunan Community College, Tambunan Village Resort Centre & Resort, Kolam, Tandarason, 89657 Tambunan, Sabah, Malaysia
[3]Malaysian Agricultural Research and Development Institute (MARDI), 13200 Seberang Prai, Pulau Pinang, Malaysia
[4]Malaysian Agricultural Research and Development Institute (MARDI), 43400 Serdang, Selangor, Malaysia

ABSTRACT

A study was conducted to evaluate the growth and yield performance of five purple sweet potato accessions on colluvium soil at Field 2, Universiti Putra Malaysia, Serdang, Selangor. The treatments comprised five purple sweet potato accessions (Accessions 1, 2, 3, 5, and 6) arranged in completely randomized design with four replications. Maximum tuber yield of purple sweet potato was highest ($p < 0.05$) in Accession 6 (34,563 kg ha^{-1}) compared to the lowest yield in Accession 3 (9,331 kg ha^{-1}). This was followed by Accession 2 (22,031 kg ha^{-1}), Accession 1 (21,094 kg ha^{-1}), and Accession 5 (22,900 kg ha^{-1}). A similar trend was observed for crop growth rate (CGR) with the highest (199 kg ha^{-1} day^{-1}) in Accession 6 and the lowest (60 kg ha^{-1} day^{-1}) in Accession 3 ($p < 0.05$). Accession 6 reached the critical leaf area index (LAI_{crit}) of 2.79 at 90% of intercepted radiation compared to the Accessions 1, 2, 3, and 5 that did not reach the LAI_{crit}. Unexpectedly, the highest total intercepted photosynthetically active radiation (PAR) was recorded by Accession 3 (340 MJ m^{-2}), whereas Accession 6 showed the lowest (309 MJ m^{-2}). In contrast, the highest radiation use efficiency (RUE) was obtained by Accession 6 with 7.58 g MJ^{-1} and the lowest was Accession 3 (2.16 g MJ^{-1}). However, there was no significant difference in RUE among the rest of the accessions, except for Accession 6. To maximize the

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E-mail addresses:
martiniupm@gmail.com (Martini Mohammad Yusoff)
jiahabdullah5168@gmail.com (Siti Nurjiah Abdullah)
ridzwan@upm.edu.my (Mohd Ridzwan Abd Halim)
wanshah24@gmail.com (Erwan Shah Shari)
arinaismail.n@gmail.com (Nur Arina Ismail)
mmyusof@mardi.gov.my (Masnira, Mohammad Yusoff)
* Corresponding author

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tuber yield, Accession 6 is the choice for cultivation in colluvium soil.

Keywords: Leaf area index (LAI), radiation use efficiency (RUE), sweet potato, total intercepted photosynthetically active radiation (PAR), tuber yield

INTRODUCTION

Sweet potato (*Ipomoea batatas*) is a dicotyledonous plant belonging to the Convolvulaceae family. The plant is a herbaceous perennial vine with alternate heart-shaped or palmate lobed leaves that is usually grown as an annual. Sweet potato ranks as the world's seventh most important food crop after wheat, rice, maize, potato, barley, and cassava (Food and Agriculture Organization of the United Nations [FAO], 2009). In Malaysia, sweet potato ranks second among the tuber crops next to cassava (Hanim, Chin, & Yusof, 2014). According to Uwah, Undie, John and Ukoha (2013), China is the largest sweet potato producer with an annual production of 100 million tonnes followed by Indonesia. The total sweet potato area in Malaysia was about 2000 ha/year (Tan et al., 2010). In the tropical, sub-tropical, and frost-free temperate climatic zones of the world, sweet potato is considered as a staple food. Hue, Chandran and Boyce (2010) reported that the varieties of sweet potato may differ in its flesh colour, storage root's skin colour, and some by origin. Its flesh ranges in colour from beige to white, purple, red, pink, violet, yellow, and orange.

Sweet potato varieties with white or pale yellow flesh are not as sweet and moist as those with red, pink, or orange flesh. Sweet potato is rich in carbohydrate, protein, vitamin A, vitamin C, potassium, iron, fiber (Mais & Brennan, 2008), β-carotene, and anthocyanins (Bovell-Benjamin, 2007). It also helps to maintain fluid and electrolyte balance in the body (Palaniswami & Peter, 2008). It is also good for treating stress and provides important minerals that help to maintain balance throughout the body during stress (Motsa, Modi, & Mabhaudhi, 2015).

Most of the sweet potatoes produced in Malaysia are consumed as supplementary food. It is also processed as snacks. Sweet potato is served mainly in the boiled and baked forms. Besides that, the roots and leaves are widely consumed as vegetable in rural and urban areas. The harvested vines are also used as cattle feed. Usually the sweet potato is consumed fresh and only a limited quantity are used in making sweet potato flour that are mixed with wheat flour in making breads and cakes.

Many areas in the states of Perak, Kelantan, and Terengganu in Malaysia are involved in sweet potato production. The area planted with sweet potato in Malaysia was 2229 ha in 2011 with the production reaching 26,582 tonnes and increased to 2505 ha with the production of 26,688 tonnes in 2013. However, the yield was lower in 2012 with 25,417 tonnes from 2386 ha planted area (Department of Agriculture, 2013).

In sweet potato cultivation, the chosen variety for cultivation plays a significant role in yield determination and improvement (Maniyam, Gangadharan, & Susantha,

2012). Jalomas, Gendut, and VitAto are the popular varieties developed by the Malaysian Agricultural Research and Development Institute (MARDI) in Malaysia, besides other high-yielding varieties such as Madu, Telong, Pontian, Bukit Naga, Serdang, Rhu Tapai dan Kuala Linggi (Maniyam et al., 2012; Tan, 2000 ; Tan et al., 2010; Zaharah & Tan, 2006). Other local varieties such as Batu Kelantan, Biru Putih, Oren, and Indon are also planted in Malaysia (Hue et al., 2010).

Research on sweet potato by organizations such as MARDI and Universiti Putra Malaysia (UPM) focuses on developing specific sweet potato variety with high yield and quality. In Malaysia, research on orange flesh sweet potato known as VitAto has been conducted by MARDI (Zaharah & Tan, 2006). It is proven that the VitAto is a suitable variety for cultivation in Kelantan and Terengganu because VitAto sweet potato was reported to grow well on bris soil (Zaharah & Tan, 2006). In addition, VitAto tubers are of high-nutrient value with high beta carotene and vitamin C. It is also a good supplementary food for Malaysians because of low-glycemic index and high-dietary fiber content (Mahmood, Ibrahim, Mohd. Nasir, Pin, & Hamzah, 2007).

Other than VitAto, there are no new selected varieties that have been released or introduced recently. Hence, identification of other high-yielding varieties such as the purple sweet potato can attract the local farmers to grow sweet potato, thus increasing their family income. Purple sweet potato also has a high nutritional value due to the presence of "anthocyanin" pigment derived from purple-coloured flesh variety of sweet potato. In addition, peonidins and cyanidins in anthocyanin have anti-oxidant and anti-inflammation properties that are very important for good digestion.

Other than planting on the bris soil, colluvium soil is also common for sweet potato planting in Malaysia. Furthermore, sweet potato vines of the vigorous varieties have high dry matter yield and protein content that could be used to feed cattle. Thus, the present study was conducted to identify the high-yielding accessions of purple sweet potato suitable to be planted on colluvium soil and to evaluate the yield performance of purple sweet potato accessions on colluvium soil.

MATERIALS AND METHODS

The study was conducted at Field 2, Faculty of Agriculture, Universiti Putra Malaysia (UPM), Serdang, Selangor (3^{o} 00′ N, and 101^{o} 42′ E). The soil type of the location of the study was colluvium soil with a flat terrain. Soil analysis was done at the Analytical Laboratory, Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor. The results of the soil analyses were soil pH was 6.18, total N was 0.11%, total P was 1.87 ppm and soil texture: sandy loam. The previous crop on the site was also sweet potato. The field experiment started on 23rd October, 2013 and ended on 23rd February, 2014. The total rainfall for the four months of the experimental period was 1040.7 mm, while the maximum and

minimum temperatures were 32.3°C and 22.8°C, respectively. The land was ploughed with a disc plough and left fallow for 14 days to ensure that the existing weeds and weed seeds in the soil were eliminated. Then a disc harrow was used to pulverize the soil followed by a rotor-ridger to loosen the soil and planting beds were then formed. Five accessions of purple sweet potato were tested in this study that were labeled Accessions 1, 2, 3, 5, and 6. The general description of these accessions is presented in Table 1.

Table 1
General description of the accessions used as planting materials for the study

Accessions	1	2	3	5	6
Mature leaf colour	Green	Green	Green with purple veins on upper surface	Green	Green
Mature leaf shape	Lobed	Lobed	Lobed	Lobed	Lobed
Tuber shape	Round elliptic	Long elliptic	Long elliptic	Long irregular or curved	Round elliptic
Tuber skin colour	White	Dark purple	Purple red	Dark purple	Dark purple
Tuber flesh colour	Light purple	Purple red	Light purple	Dark purple	Dark purple

Stem cuttings of 30 cm in length with at least seven nodes derived from plants aged 2 months were used as planting materials. These cuttings were left under the shade for 2-3 days before planting to get better root initiation. The cuttings were dipped in Benomicide™ solution for several minutes as a prevention from soil-borne disease infection. The cuttings were planted at a distance of 0.25 m between the plants and 1 m between the ridges. There were 20 plots and each plot was 6.0 × 1.0 m in size. The planting materials were obtained from Bachok Research Station of the Malaysian Agricultural Research and Development Institute (MARDI) in Kelantan. Chicken manure was applied to the experimental plots before planting at the rate of 5 tonnes ha^{-1}. Compound fertilizer NPK Blue with the ratio of 12:12:17:2 was applied at the rate of 266 kg ha^{-1}. The fertilizer was given in three split applications at the rate of 160 g $plot^{-1}$. Insects were controlled with contact insecticide Press® that contains active ingredient: profenos and organophosphate. The herbicides were used at the 37 days of planting using Roundup™ or Ecomax™ that contains active ingredient; glyphosate-isopropylammonium to control weeds in between the beds. Hand weeding was also carried out when necessary throughout the growing season. A sprinkler system was used to supply water to the plants twice a day.

Measurements

Maximum Tuber Yield and Plant Biomass. Maximum tuber yield is defined as the highest yield recorded for the whole growing season for each accession. Samples of tuber yield and plant biomass (tuber, leaves, and vines) were collected at 2-week intervals beginning at second week after planting until final harvest. Samplings were measured by taking duplicate 0.2 m^2 quadrats in each plot. For the final harvest, duplicate 0.5 m^2 quadrats were used. Samples were oven dried at 60°C to constant weight. All samples of tuber and plant biomass were weighed using a digital balance, which was examined for accurate reading prior to its use.

Total Dry Matter (TDM) Accumulation Over Time (Crop Growth Rate). Crop growth rate (CGR) is defined as = $\Delta TDM/\Delta t$, where TDM is total dry matter (kg ha^{-1}) and t is time (days after sowing) over the range of days from 5-95% of maximum TDM for all accessions.

Leaf Area Index and Radiation Interception. Leaf area index (LAI) and the fraction of radiation transmitted (I/Io) through the canopy were measured using a Plant Canopy Analyser LAI-2000 (LI-COR Biosciences, Inc., Nebraska, USA). Measurements were taken weekly from October to February starting from 14 days after sowing (DAS) until 120 DAS at final harvest. At each session, two readings of LAI were taken on each plot and the average of the measurements was obtained. The fraction of radiation intercepted (Fi) was calculated using the formula of Gallagher and Biscoe (1978):

$$Fi = 1.0-(I/Io)$$

Where, I is radiation under the canopy and Io is radiation above the canopy.

Daily incident solar radiation from the nearest Meteorological Station was used to determine total incident PAR and 50% of the incident solar radiation received was taken as PAR (Monteith, 1972). The amount of intercepted PAR by the crop (Sa) was calculated using the formula from Szeicz (1974): $Sa=Fi \times Si$

In which Fi is the fraction of radiation intercepted and Si is the total amount of incident PAR.

Total intercepted PAR was calculated as the sum daily intercepted PAR for the duration of the planting of the five accessions of sweet potato over the growing season.

Radiation Use Efficiency (RUE)

The RUE was calculated as the slope of the linear regression line between accumulated crop biomass and accumulated intercepted PAR. The regression line was forced through the origin based on the assumption that when accumulated intercepted PAR was zero, no DM was produced.

Statistical Analysis

Data of tuber yield, CGR, LAI, PAR and RUE were analyzed using analysis of variance and means separation between

accessions was determined by least significant difference (LSD) at the $p < 0.05$. Correlation analysis between TDM yield and CGR was also performed to evaluate their relationship.

RESULTS AND DISCUSSION

Maximum Tuber Yield

Among the accessions evaluated, Accession 6 had the highest maximum tuber yield at 34,563 kg ha^{-1}, followed by Accession 5 at 22,900 kg ha^{-1}, Accession 2 at 22,031 kg ha^{-1}, and Accession 1 at 21,094 kg ha^{-1} (Figure 1). The lowest maximum tuber yield was from Accession 3 at 9331 kg ha^{-1} while Accession 6 was significantly higher ($p < 0.01$) in maximum tuber yield compared with the other accessions. However, there was no significant difference among the other three accessions (1, 2, and 5) except for Accession 3.

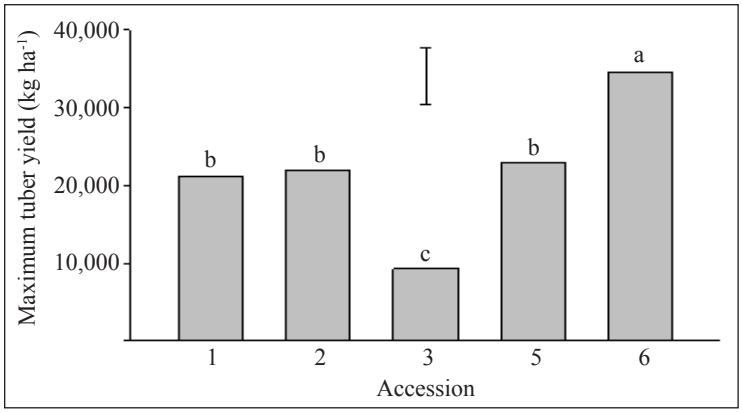


Figure 1. Maximum tuber yield of five accessions of purple sweet potato values with different letters are significantly different at $p < 0.05$

Crop Growth Rate

The pattern of TDM accumulation over time for the five accessions of purple sweet potato is shown in Figure 2. The curve fitting approximated the increase in dry matter yield over the growing period. The rapid accumulation of TDM could be seen from Accessions 6 compared with the other accessions. The highest CGR was indicated by Accession 6 with 199 kg ha^{-1} day^{-1} followed by Accession 2 with 117 kg ha^{-1} day^{-1} and Accession 1 with 106 kg ha^{-1} day^{-1}. While Accessions 3 and 5 gave the lowest CGR with 60 kg ha^{-1} day^{-1} and 82 kg ha^{-1} day^{-1}, respectively. However, no significant difference was observed among Accessions 1, 2, 3, and 5. Tuber yield is dependent on the rate of dry matter production and the distribution of dry matter within the plant (Watson, 1952). This was shown by accumulation of TDM (CGR) (Figure 2) that indicated the highest CGR was from Accession 6 with 199 kg ha^{-1} day^{-1} and the lowest CGR was from Accession 3 with 60 kg ha^{-1} day^{-1}. Van de Fliert and Braun (1999)

reported that the development cycle of sweet potato from crop establishment to harvest of the storage roots usually takes place in three phases (establishment, intermediate, and storage root bulking phase) within a time span of 90 to 120 days. The growth duration depends on the variety and environmental conditions. Accession 6 was found to form tuber earliest at 63 days after sowing (DAS) compared with Accession 3 at 78 DAS. The duration of tuber formation was considered slow since tuber could be formed as early as 4 weeks after planting. This was possibly due to the rainy season that occurred during the experimental period. The earlier formation of tuber yield in Accession 6 resulted in greater tuber yield compared to the other accessions. The variation in yield of the accessions was possibly due to genome differences among the accessions. Janssens (2001) reported that the differences in tuber yield was due to the differences in genetic constituents of genotypes of the different sweet potato accessions. This was in agreement with Ali, Wassu and Beneberu (2015) who reported that in 114 accessions evaluated, the variation in tuber yield of sweet potato might be due to genetic differences among accessions.

Although sweet potato requires moderate temperature and well-distributed rainfall for vigorous crop growth, the rainy season that had occurred during planting affected the soil texture making it compact and wet, and tended to retard tuber formation and resulted in reduced yields as suggested by Watanabe, Ozaki and Yashiki (1968). In addition, the lower maximum tuber yield of all purple sweet potato accessions except Accession 6 was due to the plants had been attacked by army worms (*Spodoptera litura*), red cotton bugs (*Dysdercus cingulatus*), and sweet potato weevils (*Cylas formicarius*). Vines and tuber were also attacked by root knot (*Meloidogyne* spp.) and *Rotylenchulus reniformis*.

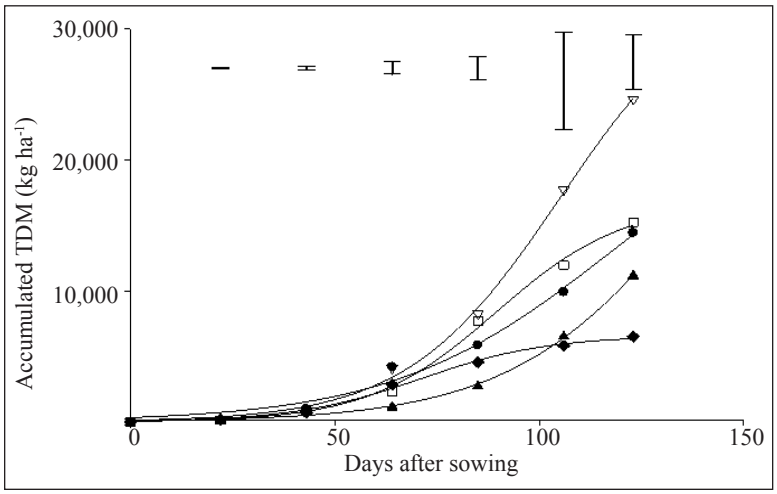
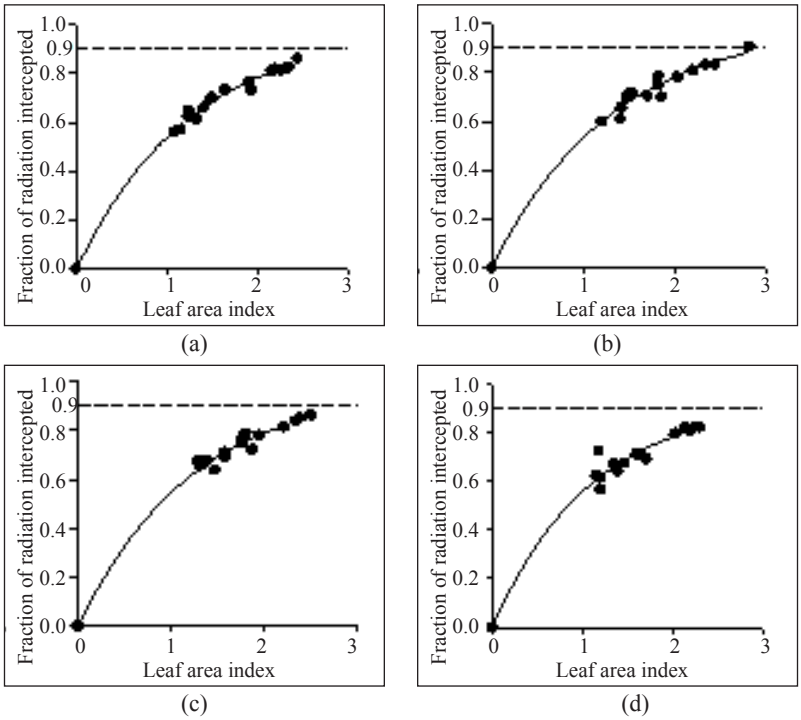
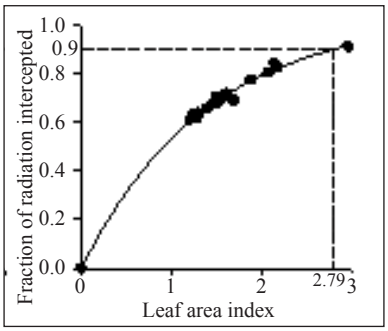


Figure 2. Accumulated total dry matter (TDM) for Accession 1 (●), Accession 2 (□), Accession 3 (◆), Accession 5 (▽), and Accession 6 (▲) of purple sweet potato.

Intercepted Radiation and Leaf Area Index (LAI)

The LAI is one of the important measurements used to determine the light interception in any crops. Light interception and photosynthesis rate are closely related to LAI (Hay & Porter, 2006). The relationship between the fraction of radiation intercepted and LAI for each accession is shown in Figure 3. From the exponential curve in Figures 3(a–e), the critical LAI (LAI_{crit}) at which 90% of intercepted PAR was calculated to be 2.79 for Accession 6 (Figure 3(e)). However, in the other accessions, radiation interception did not reach 90%, (Figures 3(a–d)), which means that they did not reach the LAI_{crit}. The LAI_{crit} for Accession 6 was achieved at 63 days after sowing (Figure 4(e)) and the other accessions could not be identified as they did not achieve the LAI_{crit} (Figures 4(a-d)). Accession 6 had the highest maximum tuber yield also due to the crop had achieved the LAI_{crit} at 2.79 (Figures 3 and 4). Critical LAI has been defined as the LAI required to intercept 90% of the incoming radiation (Brougham, 1958). Despite the ability to achieve LAI_{cri} by Accession 6, the amount of radiation intercepted by this accession was lower than the other accessions. This indicated that the amount of radiation intercepted by the crop canopy was efficiently converted to dry matter as explained by its radiation use efficiency (RUE). Radiation use efficiency is defined as crop biomass produced per unit of total solar radiation or PAR intercepted by the canopy (Monteith, 1977). Accession 6 had the highest RUE compared with the other accessions.





(e)

Figure 3. The relationship between the fractions of radiation intercepted against leaf area index of: (a) Accession 1; (b) Accession 2; (c) Accession 3; (d) Accession 5; and (e) Accession 6 of purple sweet potato

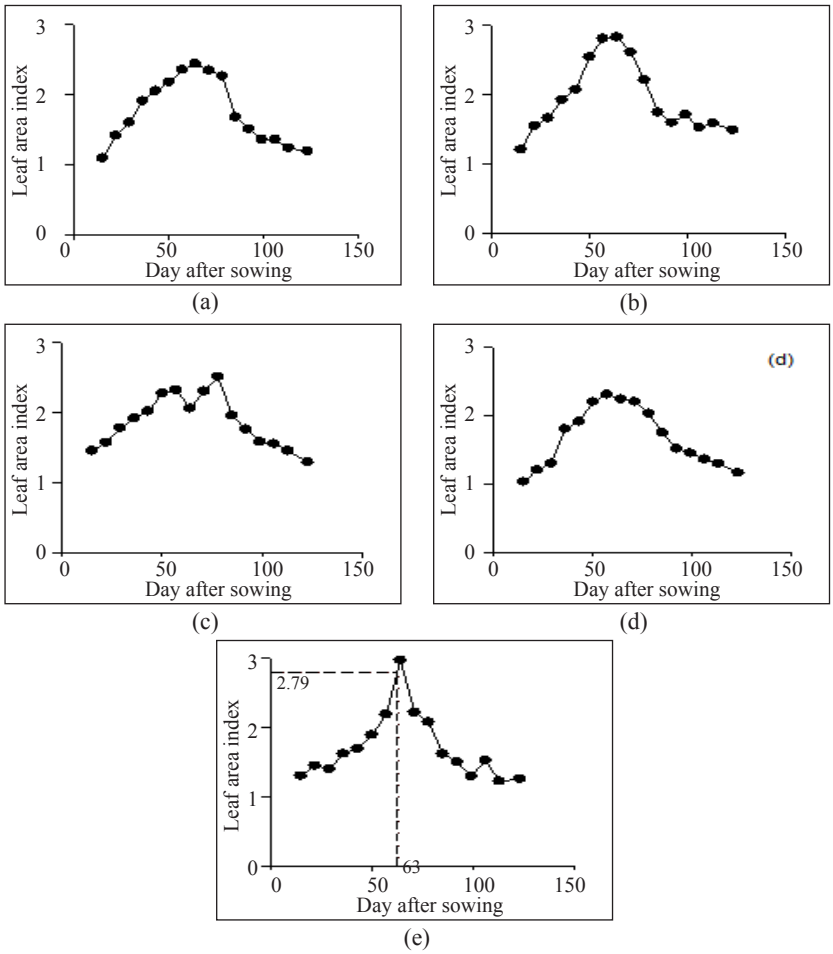


Figure 4. The relationship between the leaf area index against days after sowing of: (a) Accession 1; (b) Accession 2; (c) Accession 3; (d) Accession 5; and (e) Accession 6 of purple sweet potato

Total Intercepted Photosynthetically Active Radiation (PAR) and Radiation Use Efficiency (RUE)

Based on the analysis of variance, indicated significant differences among accessions in total intercepted PAR and RUE. Table II shows the highest total intercepted PAR was recorded for Accession 3 with 340 MJ m^{-2}; however, there was no significant difference among Accessions 1 (320 MJ m^{-2}), 2 (334 MJ m^{-2}), and 5 (324 MJ m^{-2}) except with Accession 6 (309 MJ m^{-2}). Accession 6 showed the lowest total intercepted PAR. In contrast, the highest RUE was obtained by Accession 6 with 7.58 g MJ^{-1}. However, there was no significant difference in RUE among the other accessions (Table II). This finding is in agreement with Stutzel, Aufhammer and Lober (1994) who stated that RUE differed from cultivars and species. In contrast, Pilbeam, Hebblethwaite, Nyongesa and Ricketts (1991) reported that the differences in total DM yield of grain legumes were due to the differences in both RUE and the amount of light intercepted by crops.

Table 2
Total intercepted photosynthetically active radiation (PAR) and radiation use efficiency (RUE) of five accessions of purple sweet potato

Accession	Total intercepted PAR (MJ m^{-2})	RUE (g DM MJ^{-1})
1	320^{ab}	4.08^{b}
2	334^{a}	4.36^{b}
3	340^{a}	2.16^{b}
5	324^{ab}	3.12^{b}
6	309^{b}	7.58^{a}

Means with different letters are significantly different at $p < 0.05$

Correlation Coefficient between CGR and Yield

There was a significant positive correlation between CGR and yield (Table 3). This indicated that when CGR increased, yield also increased.

Table 3
Correlation coefficient between CGR and yield

	Crop growth rate (kg ha^{-1} day^{-1})	TDM yield (kg ha^{-1})
Crop growth rate (kg ha-1 day-1)	--	0.9692***
TDM yield (kg ha-1)	0.9692***	

***Significant at $p \leq 0.001$

CONCLUSION

Based on tuber yield, CGR, and physiological parameters, Accession 6 has the highest potential among the accessions assessed. This information could form the critical criteria in assessing purple sweet potato accessions appropriate for colluvium soil. However, other information such as pest and disease tolerance and fertilizer requirement are needed to make final recommendation of the most suitable accession to be planted on colluvium soil.

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Pesticide and Heavy Metal Contamination: Potential Health Risks of Some Vegetables and Fruits from a Local Market and Family Farm in Ongkharak District of Nakhon Nayok Province, Thailand

Sirikul Thummajitsakul[1]*, Rawitsara Subsinsungnern[1], Ngamrat Treerassapanich[1], Nutthida Kunsanprasit[1], Leeyaporn Puttirat[1], Patarapong Kroeksakul[2] and Kun Silprasit[2]

[1]Faculty of Physical Therapy, Srinakharinwirot University, Ongkharak, Nakhon-Nayok, 10110 Bangkok, Thailand
[2]Faculty of Environmental Culture and Ecotourism, Srinakharinwirot University, 10110 Bangkok, Thailand

ABSTRACT

Organophosphate and carbamate pesticides were preliminarily determined in 33 vegetable samples from Ongkharak market (N = 13) and family farm (N = 20) in Sisa Krabue community at Ongkharak district of Nakhon Nayok province by a GT-test kit. More than 60% of samples contained pesticides, and eight samples had pesticides at harmful level, namely *Alpinia galanga* (Linn.) Swartz., *Coriandrum sativum* Linn., *Citrus aurantifolia* (Christm) Swing., *Ocimum sanctum* Linn., and *Carica papaya* Linn. from the Ongkharak market, and *Pandanus amaryllifolius* Roxb., *Ocimum sanctum* Linn., *Citrus aurantifolia* (Christm) Swing. from the family farm. Then, heavy metals (Cd, Pb, Fe, Cr, Zn, and Cu) were determined in eight samples by an atomic absorption spectroscopy. Our new finding showed that Pb level exceeded permissible limit in *O. sanctum* Linn., *C. aurantifolia* (Christm) Swing., *C. papaya* Linn., purchased from the local market, and *C. aurantifolia* (Christm) Swing. and *O. sanctum* Linn. from the family farm. In addition, all samples had Fe and Cr levels above permissible limits, but Zn and Cu levels were below permissible limits. Moreover, 100.0%, 62.5%, 37.5%, 37.5%, and 25.0% of the analyzed samples had target hazard quotients above 1 for Cr, Pb, Cu, Fe, and Zn, respectively, which

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E-mail addresses:
sirikul.thum@gmail.com (Sirikul Thummajitsakul)
kokdokdak@hotmail.com (Rawitsara Subsinsungnern)
nun.chh@gmail.com (Ngamrat Treerassapanich)
may_abmf@hotmail.com (Nutthida Kunsanprasit)
Leeyapornp@gmail.com (Leeyaporn Puttirat)
patarapong@g.swu.ac.th (Patarapong Kroeksakul)
kun.silprasit@gmail.com (Kun Silprasit)
* Corresponding author

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indicated potential health risks involving unsafe consumption from each heavy metal. Hazard index also indicated the health risks for female and male via consumption of each vegetable contaminated with multiple heavy metals. *O. sanctum* Linn., especially from the local market, had the highest risk.

Keywords: Family farm, health risks, heavy metals, local vegetables, market

INTRODUCTION

High-quality foods are minimally processed foods such as vegetables and fruits that contain essential nutrients (i.e., vitamins and minerals) needed at low concentration for several activities in human body. Although, metals are naturally found in vegetables, exceeding permissible limits of them can be harmful to human health (Guerra, Trevizam, Muraoka, Marcante, & Canniatti-Brazaca, 2012). They can transfer from soils to vegetables due to human activities, and accumulation of several heavy metals in rural and urban soils at excessive level can cause serious health problems of human (Chao, Xiao-Chen, Li-Min, Pei-Fang, & Zhi-Yong, 2007; Szynkowska, Pawlaczyk, Leśniewska, & Paryjczak, 2009). Using pesticides are major factors in contamination of heavy metals in exposed vegetables, such as Hg, Mn, Cu, Pb, or Zn (Hakeem, Sabir, Ozturk, & Mermut, 2015). Heavy metals can affect metabolic functions in human body by accumulation and functional disruption in organs and glands (i.e., kidneys, heart, brain, bone, and liver) including metabolic components (i.e., genetic materials and enzymes; Singh, Gautam, Mishra, & Gupta, 2011). Moreover, human health concerns, affected by heavy metals, can be indicated by target hazard quotients (THQs) and hazard index (HI). These parameters are used to evaluate the potential health risks in consumption of vegetables, exposed with heavy metals in long term (Guerra et al., 2012).

Several reports involving heavy metal contents in vegetables and human health risks have been represented. For examples, heavy metals (Fe, Mn, Zn, Cu, Ni, Cd, and Pb) have been found in common vegetables (parsley, carrot, onion, lettuce, cucumber, and green beans), and THQ of each heavy metal indicates potential health risk from consumption of the exposed vegetables for male and female (Harmanescu, Alda, Bordean, Gogoasa, & Gergen, 2011). In addition, toxic heavy metals (As, Cd, Pb, Cr, Mn, Ni, Cu, and Zn) are found in fruits and vegetables, and THQ indicates health risks of Mn and Cu through consumption of the investigated vegetables (Shaheen et al., 2016).

In Thailand, pesticides are commonly used by farmers that may lead to higher human health effects even though they have benefits in increasing agricultural products. Therefore, it causes a severe health risk in Thai people who consume the exposed vegetables and fruits. This had been reported by Wachirawongsakorn (2016) that more than 80% of vegetables from fresh market in the lower north of Thailand were contaminated with Pb and Cd above the maximum allowable concentration, and THQ indicated potential

health risk associated with consumption of the exposed vegetables for local Thai people. However, knowledge of health risks and surveillance of heavy metals in native vegetables is still limited in local areas of Nakon Nayok province, especially in Ongkharak district. Therefore, this novel knowledge is important for health promotion of human in the rural areas of developing country where they are never investigated. Therefore, our major goals were to detect a level of organophosphate and carbamate contamination, and to evaluate heavy metal contents in local vegetable samples, which related to the pesticide contamination at harmful level, from Ongkharak market and family farm in Sisa Krabue community of Ongkharak district, Nakhon Nayok province. In addition, THQs and HI were calculated and used to predict the potential risks of human health from intake of the vegetables contaminated with heavy metals (Cd, Pb, Fe, Cr, Zn, and Cu) in targeted areas.

MATERIALS AND METHODS

Sample Collection

The number of vegetable samples were obtained from Ongkharak market (N = 13) and family farm (N = 20) in Srisakrabue community, Ongkharak district in Nakhon Nayok province since October 2016 until April 2017. Seven vegetables (*Vigna unguiculata* (L.) Walp., *Eryngium foetidum* Linn., *Ocimum sanctum* Linn., *Cymbopogon citratus* (DC.) Stapf., *Alpinia galanga* (Linn.) Swartz., *Carica papaya* Linn., and *Citrus aurantifolia* (Christm) Swing.) were collected in duplicate, and different 19 vegetable types were randomly chosen. Parts of unpeeled vegetables were divided into leaves, roots, rhizomes, fruits, flowers, stem, pods, and seeds, and rinsed with deionized water, then kept at 4°C until used. Name, code, and part of each sample used in this study are shown in Table 1.

Table 1
Types of vegetables used for pesticide detection, parts of vegetables used in our study and levels of the pesticide contamination

Common name	Botanical names	Sample codes	Parts used	Sampling Area	Levels of the pesticide contamination
Yard long bean	*Vigna unguiculata* (L.) Walp.	VIW1	Pods and seeds	Ongkharak market	Not contaminated***
Bird chilli	*Capsicum flutescens* Linn.	CAF	Fruits and seeds	Ongkharak market	Not contaminated***
Culantro	*Eryngium foetidum* Linn.	ERF1	Leaves	Ongkharak market	Safe level**
White holy basil	*Ocimum sanctum* Linn.	OCS1	Leaves	Ongkharak market	Unsafe level*
Lemon grass	*Cymbopogon citratus* (DC.) Stapf.	CYC1	Leaves	Ongkharak market	Safe level**

Table 1 *(continue)*

Common name	Botanical names	Sample codes	Parts used	Sampling Area	Levels of the pesticide contamination
Cabbage	*Brassica oleracea* L. var. capitata	BRO	Leaves	Ongkharak market	Safe level**
Corainder	*Coriandrum sativum* Linn.	COS	Leaves	Ongkharak market	Unsafe level*
Galangal	*Alpinia galanga* (Linn.) Swartz.	ALG1	Rhizomes	Ongkharak market	Unsafe level*
Black pepper	*Piper nigrum* Linn.	PIN	Seeds	Ongkharak market	Safe level**
Carrot	*Daucus carota* Linn.	DAC	Roots	Ongkharak market	Not contaminated***
Cucumber	*Cucumis sativus* Linn.	CUS	Fruits	Ongkharak market	Safe level**
Papaya	*Carica papaya* Linn.	CAP1	Fruits	Ongkharak market	Unsafe level*
Lime	*Citrus aurantifolia* (Christm) Swing.	CIA1	Fruits	Ongkharak market	Unsafe level*
Leech lime	*Citrus hystrix* DC.	CIH	Fruits	Family farm	Safe level**
Pandan leaves	*Pandanus amaryllifolius* Roxb.	PAA	Leaves	Family farm	Unsafe level*
Wildbetal leafbush	*Piper sarmentosum* Roxb.	PIS	Leaves	Family farm	Safe level**
Tamarind	*Tamarindus indica* Linn.	TAI	Leaves	Family farm	Safe level**
Lime	*Citrus aurantifolia* (Christm) Swing.	CIA2	Fruits	Family farm	Unsafe level*
Cork wood tree	*Sesbania grandiflora* Desv.	SEG	Leaves and flowers	Family farm	Safe level**
Bitter cucumber	*Momordica charantia* Linn.	MOC	Leaves	Family farm	Safe level**
Galangal	*Alpinia galanga* (Linn.) Swartz.	ALG2	Rhizomes	Family farm	Safe level**
Papaya	*Carica papaya* Linn.	CAP2	Fruits	Family farm	Safe level**
Lemon grass	*Cymbopogon citratus* (DC.) Stapf.	CYC2	Leaves	Family farm	Safe level**
Brazilian pepper tree	*Schinus terebinthifolius* Raddi.	SCT	Leaves	Family farm	Safe level**
White holy basil	*Ocimum sanctum* Linn.	OCS2	Leaves	Family farm	Unsafe level*

Table 1 *(continue)*

Common name	Botanical names	Sample codes	Parts used	Sampling Area	Levels of the pesticide contamination
Yard long bean	*Vigna unguiculata (*L.) Walp.	VIW2	Pods and seeds	Family farm	Safe level**
Ivy gourd	*Coccinia grandis* Voigt.	COG	Leaves	Family farm	Safe level**
Tomato	*Lycopersicon esculentum* Mill.	LYE	Fruits	Family farm	Safe level**
Water morning glory	*Ipomoea aquatica* Forsk.	IPA	Leaves and stem	Family farm	Safe level**
Culantro	*Eryngium foetidum* Linn.	ERF2	Leaves	Family farm	Safe level**
Velvet bean	*Mucuna pruriens* (L.) DC. Var. utilis	MUP	Pods and seeds	Family farm	Safe level**
Red holy basil	*Ocimum sanctum* Linn.	OCT	Leaves	Family farm	Safe level**
Asiatic pennywort	*Centella asiatica* (Linn.) Urban.	CEA	Leaves	Family farm	Safe level**

* The pesticide contamination at unsafe level was identified by colour of the sample solution that appeared darker than colour of critical solution.
** The pesticide contamination at safe level was identified by colour of the sample solution appeared lighter than colour of critical solution, but darker than colour of control solution.
*** Sample without pesticide contamination was identified by colour of the sample solution appeared lighter than or like colour of the control solution.

Determination of Organophosphate and Carbamate Pesticides

Organophosphate and carbamate pesticides were preliminarily determined in 33 vegetable samples using GT-pesticide residual test kit (Higher Enterprises Co., LTD., Thailand) according to the method of Thoophom (1998). This method had 92.3% sensitivity, 85.1% specificity, 87.1% accuracy, 70.6% positive predictive value, and 96.6% negative predictive value for residual detection of pesticides in vegetables, and it is an easy, rapid, and low-cost way to detect the organophosphorus and carbamate pesticides in primary step. The parts of vegetables used in this study were shown in Table 1. Each fresh sample (5 g) was cut into small pieces and extracted by 5 mL of solvent No. 1 with mixing for 15 min. Next, each sample extract (1 mL) were added with 1 ml of solvent No. 2, then evaporated in an evaporation basin until all solvent No.1 residues were removed. After that, GT-1 solvent (0.50 mL) was added in each extract and incubated at room temperature for 10 min. Then, 0.25 mL of GT-2 solvent was added, followed by incubation at 37°C for 30 min, and mixing with 1 mL

of GT-3 solvent. Finally, GT-4 (0.5 mL) and GT-5 solvent (0.5 mL) were added to each reaction, respectively, then colour indication, ranging from a yellow through a dark brown of each sample solution, was compared to colour levels of control and critical solutions. The colours of sample solution indicated contamination levels of carbamate and organophosphate pesticides that they were classified into three levels according to percentage of cholinesterase inhibition: (1) If a sample solution is lighter than or like colour of the control solution, it will indicate that the pesticides are not detected in the sample; (2) If a colour of sample solution is lighter than colour of critical solution, but darker than colour of control solution, it will indicate that the pesticides are detected in the sample at a safe level for human consumption; and (3) If a colour of sample solution is darker than colour of critical solution, it will indicate that the pesticides are detected in the sample at an unsafe level.

Sample Preparation and Digestion

To determine heavy metals at unsafe level of pesticide contamination, parts of each vegetable or fruit were rinsed with deionized water and cut into small pieces for incubation at 50°C until dried. Then, each sample was grinded with a homogenizer and kept in room temperature until used.

Each powdered sample (0.5 g) was added with 70% HNO_3 for 10 mL, and digested at 100°C on a hot plate within a fume hood approximately for 1 h or until dried. Moreover, each residue was rinsed with 1% HNO_3, followed by sieving through Whatman No 1 paper, and transferring the supernatant into a 50-mL volumetric flask, and adding with 1% HNO_3 up to a 50-mL volume. Each reaction was performed in triplicate. Next, concentrations of heavy metals (Cd, Pb, Fe, Cr, Zn, and Cu) were determined in each digested sample by an atomic absorption spectrometry (Model 200 Series AA, Agilent Technologies, Malaysia). External standard method was done by dilution from standard solution at 1000 µg/mL stock of each heavy metal with 1% HNO_3, and linear standard calibration curves were generated to measure each heavy metal in individual sample. After that, each heavy metal content was compared to permissible limits found in food and vegetables according to Ministry of Public Health (1986) shown in Table 2.

Table 2
The permissible limits of each heavy metal in food and vegetables

Heavy metals	Concentration (mg/kg)
Cadmium (Cd)	3
Lead (Pb)	1
Iron (Fe)	20
Chromium (Cr)	2
Zinc (Zn)	100
Copper (Cu)	20

Target Hazard Quotients (THQ) and Hazard Index (HI)

Health risks of human from consumption of vegetables contaminated with heavy metals were estimated with a THQ and a hazard index (HI) or the sum of the hazard quotients

(Basim & Khoshnood, 2016; Guerra et al., 2012; Javed & Usmani, 2016; Wang, Qiao, Liu, & Zhu, 2012). If a THQ is below 1, it means that the exposed human is unlikely to undergo obvious risk. THQ was calculated using the following formula (1).

$$THQ = \frac{(E_F * E_D * F_{IR} * C) * 10^{-3}}{(RFD * WAB * TA)} \quad (1)$$

Where;

E_F is exposure frequency in consumption of heavy metals-contaminated vegetables (365 days/year)

E_D is exposure duration or the average lifetime of Thai people; 71.8 years for male and 78.6 years for female (Institute for Population and Social Research, 2016)

F_{IR} is food ingestion rate (kg/person/day) of Thai people; 268 g/person/day for male and 283 g/person/day for female (The Health Systems Research Institute, 2006).

C is metal concentration in vegetables or fruits (mg/kg)

R_{FD} is oral reference dose (mg/kg/day) (Cd,0.001 mg/kg/day; Pb, 0.0035 mg/kg/day; Fe, 0.7 mg/kg/day; Cr, 0.003 mg/kg/day; Zn, 0.300 mg/kg/day; Cu: 0.040 mg/kg/day) (Harmanescu et al., 2011; Chang et al., 2014).

W_{AB} is the average body weight for Thai people (68.83 kg for male and 57.40 kg for female) (Pentamwa, Sukton, Wongklom, & Pentamwa, 2013; Well et al., 2011)

T_A is the averaging exposure time for heavy metals (ED × 365 days/year)

Moreover, multiple heavy metals found in each sample can affect the risks of human health. Therefore, HI was used to determine the overall potential health risk effected by more than one heavy metal according to equation (2).

$$HI = THQ_{Cd} + THQ_{Pb} + THQ_{Fe} + THQ_{Cr} + THQ_{Zn} + THQ_{Cu} \quad (2)$$

Statistical Analysis

Number and proportions were used for explaining levels of pesticide contamination in samples. Moreover, one sample *Z* test was used to prove hypothesis that the proportion of vegetables contaminated with pesticides was more than 60% of total samples at significant level, $p < 0.05$. In addition, heavy metal contents, THQ, and HI were expressed with mean and SD. All statistical analyses were performed using PSPP program version 0.10.5 (Pfaff et al., 2013).

RESULTS AND DISCUSSION

Organophosphorus and carbamate compounds are commonly used as pesticides that are toxic to nervous system of human by inhibiting cholinesterase (ChE) enzymes (Meerdink, 1989). Nowadays, the pesticides are increasingly and commonly used in agricultural regions of Thailand to increase agricultural yields and respond to consumer needs (Chowdhury, Banik, Uddin, Moniruzzaman, Karim, & Gan, 2012). However, consumer perception in safe

foodstuffs is still restricted in Ongkharak district in Nakon Nayok province. Therefore, it is interesting to monitor organophosphate and carbamate pesticides contaminated in vegetable samples from the local market and the family farm in Srisakrabue community. The results showed 66.6%, 24.2% for pesticide contamination at safe and unsafe levels, and 9.1% for no pesticides, respectively. These results indicated that more than 60% of total samples were contaminated with organophosphorus and carbamate pesticides (Table 3). In addition, most samples from the local market (38.5% of total analyzed samples) were contaminated with the pesticides at harmful level higher than those from family farm (15% of total analyzed samples). Similarly, it has been reported that several vegetables (79.2%), namely yard long beans, chili peppers, cucumbers, and Chinese kale, are contaminated with the pesticides (Ponthas et al., 2014). Moreover, the organophosphate and carbamate compounds in soil, rice, and water samples from rice paddy fields in Nakhon Nayok province have been detected by a GT-test kit that provides percentages of pesticide contamination for 77.78%, 85.18%, and 70% of total samples, respectively (Thummajitsakul, Praditpol, Poolaoi, & Silprasit, 2015).

However, if a small amount of pesticides enters body through often eating or touching vegetables, it can be accumulated in the body and linked to more risks of human chronic diseases (Mostafalou & Abdollahi, 2013), especially people involved in pesticide exposure (i.e., consumers, farmers, and retailers). Corresponding to the report of Ponthas et al. (2014), 60% of fresh vegetable and fruit retailers in Ongkharak market have health risks from touching frequently organophosphate and carbamate pesticides in vegetables placed on sell in the local market.

Therefore, continuous monitoring of pesticides in vegetables is important for human health surveillance and pesticide applications. In our study, pesticides were found at harmful level in eight samples, namely *A. galanga* (Linn.) Swartz., *C. sativum* Linn., *C. aurantifolia* (Christm) Swing., *O. sanctum* Linn., and *C. papaya* Linn. from Ongkharak market, and *P. amaryllifolius* Roxb., *O. sanctum* Linn., and *C. aurantifolia* (Christm) Swing. from family farm (Table 3).

Table 3
Number and proportions of samples that were not contaminated and contaminated with pesticides, and one-sample z analysis for proportions of all samples contaminated with the pesticides by defining null hypothesis (H0) <60% and alternative hypothesis (H1) > 60%

Samples	Number (%)	Test Prop.	*P-value*
Not contaminated with pesticides	3 (9.1%)		
Contaminated with pesticides but safe to be consumed	22 (66.6%)	0.6	0.000
Contaminated with pesticides but not safe to be consumed	8 (24.2%)		

In previous report of Chiroma, Abdulkarim and Kefas (2007), organophosphorous pesticide, DELVAP 1000EC, could effect to increasing Cu, Cd, and Pb levels in leaves, stem, and roots of spinach, corresponding to the report of Dogheim, Ashraf, Alla, Khorshid and Fahmy (2004). Pesticides and heavy metals levels in Egyptian leafy vegetables and some aromatic medicinal plants. Food additives and contaminants (Dogheimg et al., 2004), leafy vegetables and some aromatic medicinal plants are contaminated with heavy metals (Pb, Cu, and Cd) above the maximum limits. In addition, organophosphorous pesticides (malathion and profenofos) are mostly found for 203 and 131 from 391 samples, respectively (Dogheim et al., 2004). Moreover, heavy metals (Pb, Cd, Cu, and Zn) and insecticides have been found in soil and plant products (Marković, Cupać, Đurović, Milinović, & Kljajić, 2010).

For our study, heavy metals (Cd, Pb, Fe, Cr, Zn, and Cu) in the samples was also evaluated across the unsafe level of the pesticide contamination. Our results revealed that Cd was not found in all samples. However, Pb, Fe, Cr, Zn, and Cu were found in some samples. Pb was found above the permissible limit in *O. sanctum* Linn., *C. aurantifolia* (Christm) Swing. and *C. papaya* Linn. purchased from the local market, and *C. aurantifolia* (Christm) Swing. and *O. sanctum* Linn. from the family farm ranging from 1.22-8.31 mg/ kg dry weight. Fe, Cr, and Zn were found in all analyzed samples ranging from 20.53-287.90, 6.87-13.30, and 5.23-64.77 mg/kg dry weight, respectively. These samples had Fe and Cr above permissible limits except for Zn. In addition, Cu was found in *A. galanga* (Linn.) Swartz., *C. sativum* Linn., and *O. sanctum* Linn. from the local market, and *P. amaryllifolius* Roxb. and *O. sanctum* Linn. from the family farm ranging between 7.43 and 15.67 mg/ kg dry weight that were also below the permissible limit (Table 4).

Table 4
Heavy metal contents of eight vegetables contaminated with the pesticides at unsafe level in mg/kg dry weight analyzed by the atomic absorption spectroscopy

Sample codes	Heavy metal content (Mean ± SD in mg/kg dry weight)					
	Cd	**Pb**	**Fe**	**Cr**	**Zn**	**Cu**
ALG1	*ND	*ND	41.20 ± 17.60	6.87 ± 2.36	26.43 ± 0.12	1.43 ± 0.38
COS	*ND	*ND	287.90 ± 8.25	12.73 ± 0.71	36.17 ± 4.10	10.30 ± 0.44
CIA1	*ND	6.48 ± 8.50	21.37 ± 2.20	7.80 ± 1.39	5.23 ± 1.55	*ND
OCS1	*ND	8.31 ± 0.40	213.27 ± 115.95	13.30±1.35	64.77 ± 3.50	12.40 ± 0.5
CAP1	*ND	6.11 ± 8.31	31.83 ± 4.43	6.63±0.47	8.27 ± 1.40	*ND
PAA	*ND	*ND	156.70 ± 56.57	8.17±2.40	22.53 ± 10.02	7.43 ± 0.86
OCS2	*ND	1.22 ± 0.06	125.90 ± 49.75	12.97±0.60	63.93 ± 8.70	15.67 ± 3.67
CIA2	*ND	3.04 ± 3.59	20.53 ± 4.13	5.70 ± 2.08	9.23 ± 2.70	*ND

Note: *ND: Below detection limit. Each reaction was done in triplicate.

The content of each heavy metal varies in vegetables depending on several factors such as atmospheric depositions, sampling areas, plant species and parts, metal forms, and transferring the metals from soils to vegetables (Chao et al., 2007; Sharma, Agrawal, & Marshall, 2008; Xian, 1989). In our result, Zn was the most abundant element in each investigated vegetable when compared with other heavy metals. This may involve absorption and translocation of each heavy metal from roots to shoots of vegetables, and it has been reported that Zn shows the strongest ability involving translocation from soils to vegetables (Chao et al., 2007). Moreover, correlation patterns among levels of metals and pesticides in soil have been reported that pesticides may either help to stabilize heavy metal or to degrade pesticides in soil. Acetamiprid is strongly positively associated with Cu, and negatively associated with Cr, and imidacloprid is negatively associated with Ni (Tariq, Shafiq, & Chotana, 2016).

In this study, the exposed sources of heavy metals in the vegetables and fruits were from Ongkharak market, a distribution local source of fresh-vegetables obtained from agricultural areas through Thailand, and family farm in Sisa Krabue community in Ongkharak district of Nakhon Nayok province where it was a cultivated area for consumption in family. Therefore, pesticide usage in the agricultural areas is a major factor involving the metal exposure in the vegetables. Although organic family farms are processed, they can be influenced from nearby agricultural areas where pesticides are used. However, several heavy metals are essential nutrients for plant growth at low concentration, but concentrations exceeding the permissible limits of them become toxic (Rengel, 1999).

Thus, each heavy metal content in samples was conducted to calculate THQ that was a parameter to determine potential risks of human health in long-term exposure to each heavy metal. In present study, THQ value was used to evaluate the potential health risk of each heavy metal found in each vegetable. The result showed individually obvious risk in each heavy metal except for Cd. The 87.5%, 68.8%, 62.5%, 37.5%, and 0% of total samples showed THQ below 1 for Zn, Fe, Cu, Pb, and Cr, respectively (Table 5). It indicated that most vegetables can be consumed safely from Zn, Fe, and Cu except for Pb and Cr that most samples had THQ >1. For male, the THQ values for Pb, Fe, Cr, Zn and Cu ranged from 1.35-9.23, from 0.11-1.19, from 7.40-17.26, from 0.07-0.84 and from 0.14-1.53, respectively. For female, THQ of Pb, Fe, Cr, Zn, and Cu ranged from 1.71-11.70, from 0.14-2.03, from 9.37-21.86, from 0.09-1.06, from 0.18-1.93, respectively. The sequence of THQ for male and female was Cr > Pb > Cu> Fe> Zn, respectively (Table 6).

Although the THQ values of each heavy metal showed the same sequence for both, they were higher for female. This is possible because of the differences between male and female in average lifetime, vegetable ingestion rate, and average body weight. Therefore, THQ should be calculated separately for adult male and female. In

Table 5
Percentages of vegetables that were found below and above the permissible limit of THQ for each heavy metal

	% of vegetables					
	Cd	**Pb**	**Fe**	**Cr**	**Zn**	**Cu**
THQ<1	100.0%	37.5%	62.5%	0.0%	75.0%	62.5%
THQ≥1	0.0%	62.5%	37.5%	100.0%	25.0%	37.5%
Total	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Table 6
Target hazard quotients (THQ) and hazard indexes for the eight vegetables with the contamination of heavy metals for male and female adults (ages >15 years)

	Samples	**THQ (mean ± SD)**						**Hazard Index (mean ± SD)**
		Cd	**Pb**	**Fe**	**Cr**	**Zn**	**Cu**	
Male	ALG1	ND*	ND*	0.22 ± 0.10	8.91 ± 3.07	0.34 ± 0.00	0.14 ± 0.04	10.09 ± 3.34
	COS	ND*	ND*	1.60 ± 0.04	16.53 ± 0.92	0.47 ± 0.05	1.00 ± 0.04	21.58 ± 2.83
	CIA1	ND*	7.21 ± 9.46	0.12 ± 0.01	10.12 ± 1.80	0.07±0.02	ND*	17.53 ± 10.56
	OCS1	ND*	9.23 ± 0.44	1.19 ± 0.64	17.26 ± 1.74	0.84 ± 0.05	1.21 ± 0.05	26.66 ± 6.46
	CAP1	ND*	6.80±9.24	0.18 ± 0.02	8.61 ± 0.61	0.11 ± 0.02	ND*	15.69 ± 9.65
	PAA	ND*	ND*	0.87 ± 0.31	10.60 ± 3.12	0.29± 0.13	0.72 ± 0.08	13.99 ± 1.68
	OCS2	ND*	1.35 ± 0.07	0.70 ± 0.28	16.83 ± 0.78	0.83 ± 0.11	1.53 ± 0.36	20.79 ± 0.46
	CIA2	ND*	3.38±4.00	0.11 ± 0.02	7.40 ± 2.70	0.12 ± 0.04	ND*	9.89 ± 5.22
Female	ALG1	ND*	ND*	0.29 ± 0.12	11.28 ± 3.88	0.43 ± 0.00	0.18±0.05	12.78±4.23
	COS	ND*	ND*	2.03 ± 0.06	20.93 ± 1.17	0.59 ± 0.07	1.27 ± 0.05	27.33 ± 3.59
	CIA1	ND*	9.13 ± 11.98	0.15 ± 0.02	12.82 ± 2.28	0.09 ± 0.03	ND*	22.19 ± 13.37
	OCS1	ND*	11.70 ± 0.57	1.50 ± 0.82	21.86 ± 2.22	1.06 ± 0.06	1.53±0.06	33.75 ± 8.18
	CAP1	ND*	8.61 ± 11.70	0.22 ± 0.03	10.90 ± 0.78	0.14 ± 0.02	ND*	19.87 ± 12.22
	PAA	ND*	ND*	1.10 ± 0.40	13.42 ± 3.95	0.37 ± 0.16	0.92 ± 0.11	17.72 ± 2.13
	OCS2	ND*	1.71 ± 0.09	0.89 ± 0.35	21.31 ± 0.99	1.05 ± 0.14	1.93 ± 0.45	26.32 ± 0.58
	CIA2	ND*	4.28 ± 5.06	0.14 ± 0.03	9.37 ± 3.42	0.15 ± 0.04	ND*	12.52 ± 6.61

addition, if THQ is above 1, it reflects the level of health concern in exposed people (Harmanescu et al., 2011).

However, more than one heavy metals in each vegetable can multiply to potential health risks of exposed people. Therefore, hazard index (HI) values of male and female, the sum of THQ of each heavy metal, were calculated. Similarly to THQ, HI should also not more than 1 (Javed & Usmani, 2016). In our results, HI values for male and female ranged from 9.89-26.66 and 12.52-27.33, respectively (Table 6). Although it indicated that HI was higher for female, human health could be affected via unsafe consumption of individual vegetable contaminated with multiple heavy metals. The sequence of the HI for male and female was *O. sanctum* Linn. (Ongkarak market) > *C. sativum* Linn. (Ongkarak market) > *O. sanctum* Linn.(family farm) > *C. aurantifolia* (Christm) Swing. (Ongkarak market) > *C. papaya* Linn. (Ongkarak market) > *P. amaryllifolius* Roxb. (family farm) > *A. galanga* (Linn.) Swartz. (Ongkarak market) > *C. aurantifolia* (Christm) Swing. (family farm), respectively.

Therefore, our new finding indicated that contamination of the pesticides in investigated vegetables may affect heavy metal contents in exposed vegetables. Furthermore, the new data involving human health risks and metal concentrations in local areas may be useful to promote human health and in decision about consumption of the vegetables in a local market or family farm. Furthermore, the major information can be used to manage environmental surveillance, awareness among farmers to grow safe vegetables for supply local markets, and health impact of human in the developing country.

CONCLUSION

Organophosphate and carbamate pesticides were investigated in 33 vegetable samples. More than 60% of total samples were contaminated with pesticides in organophosphorus and carbamate group. Then, this study monitored heavy metals in eight vegetables found on harmful level of the pesticide contamination, followed by THQ and HI calculation. Fe, Zn, Cr, Cu, and Pb were mostly found in the vegetables at high level, and most samples had heavy metals above the permissible limits. Furthermore, THQ provided information in safe consumption from Cd of all investigated vegetables, but there are still health risks of unsafe vegetable consumption from other heavy metals for both male and female. Moreover, human health risk from consumption of each vegetable contaminated with multiple heavy metals indicated that *O. sanctum* Linn. from the local market was the highest risk.

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Characterisation and Effect of Protectants on Preservation of *Bacillus methylotrophicus* UPMC 1166 Isolated from Liquid Biofertiliser

Musliyana Mansor[1], Tan Geok Hun[1,2*], Nor Umaira Abu Asan[1] and Raha Abdul Rahim[1,3]

[1]Microbial Culture Collection Unit, Institute of Bioscience, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[2]Faculty of Agriculture, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[3]Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia

ABSTRACT

UPMC 1166 bacterial strain was isolated from SG1 liquid biofertiliser and has proven to have an ability to produce indole acetic acid (IAA) and fixed atmosphere nitrogen. The objectives of this research were to characterise UPMC 1166 isolate, to determine the growth kinetics, and effect of different protectants for preservation. UPMC 1166 were characterised phenotypically and genotypically. The growth kinetics was determined using viable cell count and optical density methods. The effect of different protectants on the viability of UPMC 1166, subjected to freeze–drying and freezing at −80°C, was studied. UPMC 1166 belonged to Gram-positive bacteria (with the size of 0.49 – 0.52 × 1.56 – 2.34 µm), catalase positive, rod-shaped with the arrangement of single or paired bacilli, endospore forming and creamy white pigmentation colonies. Based on API biochemical test kit confirmed that UPMC 1166 was under the *Bacillus* genus. From BLAST, UPMC 1166 showed pairwise sequence similarity range of 99.0% and is closely related to *Bacillus siamensis*, *Bacillus amyloliquefaciens, Bacillus vallismortis*, *Bacillus subtilis, Bacillus mojavensis*, and *Bacillus atrophaeus.* 16S rRNA gene sequence used for phylogenetic tree analysis suggested that UPMC 1166 is *Bacillus methylotrophicus*. To obtain the maximum viability after preservation, it is important to harvest cells during the late logarithmic phase of growth and to choose a suitable protective agent. UPMC 1166 needs approximately 16

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E-mail addresses:
musliyana@upm.edu.my/musliyana@gmail.com (Musliyana Mansor)
geok_hun@upm.edu.my (Tan Geok Hun)
nor_umaira@upm.edu.my (Nor Umaira Abu Asan)
raha@upm.edu.my (Raha Abdul Rahim)
* Corresponding author

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h to reach the end of the logarithmic phase; consisting of a lag phase up to 2 h and the logarithmic growth that lasted up to 14 h before entering the stationary phase. During freeze–drying, the maximum protection for UPMC 1166 was achieved by using 10% skimmed milk with 1% sodium glutamate, and 5% trehalose. Maximum protection of cells during −80°C cryopreservation was achieved with 10% dimethyl sulfoxide (DMSO). A suitable selection of protectant seemed to be important to acquire maximum cells viability for long-term preservation. Resistance potential of bacterial strains toward preservation procedures is useful from a research and commercial point of view.

Keywords: Bacillus methylotrophicus, biofertiliser, freeze–drying, freezing, gram-positive bacteria, growth kinetics, logarithmic phase, preservation

INTRODUCTION

Farmers used organic fertilizers as nutrient supply before the introduction of chemical fertilizers. Due to the enhancement and rapid productivity of plant growth, farmers have depended on chemical fertilizers since World War II (Ward, 2009). Besides the important role of sustaining yields and ensuring level of adequate profit, a long-term application of chemical fertilizer unfortunately causes food safety issues, human health, economical factor, and environmental damages such as pollution (water, soil, and air), soil infertility, and disease outbreak (Othman & Jafari, 2014; Patil, Patil, & Pathade, 2012). Recently, the awareness regarding the safety issue of utilising chemical fertilizers has increased. Biofertiliser consists of living beneficial microorganisms that enhance plant growth by supplying phytohormones or increasing primary nutrients, and also acting as a complementary source for chemical fertilizers (Naher, Panhwar, Othman, Ismail, & Berahim, 2016). With the presence of natural microorganism, biofertiliser provides the supplements required by plants and serves to increase soil quality (Vessey, 2003).

Plant growth promoting rhizobacteria (PGPR) are characterized as a growth enhancer and plant roots colonist (Zahir, Arshad, & Frankenberge, 2003). The bacteria species of *Bacillus, Enterobacter, Pseudomonas*, and *Erwinia* are the largest group of PGPR (Grobelak, Napora, & Kacprzak, 2015). *Azospirillium, Azotobacter, Burkolderia, Rhizobium*, and *Serratia* are some other bacteria species that have successfully been commercialized (Chauhan, Bagyaraj, Selvakumar, & Sundaram, 2015). Other types of beneficial microorganisms used frequently as biofertiliser element are nitrogen fixers, and phosphorus solubiliser and potassium solubiliser, or a combination of moulds or fungi (Mohammadi & Sohrabi, 2012).

Methylotropic is the characteristic of bacteria that enables plants to grow in C1 compounds (such as methanol, methane, methylated amines). *Bacillus methylotrophicus* CBMB205^T is categorised as a methylotropic bacterium and has the ability to use methanol as a carbon source (Madhaiyan, Poonguzhali, Kwon, & Sa,

2010). Patent deposit strain, called *Bacillus methylotrophicus* UTM401 (CGMCC 5927), was isolated from sewage sludge (Anonymous 1, 2012). The strain was inoculated into an organic waste, fermented and used as biofertiliser that is environmental friendly, stable in viability, incurred low-cost production and showed significant yield on Chinese cabbage (Anonymous 1, 2012).

In microbiology, preservation techniques (whether it is short- or long-term preservation methods) are imperative. Different type of microorganisms was applied with varying methods of preservation to ensure the guarantee of optimal viability, survival, purity and storage of the strains (WFCC Guidelines, 2010). Usually, two different methods should be implemented for each strain for storage to minimise the possibility of strain loss. Some methods of storage to help decrease the risks of genetic change are: (1) freeze–drying; (2) ultra-low temperature (liquid nitrogen); (3) freezers (temperature of −140°C or lower) (WFCC Guidelines, 2010). During freezing, storage and thawing process of preservation, cryoprotectants (protective compound) act as a protection to microorganisms that could remove most of the multiple damage factors (Tedeschi & De Paoli, 2011; Uzunova-Doneva & Donev, 2005).

This experiment was carried out to characterize UPMC 1166 isolate, to determine the growth kinetics, and effect of different protectants for preservation. UPMC 1166 was isolated from a liquid biofertiliser product that was proven to have the ability as a nitrogen fixer and produce indole acetic acid (IAA) (Nazaruddin, 2014). Gram stain and API biochemical test kit were applied for characterisation process phenotypically. 16S ribosomal ribonucleic acid (rRNA) gene sequencing and construction of phylogenetic tree were used in order to identify and confirm the species of the strain genotypically. Pattern estimation of growth was obtained from the growth kinetics. The bacterial cells were in its most ideal state to be harvested during the late logarithmic phase before preservation, to acquire optimal cell viability upon recovery (ATCC Bacterial Culture Guide, 2015). The effect of various protectants on viability of UPMC 1166, subjected to freeze–drying and cryopreservation at −80°C, was studied.

METHODS

Phenotypic Characterisation

Bacterial isolate UPMC 1166 was isolated from SG1 liquid biofertiliser from Agricultural Department of Kelantan and the isolate was chosen for further study based on the ability to produce IAA and fixed nitrogen (Nazaruddin, 2014). During the experiments, the isolate was incubated for 18 to 24 hours at 30°C.

The colony morphology was studied by culturing the isolate on nutrient agar (Merck, Germany) and incubated at 30°C. Gram stain was performed by using a Gram staining kit (Merck, Germany) and the images were observed using light microscope (Leica, Germany). The observation of colony morphology and Gram stain was done after 24, 48, and 72 h of incubation. The

biochemical characteristics of the *Bacillus* genus were tested by using combination of API 50CH and API 20E system test kit (BioMérieux SA, France). The catalase activity was determined by dropping 40 to 50 µL of 3% hydrogen peroxide (H_2O_2) (Merck, Germany) onto the cultures and assessing bubble production was observed.

Identification of Bacterial Isolate using 16S rRNA Gene Sequencing

UPMC 1166 was cultured overnight in nutrient broth (Merck, Germany) for 18 to 24 h at 30°C and the genomic DNA was extracted using standard protocols with some modifications (Sambrook, Russell, & Maniatis, 2001). The 16S rRNA gene sequence was amplified from the genomic DNA by polymerase chain reaction (PCR) using thermal cycler (peqSTAR, Germany). The PCR for total volume of 25 µL per reaction was carried out as follows: deionized water (14 µL); 10× reaction buffer containing 15 mM MgCl2 (Lucigen, USA) (2.5 µL); 2.5 mM dNTP mix PCR Grade (Lucigen, USA)(2.0 µL); 100 pmol/µL forward universal primers (0.25 µL); 100 pmol/µL reverse universal primers (0.25 µL); 5 U/mL Taq Polymerase (Lucigen, USA) (0.5 µL) and genomic DNA as template (50 to 200 ng)(5 µL). The PCR was carried out using forward primer 8F (5′ GAG TTT GAT CCT GCT CAG 3′) and reverse primer 1492R (5′ GTT ACC TTG TTA CGA CTT 3′) (Tan & Nazaruddin, 2015). The thermal cycling conditions involved preheating at 94°C for 2 min, followed by denaturation (35 cycles at 94°C for 30 s); annealing (52°C for 30 s); extension (72°C for 1 min), and final elongation (72°C for 10 min). The PCR product was purified using HiYield™ Gel/PCR DNA Mini Kit (Real Biotech, Taiwan) and sent for sequencing (1st BASE Laboratories Sdn. Bhd.). Same forward and reverse primers were used for sequencing.

The sequences were aligned using BioEdit 7.2.4 (Hall, 1999) and ClustalW (Thompson, Higgins, & Gibson, 1994) software. The aligned partial 16S rRNA gene sequence (1403 bp) was compared with the genes from Basic Local Alignment Search Tool (BLAST), NCBI Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple alignments and phylogenetic tree were done using Mega 7.0 (Kumar Stecher, & Tamura, 2016). The phylogenetic tree was constructed with the following setting: analysis by standard neighbour-joining method (Saitou & Nei, 1987) and bootstraps of 1,000 replicates (Felsenstein, 1985).

Growth Kinetics

The growth kinetics were obtained by viable cell counting in colony forming unit (CFU) and optical density (OD) measurement of liquid culture at 600 nm (OD_{600}). The experiments were done using protocols by Pepper and Gerba (2004) with some modifications.

A loop of UPMC 1166 bacterial strain was aseptically transferred to 10-mL nutrient broth. The culture was incubated overnight at 30°C (Infors HT, Switzerland). Sixty microliters of the overnight culture was

inoculated in the flask containing 60 mL of nutrient broth. Experiment was started from 0 h with 1 mL of culture was used for the measurement of OD of the bacteria at 600 nm using spectrophotometer (CE 1021 Cecil, UK), and another 100 µL was removed aseptically for serial dilution (viable cell counting). The flask was then incubated with constant shaking at 200 rpm at 30°C. Every 2 h interval and up to 20 h, 100 µL of the culture suspension was removed for viable cell counting (CFU/mL) and 1 mL of culture was used for OD_{600}. As a control, 60 mL of nutrient broth without inoculation was also incubated.

For serial dilution, sterilized water was used in this study (Miyamoto-Shinohara, Sukenobe, Imaizumi, & Nakahara, 2008). Hundred microliters of bacterial suspension was transferred to 900 µL of sterilized water to obtain a dilution of 10^{-1}. Serial dilution was made starting from 10^{-1} to 10^{-6}, mixed well and 100 µL of each dilution was spread using hockey stick on the nutrient agar (Merck, Germany) in triplicates. All the nutrient agar plates were incubated at 30°C for overnight before counting. Regarded as being statistically reliable, the colonies between a range of 30 and 300 on the agar plate were counted (Hogg, 2005). By knowing the number of colonies, dilution factor and volume plated, the calculation of CFU/mL was done using the following equation (Ravishankar, 2004):

$$\text{CFU/mL} = \frac{\text{Number of colonies}}{\text{Dilution factor} \times \text{Volume plated}}$$

Protectants

The protectants used for this experiment can be referred in Table 1. Nutrient broth (Merck, Germany) was used as a control. Thirty percentage of stock solutions were prepared for all protectants [except for 10% (w/v) skimmed milk with 1% (w/v) sodium glutamate] and later were diluted with nutrient broth to obtain the required working concentration.

Table 1
Protectants used in this experiment

Group of Protectants	Protectants
Sugar	Trehalose (Sigma, USA), sucrose (Fisher Chemical, UK), glucose (Merck, Germany) and fructose (Sigma, USA). 5%, 10%, and 15% (w/v) of sugar solution were prepared for each
Combined protectants	10% (w/v) skimmed milk (Merck, Germany) added with 1% (w/v) sodium glutamate (Merck, Germany)
Triols	10% and 15% (v/v) glycerol (Fisher Chemical, UK)
Sulphoxides	5% and 10% (v/v) dimethylsulphoxide (DMSO) (QReC, New Zealand)

Sample Preparation

Sample preparation was carried out according to manual by ATCC Bacterial Culture Guide (2015) with some modifications. UPMC 1166 was grown in 60 mL nutrient broth at 30°C and harvested after 16 h of incubation. Three milliliters of culture suspensions were transferred into Falcon tubes (Fisher Scientific, USA) and centrifuged at 4000

rpm for 15 min at 10°C (5415 R Centrifuge, Eppendorf, Germany). Supernatants were discarded, and the pellet was suspended with the protectants.

Freeze–drying Technique

Freeze-drying was carried out according to protocol by Microbial Culture Collection Unit UPM (2009) and freeze-dryer (Virtis, USA) manufacturer's manual with some modifications. Two-hundred microliters of bacterial suspensions were placed into 1 mL pre-scored ampoules (Wheaton, USA). The ampoules were transferred to −20°C freezer (Liebherr, Germany) for 1 h and then to −80°C freezer (Sanyo, Japan) for another hour. The primary and secondary freeze–drying using freeze-dryer was carried out with a condenser at a temperature of −50°C and vacuum condition at 100 mTorr. The ampoules were sealed to maintain the vacuum condition. Tesla coil spark tester (Electro-Technic Products Inc, USA) was used to check the ampoules for vacuum leakage. The preserved cultures were recovered after 2 weeks of preservation by adding 0.2 mL of nutrient broth. Mixed well and let it stand for 15 min at room temperature to equilibrate. The suspensions were serially diluted with sterilized water and spread on nutrient agar plates.

Freezing at −80°C Technique

Cryopreservation technique was performed according to a manual by ATCC Bacterial Culture Guide (2015) with some modifications**.** Eight-hundred microliters of bacterial suspensions were placed into 1.8 mL cryotubes (Nalgene, USA). Caps were sealed with Parafilm and incubated at room temperature to equilibrate in the protectants for a minimum period of 15 min but no longer than 40 min. The cryotubes were frozen at −20°C freezer for 1 h before transferring to −80°C deep freezers. The recovery was made after 2 weeks of preservation. The cryotubes were warmed as quick as possible by thawing at water bath (30°C) for approximately 2 min or until all samples were melted. The suspensions were serially diluted with sterilized water and spread on nutrient agar plates.

Survival Rate Determination

Bacterial viable cell count was conducted for bacterial suspensions before and after preservation. Change in viability after preservation is expressed as survival rate. The survival rate was calculated as follows (Peiren et al., 2015):

$$\text{Survival rate (\%)} = \frac{\text{Log (CFU/mL) after preservation}}{\text{Log (CFU/mL) before preservation}} \times 100$$

RESULTS AND DISCUSSION

Phenotypic Characterisation

UPMC 1166 strain was deposited at Microbial Culture Collection Unit (UNiCC), Institute of Bioscience, Universiti Putra Malaysia. The morphology of UPMC 1166 was observed after 24, 48, and 72 h of incubation at 30°C. UPMC 1166 is a

Gram-positive bacterium with the size of 0.49 – 0.52 × 1.56 – 2.34 µm, rod-shaped with single or in pair bacilli arrangement (Figure 1). After 24 h of incubation, the size of the colonies were 3 to 5 mm. However, it expanded to 5 to 7 mm after 48 h of incubation. The isolate showed the formation of endospores during the 48 h observation. The colonies of UPMC 1166 had creamy white pigmentation, raised elevation, irregular form, bumpy and shiny appearance, undulate margin, translucent and gummy structure. UPMC 1166 showed production of bubbles during application of H_2O_2 indicating catalase positive. Morphological characteristics of UPMC 1166 such as Gram-positive bacterium, rod-shaped cell, catalase positive and exhibited with one endospore in one cell, showed similarities with characteristics of *Bacillus* species (Logan & Vos, 2015). The morphology of colonies for both within and between *Bacillus* species, surely with strong effect of medium component and other incubation environment, show a very broad range (Logan & Vos, 2015).

Biochemical test results from API 50 CHB and API 20E kit obtained from APIWEB (https://apiweb.biomerieux.com) showed a very good identification result up to *Bacillus* genus. UPMC 1166 was identified (closest matched species) as *Bacillus subtilis/amyloliquefaciens* with 99.9% similarity. Some of the biochemical test results for UPMC 1166 were compared with *Bacillus methylotrophicus* CBMB205^T (Madhaiyan et al., 2010) in Table 2.

Molecular Identification based on 16S rRNA Gene Sequence

From the BLAST results, UPMC 1166 was closely related to *Bacillus siamensis*, *Bacillus amyloliquefaciens, Bacillus vallismortis*, *Bacillus subtilis, Bacillus*

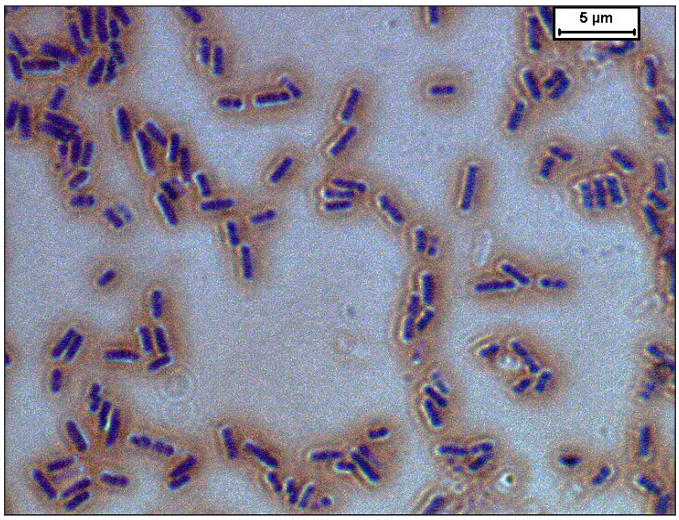

Figure 1. Gram-stain image of UPMC 1166. 24 h of incubation. 100× magnifications. Gram-positive, small size (0.49 – 0.52 × 1.56 – 2.34 µm), rod shape with single or in pair bacilli arrangement

mojavensis, and *Bacillus atrophaeus* showing pairwise sequence similarities up to 99.0%. UPMC 1166 results also revealed that the isolate was phylogenetically related to the genus *Bacillus* and showed a close relationship with *Bacillus methylotrophicus* (Figure 2). UPMC 1166 was grouped together and monophyletic with *Bacillus methylotrophicus* CBMB205^T and *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42^T with bootstrap value of 55%. *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42^T was recategorised as *Bacillus methylotrophicus* by Dunlap, Kim, Kwon and Rooney (2015) because results of phenotypic and genotypic analyses indicated that the strain showed highly similarity with *Bacillus methylotrophicus* CBMB205^T.

In this study, combination of API 50 CHB and API 20E biochemical test kit did not give the same species outcomes as the phylogenetic tree analysis, but produced good results up to the *Bacillus* genus level. This exhibited that the API system was not

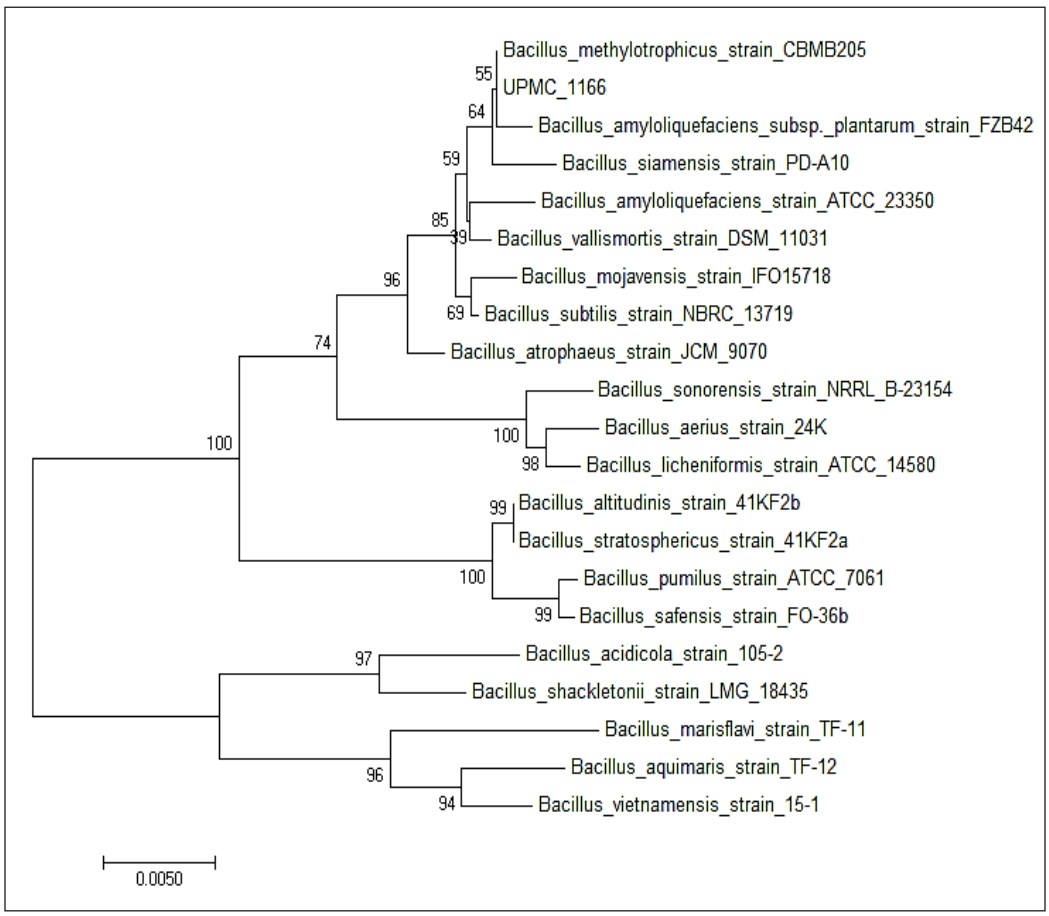

Figure 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence comparison for UPMC 1166 using Mega 7.0. Bar is 0.005 nucleotide substitution rate per site

a dependable test for characterisation up to species level. The commercial test kit databases that carried out for identification of bacterial species was limited, relying on the number of strains in the database and also the variety of phenotype of strains tested (Janda & Abbott, 2002). 16S rRNA gene sequencing has different advantages compared to phenotypic characterisation procedures such as results are not limited to a particular group of bacteria because public online database comprises the whole spectrum of phylogenetic diversity and novel species can be allocated to a group of related species (Bosshard et al., 2006).

Characteristics Comparison of Strain UPMC 1166 and *Bacillus methylotrophicus* CBMB205^T

The differential characteristic of strain UPMC 1166 and *Bacillus methylotrophicus* CBMB205^T are summarized in Table 2. From the previous research, the species of *Bacillus methylotrophicus* CBMB205^T was reported by Madhaiyan et al. (2010) and isolated from rice rhizoplane. UPMC 1166 and *Bacillus methylotrophicus* CBMB205^T showed similarity in some of the characteristics such as Gram-positive bacteria, arrangement that single or in-pair, endospore-forming, colonies with creamy

Table 2
Differential characteristics of isolate UPMC 1166 and Bacillus methylotrophicus *CBMB205^T*

Characteristic	UPMC 1166	*Bacillus methylotrophicus* CBMB205^T (Madhaiyan et al., 2010)
Gram results	Positive	Positive
Shape	Rods (0.49 – 0.52 × 1.56 – 2.34 μm)	Rods (0.63 - 0.64 × 1.8 – 2.7 μM)
Arrangement	Singly or in pairs	Singly or in pairs
Endospore-forming	Yes	Yes
Pigmentation	Creamy white	Creamy white
Motility	nd	Yes
Some biochemical test results:		
L-Arabinose	–	+
D-Mannose	–	+
L-Rhamnose	–	+
D-Sorbitol	–	+
N-Acetylglucosamine	–	+
Potassium gluconate	–	+
Trisodium citrate	–	+
Nitrate reduction to nitrite	–	+

Note. Both isolates were positive for catalase production. In biochemical tests, both taxa were positive for glycerol, D-ribose, D-glucose, inositol, D-mannitol, esculin ferric citrate, D-cellobiose, glycogen and gelatin. Both taxa were negative for L-fucose, potassium 2-ketogluconate, potassium 5-ketogluconate, arginine dihydrolase, urease and indole production. Data of UPMC 1166 were obtained from this research. +, growth; -ve, no growth; nd, no data.

white pigmentation, catalase positive and producing IAA. However, *Bacillus methylotrophicus* CBMB205^T produced a negligible amount of IAA (Madhaiyan et al., 2010).

Future work can be done to reconfirm the characteristic of the strains and obtain further information on other abilities such as morphology observation on motility, methanol-utilising ability, antagonistic activity, and so on.

Growth Kinetics

The growth kinetics results for UPMC 1166 were compared between viable cell quantification and OD_{600}. Both methods yielded similarity growth kinetics (Figure 3). The growth kinetics showed the lag phase up to 2 h, and the logarithmic growth lasted up to 14 h, which followed by entering the stationary phase. UPMC 1166 has the tendency to form clumps at 10th hour of incubation. Bacterial cells harvested during the late logarithmic phase were important to acquire optimal cell viability consequent to recovery after preservation process (ATCC Bacterial Culture Guide, 2015). During late logarithmic phase and entering early stationary phase, older cells grown in this phase would activate a starvation response that was an initiation of internal protection mechanism system against stress and develop an ability to survive under extremely critical environment (Morgan, Herman, White, & Vesey, 2006; Pletnev, Osterman, Sergiev, Bogdanov, & Dontsova, 2015).

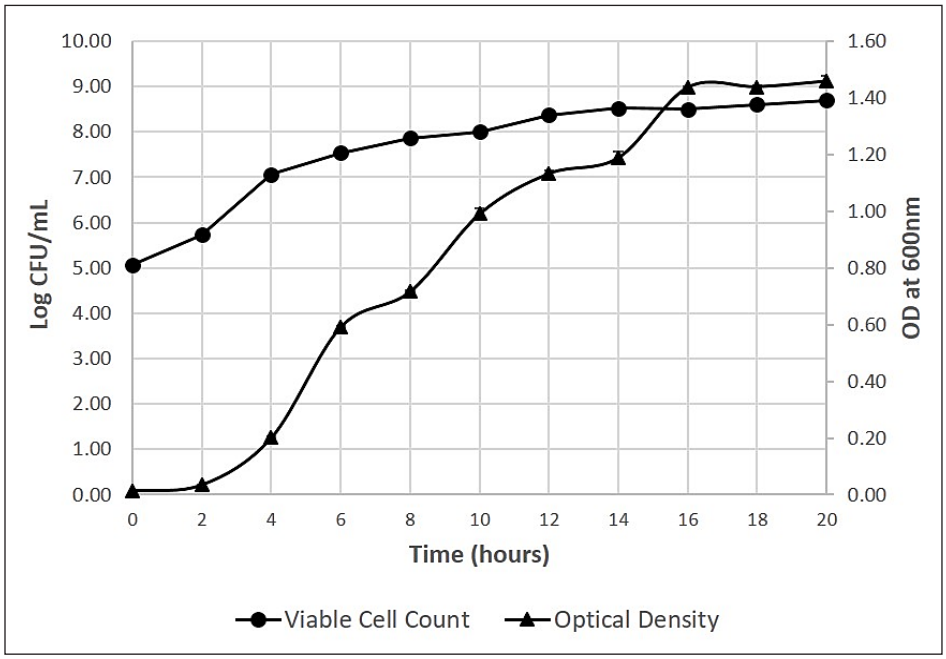

Figure 3. Bacillus methylotrophicus UPMC 1166 growth kinetics determined by viable cell count and optical density. The test was carried out using three replicates

Viable Cell Count before Preservation Process

For freeze–drying and freezing of UPMC 1166, 17 various protectants with different concentrations were used in this study. During the late logarithmic phase, the cell viability was approximately at 1×10^8 CFU/mL (Figure 3). Before preservation, the viable cell counts were carried out to obtain the results of cells' viability of the suspended cultures (Figures 4 and 5). The control (nutrient broth) showed the result of 8.31 log CFU/mL. All protectants showed cell viability of more than 8.0 log CFU/mL except for 5% (w/v) glucose, 15% (w/v) glucose, 5% (w/v) fructose, 10% (w/v) fructose, and 15% (w/v) fructose. Lowest cell viability with 7.19 log CFU/mL was obtained by 15% (w/v) fructose, while 15% (v/v) glycerol displayed the highest cell viability of 8.32 log CFU/mL. Before preservation, the cells recovery was greater if the initial number of cells is higher (Tedeschi & Paoli, 2011). Most of UPMC 1166 with mixture of protectants obtained viability of more than 8.0 log CFU/mL before preservation was possibly related to the ability of bacterial cell wall adapting to the protectants.

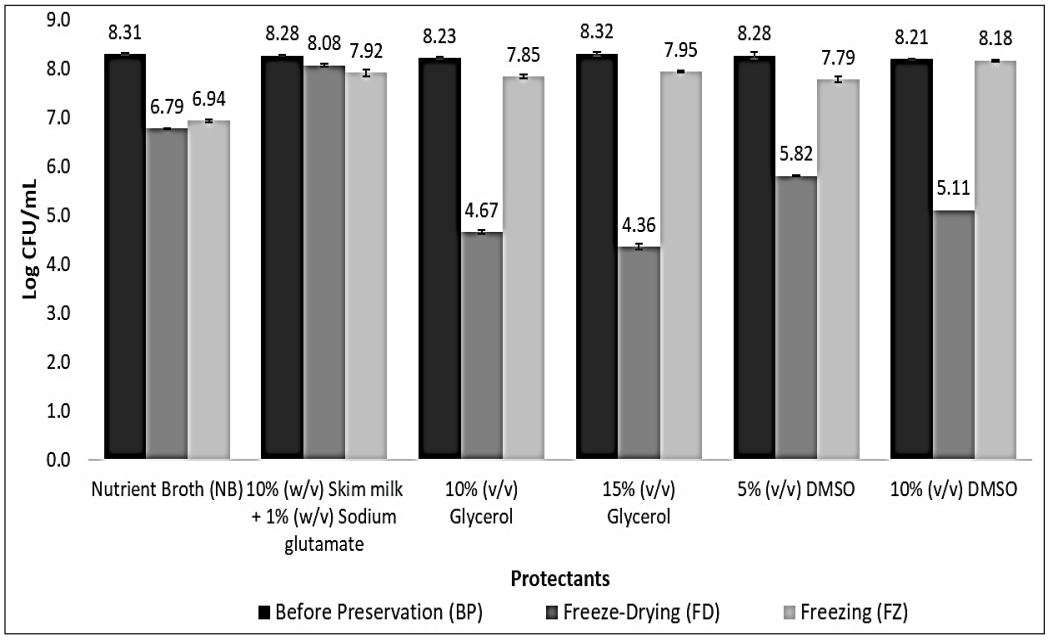

Figure 4. Effect of various protectants [10% (w/v) skimmed milk with 1% (w/v) sodium glutamate, glycerol and DMSO] on viability of *Bacillus methylotrophicus* UPMC 1166 preserved by freeze-drying and freezing technique. The test was done using three replicates

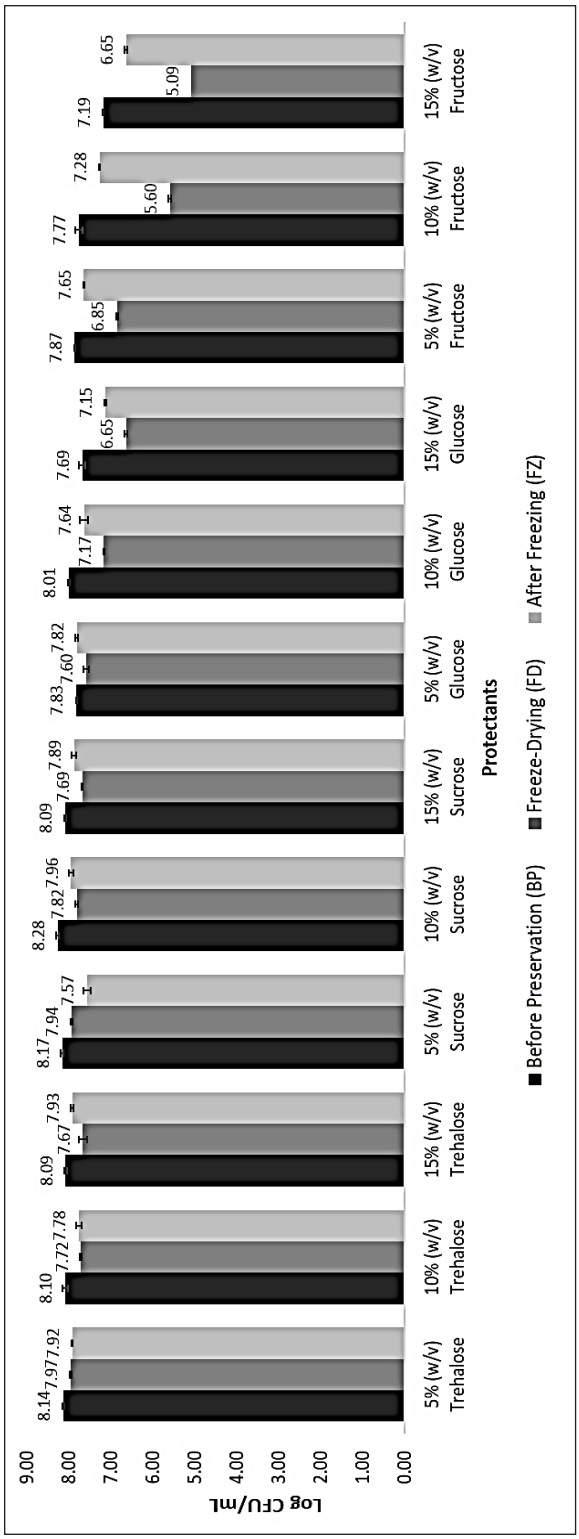

Figure 5. Effect of various protectants (sugar) on viability of *Bacillus methylotrophicus* UPMC 1166 preserved by freeze-drying and freezing technique. The test was done using three replicates

Effects of the Protectants on *Bacillus methylotrophicus* UPMC 1166 Viability Preserved by Freeze-drying and Freezing Method

Figures 6 and 7 showed the survival rate of UPMC 1166 toward various protectants after freeze–drying and freezing process. From this study, a maximum protection of UPMC 1166 during freeze–drying was achieved with 10% (w/v) skimmed milk with 1% (w/v) sodium glutamate and 5% (w/v) trehalose. Maximum protection of UPMC 1166 during freezing at −80°C was achieved with 10% (v/v) DMSO. DMSO at 10% (v/v) was proposed to be utilised as protectant in the future work for freezing process because it could maintain the viability of 8.0 log CFU/mL before freezing and gave 100% survival rate after freezing. Although 5% (w/v) glucose achieved 100% viability during freezing, it did not showed the viability of more than 8.0 log CFU/mL before preservation. The summary results of protectants' effect before and after preservation were obtained in the percentage of survival rate and log CFU/mL (refer Table 3).

After freeze–drying process for UPMC 1166, disaccharides (trehalose and sucrose) provided better viability than monosaccarides (glucose and fructose). Trehalose and sucrose were very good protective agents toward proteins and membranes in bacteria during freeze–drying (Leslie et al., 1995). When the cell wall was protected and preserved by the protective agent and also with its ability of intracellular trehalose uptake, critical intracellular macromolecules could possibly be protected from inside against injuries during the freezing and freeze–drying procedure (Peiren et al., 2015).

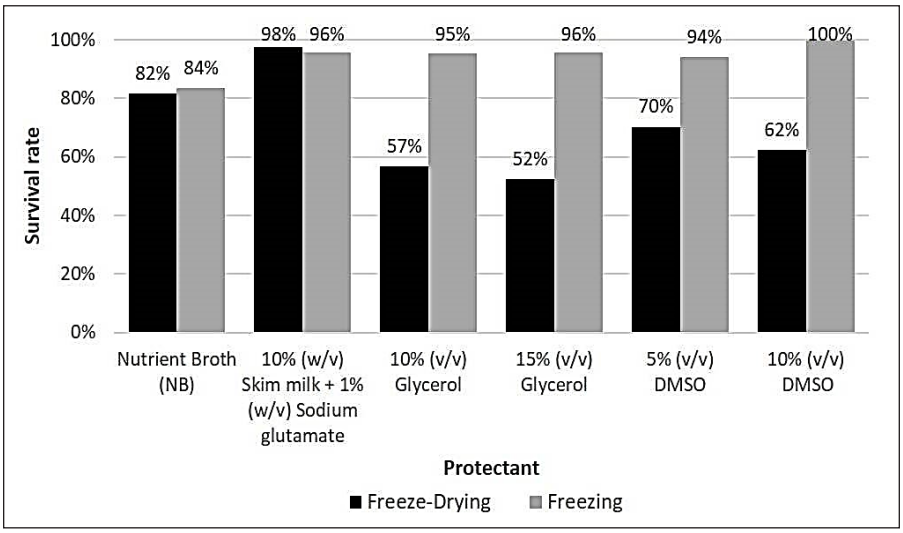

Figure 6. Effect of various protectants [10% (w/v) skimmed milk with 1% (w/v) sodium glutamate, glycerol and DMSO] on the survival rate of *Bacillus methylotrophicus* UPMC 1166 preserved by freeze–drying and freezing technique. The test was done using three replicates.

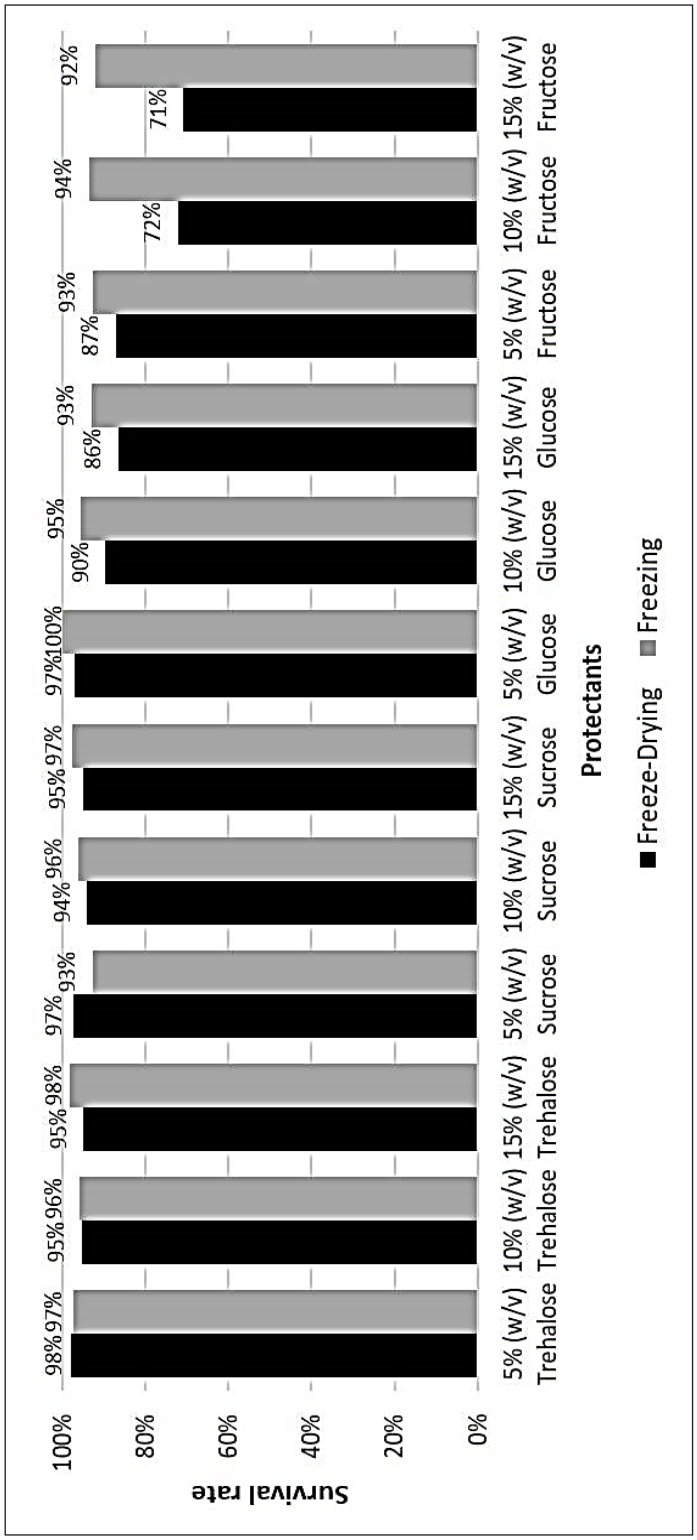

Figure 7. Effect of various protectants (sugar) on Survival Rate of *Bacillus methylotrophicus* UPMC 1166 preserved by freeze-drying and freezing technique. The test was carried out using three replicates

Table 3
Summary of preservation results for UPMC 1166

Observation	Results
Growth kinetics: Late logarithmic phase	16 hours
Before preservation:	
- Control (nutrient broth only)	8.31 log CFU/mL
- Protectants: Cell viability more than 8.0 log CFU/mL	All protectants except for except for 5% (w/v) glucose, 15% (w/v) glucose, 5% (w/v) fructose, 10% (w/v) fructose and 15% (w/v) fructose
- Protectants: Highest log CFU/mL	15% (v/v) glycerol (8.32 log CFU/mL)
- Protectants: Lowest log CFU/mL	15% (w/v) fructose (7.19 log CFU/mL)
Freeze-drying results:	
- Control (nutrient broth only)	82%
- Protectants: Survival rate more than 90%	All protectants except for 10% (v/v) glycerol, 15% (v/v) glycerol, 5% (v/v) DMSO, 10% (v/v) DMSO, 15% (w/v) glucose, 5% (w/v) fructose, 10% (w/v) fructose and 15% (w/v) fructose
- Protectants: Highest survival rate	10% (w/v) skimmed milk with 1% (w/v) sodium glutamate, and 5% (w/v) trehalose (98% survival rate)
- Protectants: Lowest survival rate	15% (v/v) glycerol (52% survival rate)
Freezing at −80°C results:	
- Survival rate of control (nutrient broth only)	84%
- Protectants: Survival rate more than 90%	All protectants showed survival rate of more than 90%
- Protectants: Highest survival rate	10% (v/v) DMSO and 5% (w/v) glucose (100% survival rate)
- Protectants: Lowest survival rate	15% (w/v) fructose (92% survival rate)

In this study, after the freeze–drying procedure, 10% (w/v) skimmed milk with 1% (w/v) sodium glutamate displayed a compact or small porous physical formation but DMSO and all sugars showed a large porous physical and glass-like formation. Both 10% and 15% (v/v) glycerol remained as a gel-like residue after freeze–drying procedure, which possibly influenced the viability of the cells as it gave the lowest survival rate after the freeze–drying procedure. The low survival rate of UPMC 1166 when glycerol was utilised as protectant was possibly due to freeze–thawed process before drying, or the cells were probably sensitive in the presence of unfrozen glycerol during the whole long procedure of primary and secondary drying. Ten percentage (v/v) and 15% (v/v) glycerol providing very good protection to freezing at −80°C but showed less protection to freeze–drying.

The connection between protectants's crystallization and their ability to protect bacterial cells during preservation was not investigated in this study. During the

initial freezing of the bacterial cell wall, ice crystals begin to develop and concentration of solute (osmotic stress) in the suspension increases (ATCC Bacterial Culture Guide, 2015). If excessive water remains inside the cell, injury happens due to ice crystal formation (Zhao & Zhang, 2005). Slow cooling rates were recommended as this will result in less formation of internal ice crystal, therefore letting for more efficient water sublimation from frozen sample (ATCC Bacterial Culture Guide, 2015). Survival of freeze–drying demonstrate the cells' ability to resist the rapid freezing and drying effect (Miyamoto et al., 2008).

CONCLUSION

From morphological observation, biochemical test and molecular identification concluded that bacterial isolate UPMC 1166 comes under the *Bacillus* group of species and suggested (closely related to known or identified species) to be *Bacillus methylotrophicus*. Identification of bacteria is essential to differentiate one type of bacteria from the others. *Bacillus methylotrophicus* UPMC 1166 would be also tested as a biofertiliser on crops to observe the effectiveness and yield for future work.

When subjected to freeze–drying and −80°C freezing, the recovery of cells for UPMC 1166, are dependent on the protectant agent used. To obtain maximum cells viability for long-term preservation, a suitable selection of protectants is important. To get the maximum viability for the strain, it is also important to ensure that the initial cell load should be at least 1×10^8 CFU/mL and the cells should be harvested during the late logarithmic phase of growth. Resistance potential of bacterial strains toward preservation procedures is functional from a research and commercial point of view.

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Prebiotic Potential of Xylooligosaccharides Derived from Cassava Dregs in Balb/c Mice Colon

Ani Harfilia Hafidah[1], Erma Sulistyaningsih[2,3], Wuryanti Handayani[4] and Anak Agung Istri Ratnadewi[3,4]*

[1]Graduate School of Biotechnology, University of Jember, 68121 Jember, Indonesia
[2]Faculty of Medicine, University of Jember, 68121 Jember, Indonesia
[3]Laboratory Center for Development of Advanced Sciences and Technology (CDAST), University of Jember, 68121 Jember, Indonesia
[4]Department of Chemistry, Faculty of Mathematics and Natural Science, University of Jember, 68121 Jember, Indonesia

ABSTRACT

Xylooligosaccharides (XOS) are polymers of the sugar xylose bound by β(1→4) glycoside bonds. XOS have potency as a prebiotic and can be produced from agricultural waste such as cassava dregs. The purpose of this study was to examine prebiotic potential of XOS derived from cassava dregs from hydrolysis reaction catalyzed by endo-β-1,4-D-xylanase. The prebiotic activity of XOS derived from cassava dregs was examined by the number of *Bifidobacterium*, *Lactobacillus*, and *Escherichia coli* in Balb/c mice colon, the fermentation products of *Bifidobacterium* and *Lactobacillus* including changes in pH in the colon and short chain fatty acids (SCFAs) produced by the bacteria as well as the concentration of Ca^{2+} excreted through mice faeces. This study administered XOS derived from cassava dregs at 0.5 and 1.0 g (kg.BW) for 14, 21, and 28 days. The negative control group was Balb/c mice without XOS derived from cassava dregs. The results showed that feeding with XOS derived from cassava dregs at 0.5 and 1.0 g/(kg.BW) could increase the number of both *Bifidobacterium* and *Lactobacillus* and decrease the number of *E.scherichia coli*. The main fermentation products of *Bifidobacterium* and *Lactobacillus* were SCFAs such as acetic acid and butyric acid with respective concentrations of 11.57 and 2.97 mM. The pH in the colon of treatment groups was more acidic than that of in the control groups. The Ca^{2+} absorption

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E-mail addresses:
liahafidah@yahoo.co.id (Ani Harfilia Hafidah)
sulistyaningsih.fk@unej.ac.id (Erma Sulistyaningsih)
wuriyanti.handayani@gmail.com (Wuryanti Handayani)
istri_dewi.fmipa@unej.ac.id (Anak Agung Istri Ratnadewi)
* Corresponding author

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was increased by 0.27% (w/v) that is characterized by decreased concentration of Ca^{2+} in Balb/c mice faeces of treatment groups. This study has led to a conclusion that XOS derived from cassava dregs of xylan hydrolyzed by endo-β-1,4-D-xylanase has prebiotic effect.

Keywords: Cassava, endo-β-1,4-D-xylanase, prebiotic, xylooligosaccharides

INTRODUCTION

A prebiotic is defined as a nondigestible food ingredient that can stimulate the growth and/or activity of limited number of bacteria in the colon (Saier & Mansour, 2005). Many oligosaccharides have been reported to have prebiotic properties, such as galactooligosaccharides, fructooligosaccharides, and inulin (Merrifield et al., 2010). Moure, Gullon, Dominguez and Parajo (2006) reported that xylooligosaccharides (XOS) derived from a variety of xylan had been proposed as excellent candidates for new generation prebiotics. XOS are polymers of the sugar xylose bound by β(1→4) glycoside bonds. XOS are naturally found in bamboo shoots, fruits, vegetables, milk, and honey (Ebringerova & Heinze, 2000; Gibson & Fuller, 2000; Okazaki, Fujikawa, & Matsumoto, 1990). XOS can be generated from the hydrolysis of xylan by endo-β-1,4-D-xylanase (EC. 3.2.1.8). Endo-β-1,4-D- xylanase cuts backbone of xylan randomly and generates XOS and little of xylose (Polizeli et al., 2005). Ratnadewi, Santoso, Sulistyaningsih and Handayani (2016) has succeeded extracting xylan from cassava dregs as much as 6.23% and yielded hydrolysis products such as xylotriose, xylotetraose, and xylopentaose enzymatically using the endo-β-1,4-D-xylanase from *Bacillus* sp.

XOS have high stability under acidic conditions and high temperatures. They can improve the quality of food by giving a change in taste and physico-chemical characteristics as well as stimulate the activity of *Bifidobacterium* in the intestine (Nakano, 1998; Suwa et al., 1999). In addition, XOS can decrease cholesterol and increase the absorption of Ca^{2+}. Specific XOS can increase the population of good bacteria (probiotics) in the colon of the elderly and pregnant women (Okazaki et al., 1990; Rycroft, Jones, Gibson, & Rastall, 2001). Compared to fructooligosaccharide (FOS), XOS are more effective in improving bowel health (Hsu, Liao, Chung, Hsieh, & Chan, 2004). XOS, composed of two to seven xyloses unit attached by the β(1→4) glycoside bond, caused difficulties in hydrolysis by enzymes in the digestive tract enhancing the growth of *Bifidobacteria* with high selectivity (Fedorak & Madsen, 2004; Guan, Zhou, & Wang, 2011; Manning & Gibson, 2004). According to Gullon, Moura, Esteves, Dominguez and Parajo (2008), the percentage of total consumption of XOS by *Bifidobacterium adolescentis* after 24 h was approximately 77%. The highest percentage of consumed XOS is xylotriose (90%), followed by xylobiose (84%), xylotetraose (83%), and xylopentaose (71%). Studies on human showed that XOS intake could increase

the number of probiotic bacteria in the large intestine, where xylobiose (X2) is kept for 24 h before excreted in faeces and urine. Xylobiose is not hydrolyzed both by enzymes in the saliva and pancreas and by gastric acid, instead it acts as a substrate by the probiotic bacteria (Garcia & Lopez, 2013).

Cassava dregs as a byproduct of cassava processing are easily found in Indonesia, especially in East Java (Indonesian Statistics Board, 2017). Recent studies on the *in vitro* have shown that XOS derived from cassava dregs have potential prebiotic to enhance the growth of *Lactobacillus acidophilus* up to 8.61 log CFU/mL and produce short chain fatty acids (SCFAs) such as acetic acid, propionic acid, isobutyrate acid, n-butyric acid, isovaleric acid, and n-valeric acid (Ratnadewi et al., 2017). In this study, the *in vivo* prebiotic activity of XOS derived from cassava dregs was determined by calculating the number of *Bifidobacterium*, *Lactobacillus,* and *E. Coli* in the Balb/c mice colon, measuring the pH of colon, assessing fermentation products of *Bifidobacterium* and *Lactobacillus* in the form of SCFA such as butyric acid, propionic acid, acetic acid and lactic acid, and calculating the Ca^{2+} ion concentration in Balb/c mice faeces.

MATERIALS AND METHODS

Xylooligosaccharides Derived from Cassava Dregs

This study employed xylooligosaccharides (XOS) from hydrolysis of cassava dregs xylan by endo-β-1,4-D-xylanase as the main material (Ratnadewi et al., 2016). Endo-β-1,4-D-xylanase was purified by precipitation with ammonium sulfate at a concentration corresponding to 50% saturation and by dialysis. The purity level of endo-β-1,4-D-xylanase was 6.3-fold purer than the crude extract of endo-β-1,4-D-xylanase.

Animals and Treatments

As many as 27 male Balb/c mice weighing 29- 31 g were housed for 14, 21, and 28 days after receipt. Every three mice were housed in suspended stainless steel cage under a 12- h cycle of light and darkness. The animal use protocol was reviewed and approved by the Ethical Committee of Faculty of Medicine University of Jember.

Nine groups of Balb/c mice were randomly divided into three control groups, three low-dose groups [0.5 g/(kg. BW)] and three high-dose groups [1.0 g/(kg.BW)]. XOS derived from cassava dregs were given for 14, 21, and 28 days, once a day by intragastric administration**,** while the negative control group did not receive any XOS derived from cassava dregs.

Sample Collection

The different doses of XOS were given for 14, 21, and 28 days. At the end of the experimental period, faeces were collected to analyze Ca^{2+} concentration and then Balb/c mice were euthanized by chloroform 90%. A ventral midline incision was made and the colon was excised. Immediately

after removal, the colon contents were collected, pH was measured, and as much as 0.4 g aliquot was processed for SCFA analysis. The remaining colon contents were immediately placed into a sterile assay tube for bacterial calculation.

Bacterial Enumeration

Samples for enumeration of selected genera of *Bifidobacterium*, *Lactobacillus* and *E. coli* were serially diluted 7-fold with physiologic salt solution immediately after collection. As much as 100 µl of the dilutions were inoculated onto duplicate plates using selective media for the enumeration of different bacteria. Bacteria were counted on BD^{TM} Bifidobacterium Agar, BBL™ Eosin Methylene Blue (EMB) Agar, and BBL™ LBS (*Lactobacillus* Selection). Plates were incubated for 24 or 72 h. After incubation, single colony was counted, and the results were expressed as the log values of the colony forming unit (CFU) per mL of wet of colon content.

SCFA Analysis

Extraction of SCFA was carried out through acidification 0.05 g of colon contents using 0.05 mL of H_2SO_4. Next, SCFAs were extracted by adding 0.6 mL of diethyl ether, and agitated and centrifuged for 30 s at 14,000 rpm. The organic phase was taken for further analysis of SCFA concentrations by gas chromatography (Garcia & Lopez, 2013).

Analysis of Ca^{2+} Concentration in the Faeces

Ca^{2+} concentration in the faeces of Balb/c mice colon was determined by previous method (Coudray et al., 2005). As much as 1.0 g of sample was added to 1 mL $HClO_4$ 60% pure analysis and 5 ml HNO_3 65% pure analysis, and incubated overnight. The next day, the sample was heated by hot plate (Stuart MC 152) at 100°C for 90 min, then the temperature was gradually increased to 130, 150, 170, and 200°C, each for 1 h to form white clouds. Destruction was completed with the formation of a white precipitate or the remainder of the clear solution of about 1 mL. The extract was cooled and then diluted with deionized water to 10 mL, then shaked. Clear extract was measured by Atomic Absorption Spectrometry (AAS) (Perkin-Elmer 420, Norwalk, CT USA) at wavelength of 422 for calcium (Ca).

RESULTS AND DISCUSSION

Bacterial Concentration

Microbiota concentration in Balb/c mice colon of different dose of XOS derived from cassava dregs for 14, 21, and 28 days was shown in Table 1. The results showed that the administration of XOS derived from cassava dregs at 0.5 and 1.0 g/(kg.BW) in Balb/c mice increased the number of *Bifidobacterium* by 1.5%, 4%, and 10% in the feeding for 14, 21, and 28 days, respectively ($P < 0.05$). The number of *Lactobacillus* was also increased by 1.5%, 5%, and 11.5% on feeding at 0.5 and 1.0 g/(kg.BW) XOS derived from cassava

dregs for 14, 21, and 28 days, respectively (P<0.05). According to Wei et al. (2013), feeding a commercial XOS (Shandong factory Long-li Biotechnology Co., Ltd.) 0.5 and 1.0 g/ (kg.BW) for 14 days can promote the growth of *Bifidobacterium* and *Lactobacillus* by 5% ($P < 0.05$) and 9% ($P < 0.05$), respectively. Whereas, in this study to achieve an increase in the number of *Bifidobacterium* and *Lactobacillus* by 5% and 9% ($P < 0.05$) took 7-14 days longer.

The total growth of *E. coli* in the study decreased by 1%, 3%, and 8% ($P < 0.05$) on feeding at 0.5 and 1.0 g/(kg. BW) XOS derived from cassava dregs for 14, 21, and 28 days, respectively, but the difference was not statistically significant. It indicates that XOS derived from cassava dregs is effective in increasing the growth of *Bifidobacterium* and *Lactobacillus* in the Balb/c mice colon.

Table 1
Microbiota concentration in the Balb/c mice colon

Sample	Time	Number of bacteria (log CFU/ ml)		
		Bifidobacterium	*Lactobacillus*	*Escherichia coli*
Control	14th day	7.67±0.02	7.37±0.02	7.77±0.00
XOS 0.5 g /(kg.BW)		7.78±0.00#	7.44±0.00	7.61±0.01
XOS 1.0 g /(kg.BW)		7.83±0.01#	7.56±0.07	7.63±0.00
Control	21th day	7.60±0.02	7.26±0.01	7.82±0.01
XOS 0.5 g /(kg.BW)		7.99±0.00	7.63±0.02	7.54±0.01
XOS 1.0 g /(kg.BW)		8.22±0.01	7.80±0.01	7.58±0.01
Control	28th day	7.36±0.01	7.00±0.00	7.95±0.01
XOS 0.5 g /(kg.BW)		8.33±0.01	8.15±0.01	7.24±0.02
XOS 1.0 g /(kg.BW)		8.39±0.00	8.21±0.01	7.00±0.06

Note: # indicates the data are not significantly different ($P < 0.05$)

pH of Balb/c Mice Colon

The administration of XOS derived from cassava dregs at 0.5 and 1.0 g/ (kg.BW) in Balb/c mice for 14, 21, and 28 days significantly decreased the pH of Balb/c mice colon ($P < 0.05$), compared to control (Table 2). A decrease in the pH of Balb/c mice colon generated the growth of probiotic bacteria (*Bifidobacterium* and *Lactobacillus*) (Hsu et al., 2004). The decline in the colon pH was concomitant with the increase of probiotic bacteria growth, as shown in Table 1. This finding is in accordance with the previous study by Hsu et al. (2004). Probiotic, bacteria such as *Bifidobacterium* and *Lactobacillus*, produces lactic acid and SCFA as metabolic products of carbohydrate fermentation that is not digested in the gut. SCFA can maintain the homeostasis and decrease the intestinal pH (Jan et al., 2002).

Table 2
pH of Balb/c mice colon

Sample	Time	pH of Colon
Control		7.1
XOS 0.5 g/(kg.BW)	14th day	6.34
XOS 1.0 g/(kg.BW)		6.62
Control		7.28
XOS 0.5 g/(kg.BW)	21th day	5.95
XOS 1.0 g/(kg.BW)		6.6
Control		7.24
XOS 0.5 g/(kg.BW)	28th day	5.85
XOS 1.0 g/(kg.BW)		6.57

SCFA Concentrations

Fermentation of prebiotic by probiotic bacteria in the colon produces SCFA. SCFA is a key product that can maintain intestinal health, gut morphology, and function (Roy, Kien, Bouthillier, & Levy, 2006; Scheppach, 1994). Commonly produced SCFA are acetic acid, propionic acid, and butyric acid (Jan et al., 2002). In this study, feeding Balb/c mice with 0.5 and 1.0 g/(kg. BW) XOS derived from cassava dregs for 14 and 21 days resulted in the production of SCFA such as acetic acid, propionic acid, isobutyric acid, n-butyric acid, isovaleric acid and n-valeric acid (Table 3). The dominant SCFA produced were acetic acid and butyric acid resulted from feeding 1.0 g/ (kg.BW) XOS derived from cassava dregs for 21 days with concentrations of 11.57 and 2.97 mM, respectively.

The similar result of acetic acid concentrations was reported by Pan, Chen, Wu, Tang, and Zhao (2009), assessing the fermentation of 90% cytooligosaccharides (COS) (Weikang, Shanghai, China) by probiotics. The concentrations of acetic acid and butyric acid found in Pan's et al. (2009) study were 37.13 and 3.64 μmol g^{-1}, respectively (1 Molar solution = 1×10^{-3} mol g^{-1}). The present study proves that XOS derived from cassava dregs is effective in increasing the growth of probiotic (*Bifidobacterium* and *Lactobacillus*) in the Balb/c mice colon, and cause the increase of fermentation product (acetic acid and butyric acid).

Butyric acid serves as an energy source for the colon by introducing the butyrate that produces strain *Butyrivibrio fibrisolvens* into germ-free mice or by adding butyrate to isolated colonocytes of germ- free mice. They rescued the colonocytes from both the deficit in mitochondrial respiration and autophagy. In the presence of an inhibitor for fatty acid oxidation, butyrate was unable to suppress autophagy. Those phenomena indicate that the rescue was due to butyrate acting as an energy source rather than as a regulator (Donohoe et al., 2011) and decreased the luminal pH. At the molecular level, butyric acid acts as an inhibitor of histone deacetylase promoting epigenetic hyperacetylation of histone proteins and non-histone that regulates the expression of cell cycle regulation CDKN1A, and alter DNA methylation resulting in increased accessibility of transcription factors to nucleosomal DNA (Hamer et al., 2008; Jan et al., 2002; Li et al., 2012; Sanderson, 2004; Scheppach, 1994; Smith, Yokoyama, & German, 1998). Butyric acid can also induce the differentiation of cells, suppress

the proliferation and increase apoptosis to remove the damaged DNA in cells that may develop into malignant cells both *in vitro* and *in vivo* (Leu, Brown, Hu, & Young, 2003; Li & Elasser, 2005; Medina et al., 1997).

Fermentation of XOS derived from cassava dregs by probiotics in the Balb/c mice colon produces branched-short chain fatty acids (BSCFAs) such as isobutyric, and isovaleric acids. Isobutyric and isovaleric acids were produced from the fermentation of branched amino acids, valine, leucine, and isoleucine, derivations of indigestible protein-reaching colon (Lynch & Adams, 2014; Yao, Muir, & Gibson, 2016). According to Heimann, Nyman, Palbrink, Petersson and Degerman (2016), BSCFA such as isobutyric and isovaleric acids have an effect on adipocyte and glucose metabolism that may contribute to improve insulin sensitivity. It implies that the intake of XOS derived from cassava dregs in mice Balb/c colon produce fermentation products such as SCFA and BSCFA that can improve health.

Table 3
SCFA concentrations in the Balb/c mice colon

Samples	Time	SCFA (mM)					
		C_2	C_3	iC_4	nC_4	iC_5	nC_5
Control		8.99	5.00	1.44	3.09	1.67	2.10
XOS 0.5 g/(kg.BW)	14th day	6.79	1.73	0.22	0.56	0.53	0.15
XOS 1.0 g/(kg.BW)		*	*	*	*	*	*
Control		9.93	7.16	1.02	1.92	1.84	0.83
XOS 0.5 g/(kg.BW)	21th day	57.26	4.43	0.80	1.45	0.43	0.26
XOS 1.0 g/(kg.BW)		11.57	4.2	0. 55	2.97	0.86	0.13

Note: (*) The sample is dried
C_2 (acetic acid), C_3 (propionic acid), $_iC_4$ (iso-butyric acid)
$_nC_4$ (n-butyric acid), $_iC_5$ (iso-valeric acid), $_nC_5$ (n-valeric acid)

Concentrations of Ca^{2+} in Balb/c Mice Faeces

The SCFA produced by probiotics in the gut lumen contribute to decrease pH in the colon, which is associated with an increase in dissolved calcium absorption, especially in the caecum. This is because SCFA affects the transcellular absorption of calcium by modifying the exchange of intracellular H^+ for Ca^{2+} in the distal colon (Van den Heuvel et al., 1999). Butyric acid can stimulate the intestinal epithelial cells, promote colon motility, and increase its absorptive capacity (Canani et al., 2011; Zhang et al., 2010). In this study, feeding with XOS derived from cassava dregs at 1.0 g/(kg.BW) for 14 days resulted in an increased concentration of Ca^{2+} absorption in the intestinal epithelial cells indicated by the decreased concentration of Ca^{2+} in

the faeces. The Ca^{2+} concentration in the control group faeces was found higher than that of in the treatment group 1.57% (w/v) to 1.34% (w/v), respectively. Based on the data in Table 4, XOS derived from cassava dregs at 1.0 g/(kg.BW) stimulated better in Ca^{2+} absorption in the intestine than corn bran arabinoxylans (Lopez et al., 1999). A recent study found higher Ca^{2+} released in mice faeces fed with corn bran arabinoxylans than that of from the control group, 79.6 and 52.7 mg/d, respectively. This finding proved that Ca^{2+} absorption was higher in the intestinal epithelial cells of Balb/c mice with XOS derived from cassava dregs intake.

Table 4
Concentrations of Ca^{2+} in Balb/c mice faeces

Sample	Time	Results of analysis % (w/v)
Control		1.57
XOS 0.5 g/(kg.BW)	14th day	1.70
XOS 1.0 g/(kg.BW)		1.34
Control		1.57
XOS 0.5 g/(kg.BW)	21th day	2.42
XOS 1.0 g/(kg.BW)		2,09
Control		1.57
XOS 0.5 g/(kg.BW)	28th day	2.19
XOS 1.0 g/(kg.BW)		2.24

CONCLUSION

XOS derived from cassava dregs could increase the growth of both *Bifidobacterium* and *Lactobacillus* and decrease the growth of *E. coli* in Balb/c mice colon. XOS derived from cassava dregs produced fermentation products such as SCFA and BSCFA resulting in a decreasing pH of the colon. In addition, the resulted butyric acid plays a role in the increased uptake of Ca^{2+} in the intestine characterized by the decrease of Ca^{2+} concentration in Balb/c mice faeces.

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Traits Performance and Heterosis Estimation in F_1 Rice Generations Crossed between Basmati 370 and Selected Malaysian Rice Varieties

Nur Suraya Abdullah[1,2]*, Mohd Yusoff Abdullah[2], Mohd Bahagia Abdul Ghaffar[3], Asmah Awal[2], Noorshilawati Abdul Aziz[1] and Shamsiah Abdullah[2]

[1]Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (UiTM) Pahang, 26400 Bandar Jengka, Pahang, Malaysia
[2]Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (UiTM) Shah Alam, 40450 Shah Alam, Selangor, Malaysia
[3]Rice and Industrial Crop Research Centre, MARDI Seberang Perai, 13200 Kepala Batas, Penang, Malaysia

ABSTRACT

The assessment in 13 rice genotypes that consisted of MR219, Mahsuri Mutant, Basmati 370, MRQ76, MRQ74, MRQ50, and Mahsuri Mutant 98 as parental lines, and six rice combinations were conducted in MARDI Seberang Perai, Penang. This assessment analyzed the variability among parental lines and F_1 generations, including heterosis mechanism in rice combinations for the identification of better traits related to grain quality and yield. The parental lines, Basmati 370, MRQ50, MRQ74, MR219 and Mahsuri Mutan have long and slender grains. This characteristic is desired in breeding for grain quality traits. It was found that cross Basmati 370 × MR219 showed the best performances in most of the grain quality traits with grain shape >3 mm. Besides that, it also showed significant differences as compared to other. The findings included that the cross had the highest value in plant height (120.2 cm), panicle length (30.4 cm), thousand grain weight (26 g), and flag leaves length (42.4 cm). Pearson correlation analysis showed that strong positive relationship and a significant association were found between traits panicle fertility and tiller number (0.843), and traits milled grain length and length/breadth ratio (0.768). Heterosis analysis showed that RU14387 posed positive heterosis value in most of the traits studied except for the panicle fertility and filled grain per panicle traits when compared with better parent. RU14387 also recorded

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E-mail addresses:
nsa@pahang.uitm.edu.my (Nur Suraya Abdullah)
yusoff_abdullah@salam.uitm.edu.my (Mohd Yusoff Abdullah)
bahagia@mardi.gov.my (Mohd Bahagia Abdul Ghaffar)
asmah138@salam.uitm.edu.my (Asmah Awal)
noorshilawati@pahang.uitm.edu.my (Noorshilawati Abdul Aziz)
shamsiah3938@salam.uitm.edu.my (Shamsiah Abdullah)
* Corresponding author

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positive heterosis over standard check variety in length/breadth ratio and milled grain length compared to standard check variety. The information gained from this study can be a great foundation for future rice development study of rice lines with better grain quality and high yield.

Keywords: Basmati 370, grain quality, heterosis, rice breeding

INTRODUCTION

Rice is the most important food crop consumed by more than four billion people throughout the world and the demand has been increased continuously in the world population (Bouman & Reyes, 2017). Currently, changes in lifestyle, education, income, eating habits, healthy diets concerns, and taste preferences have caused the trend in rice production and consumption to change and most of the rice producers and consumers have also demanded on rice with higher-quality traits. According to Nirmaladewi, Padmavathi, Suneetha and Babu (2015), to meet consumer and industry preferences as well as market demand; improving milling, cooking, eating, and processing qualities is crucial in rice breeding for quality as well as high-yielding performance. Sran, Pandey and Kumar (2016) stated that the improvement of rice quality could be achieved through heterosis breeding.

Heterosis is defined as the superiority of the first generation (F_1) hybrids over its parents (Virmani et al., 1997) and the occurrence of heterosis is determined as a success and a great achievement in rice breeding program (Tiwari, Pandey, Giri, & Dwivedi, 2011). Heterosis in rice was first discovered by Jones in 1926 who found the great performances of grain yield and culm in F_1 population compared to their parental lines. The values of heterosis can be observed through hybrid performances over their mid-parents' (MPH), better parents (BPH), and standard check variety (STH) (Rahimi, Rabiei, Samizadeh, & Kafi Ghasemi, 2010; Yildrim, Gezginc, Paksoy, 2014). The value of heterosis either positive or negative depends on the degree of selected parental lines (Rahimi et al., 2010; Bhatti, Pandey, & Singh, 2015) and both indicate significant information of preferred traits for quality and gene action that is beneficial for rice development program (Raju, Kumar, Raju, & Srijan, 2014; Reddy, Raju, Srayani, Reddy, & Reddy, 2012). Rahimi et al. (2010) reported significant heterosis in all traits studied as compared to standard check variety Dorfak, and the highest heterosis value was found in grain yield. High degree of heterosis over standard check variety in different quality traits of some cross combinations have been claimed by Sran et al. (2016). The finding showed the highest value in general combining ability (GCA) and served as a guidance in the determination of suitable parents as good combiners for quality traits. Venkanna, Raju, Lingaiah and Rao (2014) have revealed significant positive heterosis for yield and grain quality traits in crosses JGL 3844 × JGL 11690, Erramallelu × LGL 11690, BPT 5204 × JGL 11690, and MTU 1001 × JGL 1798. The same findings were also claimed

by Krishna et al. (2016) in crosses NLR 145 × Sumathi, Akshyadhan × Pusa 1122, and RNR 2354 × Basmati 370. In contrast, Faiz, Sabar, Awan, Tjaz and Manzoor (2006) revealed negative value in heterobeltiosis in plant height and spikelets sterility that is desirable for those particular traits. However, the study also reported positive heterobeltiosis and in desirable traits such as grain yield, number of productive tillers and number of filled panicles. The highly significant negative value also was found by Ariful, Khaleque, Golam, Khaliq and Mannan (2015) in plant height of five male plants that were important for developing dwarfing characters.

Consumers mostly described high-quality rice based on grain appearance, shape, size, cooking properties, flavor, and taste. Rice with pleasant aroma, short or long slender grain normally fetched higher prices in market. Kernel length, kernel width, and kernel length/breadth ratio are among the important components in physical quality traits (Reddy et al., 2012). Based on the standard rice classification system by the International Rice Research Institute (IRRI), length of rice grain ranges from 5.51-6.60 mm is considered as medium grain, while a range of 6.61-7.5 mm is considered as long grain (Cruz & Khush, 2002). Likewise, rice with grain shape less than two mm (<2 mm) is considered as bold grain while grain-shaped rice ranged from 2.1-3.0 mm and more than 3 mm (>3.0 mm) are classified as medium and slender shape. Grain shape is determined from the proportion of length to width of grain (Graham, 2002). Although Malaysia has released more than 40 rice varieties including special variety with characteristic pleasant aroma, long grain, high yield and resistance to pest and diseases (Jamal, Kamarulzaman, Abdullah, Ismail, & Hashim, 2014), but some of the variety, such as aromatic rice is reported to have undesirable agronomic characters such as low yield and susceptible to pest and disease (Golam et al., 2011). Thus, exploitation through heterosis breeding is one of the alternatives to improve crop performances. Nevertheless, the studies on heterosis breeding for grain quality improvement among Malaysian rice varieties with other specialty variety are still lacking. Therefore, by means to provide significant information and essential knowledge for future grain quality and yield traits improvements as well as varietal development, the breeding and study on heterosis were conducted among selected Malaysian rice varieties with Indian traditional rice, Basmati 370. Basmati 370 was selected as parent due to its pleasant aroma and elongation of cooked rice. The main objective of this study was to analyze the variability in grain quality and yield traits among parental lines and F_1 generations including heterosis mechanism in rice combinations for identification of better traits related to grain quality and yield.

MATERIALS AND METHODS

Plant Materials and Hybridisation

The experiment was conducted over two seasons (main season and off season) at Malaysian Agricultural Research and

Development Institute (MARDI), Seberang Perai, Penang (Latitude 05° 21'N and Longitude 100° 24'E). Parental lines consist of local Malaysian rice varieties: MR219, MRQ76, MRQ74, MRQ50, Mahsuri Mutant 98, Mahsuri Mutant, and traditional Indian rice Basmati 370 (B370) were used in this study. The hybridization among selected varieties was conducted under closed area according to the method described by Jenning, Coffman and Kauffman (1979). Crosses plants were kept under glasshouse with good exposure to sunlight. The F_1 seeds were harvested when the flag leaves turned yellowish and reached maturity stage, and the seeds were kept in a cool temperature 4°C to maintain its viability before use for the next experiment. Hybridization programs produced six F_1 rice combinations named as RU14701, RU14702, RU14703, RU14704, RU14705, and RU14387 (Table 1).

Table 1
Rice combinations and its F_1 generation

Female		Male	F_1 Generation
Basmati 370	×	MRQ50	RU14701
Basmati 370	×	MRQ76	RU14702
Basmati 370	×	MRQ74	RU14703
Basmati 370	×	Mahsuri Mutan	RU14704
Basmati 370	×	Mahsuri Mutan 98	RU14705
Basmati 370	×	MR219	RU14387

Raising of F_1 Generations

The F_1 seeds were placed in petri dishes and heated in an oven for 24 h at a temperature of 37.5°C. Then, distilled water with a few drops of fungicide was added and the seeds were kept for 48 h. After that, distilled water was removed and seeds were allowed to germinate under room temperature. Germinated seeds were then sown in the glasshouse. Rice seedling at 25 days old was then transplanted into field plots with a total area 101.31 m^2 at a spacing of 0.3 m × 0.3 m between rows and plants, and 0.5 m between plots and replicated in a randomized complete block design with three replicates. Fifty seedlings of each parental lines and F_1 plants were planted with single seedling per hill in each row. Fertilizers were applied in experimental plot according to the standards rates stated by MARDI (2002) whereas NPK (17.5:15.5:10) and urea (46% N) were applied at 15 days and 30 days after planting at the rates of 2.55 kg and 0.85 kg, respectively. NPK (17:3:20:2:0.8 S+TE) was applied at 45 days and 70 days at the rates of 1.6 kg, respectively.

Grain quality traits consisted of milled grain length (MGL), milled grain width (GW), length/breadth ratio (LB), and filled grain per panicle (FGP) and selected yield component, such as heading days at 70% (HD), plant height (PL), panicle length (PL), fertile panicle per plant (FP), tiller number per plant (TN), flag leaf length (FLL), flag leaf width (FLW), weight of thousand rice grains (TGW) on 10 randomly selected plants were evaluated according to standard evaluation system rice described by Scshu

(1988) and IRRI (Cruz & Khush, 2002). The harvested seeds that represent F_2 generation were kept under 4°C for future analysis.

Statistical Analysis

The data was analyzed by analysis of variance (ANOVA) using SPSS software version 23. Mean comparisons were made using Duncan New Multiple Range Test (DNMRT) and means were statistically significant when $P<0.05$. Interrelationships among traits studied were made using Pearson correlation coefficient at level 0.01 and 0.05 while the coefficient of variation was calculated by divided value of standard deviation with a mean of each trait. Heterosis was expressed as an increase or decrease of percentage in the performances of F_1 generations and was calculated based on the differences of F_1 generations from mid-parent (MPH), better parents (BPH), and standard variety (STH) following the method described by Virmani et al. (1997). MRQ76 was used as a standard check variety for quality traits studied in this research.

Mid parent heterosis (MPH)
$= [(F_1 - M.P)/M.P] \times 100$

Better parent heterosis (BPH)
$= [(F_1 - B.P) / B.P] \times 100$

Standard heterosis (STH)
$= [(F_1 - S.T) / S.T] \times 100$

Where,
F_1 = Mean value of F_1 progeny
M.P = Mean value of parents
B.P = Mean value of better parent
S.T = Mean value of standard check variety

RESULTS AND DISCUSSION

Mean performances of grain quality traits and selected yield components of parental lines and F_1 generations were summarized in Table 2. The results of the study indicated that parental lines consisting of Basmati 370 (6.8 mm), MRQ50 (6.9 mm), MRQ74 (6.7 mm), MR219 (6.7 mm), and Mahsuri Mutan (6.6 mm) showed long grain length while another two parents consisting of MRQ76 (6.5 mm) and Mahsuri Mutan 98 (6.6 mm) showed medium grain length. Among six crosses, three crosses consisting of RU14701 (Basmati 370 × MRQ50), RU14703 (Basmati 370 × MRQ74), and RU14387 (Basmati 370 × MR219) showed long grain length with value 6.9 mm, 6.6 mm, and 6.8 mm, respectively. However, there was no significant differences recorded when compared with their parental lines. In contrast, cross RU14704 (Basmati 370 × Mahsuri Mutan) showed the lowest grain length (6.1 mm) and it differed significantly with their parental lines. Grain width of parental lines ranged from 1.6 mm to 2.0 mm, while in crosses ranged from 1.7-2.0 mm. Among the six crosses plants, three combinations consisting of RU14701, RU14703, and RU14387 showed the best performances in grain shape.

Filled grain per panicle also contributed to the good quality of rice. The highest filled grain per panicle was found in parent MRQ76 followed by MR219 and MRQ74. The lowest filled grain was recorded in

MRQ50. In crosses rice plant, the highest filled grain (136) was recorded in cross RU14387 from the combination of parents Basmati 370 and MR219 followed by cross RU14702 (110.3) from the combination of Basmati 370 and MRQ76. The lowest filled grain per panicle was recorded in cross RU14701, from the combination of Basmati 370 and MRQ50. Juliano (1993) stated that grain length and width were considered as important traits compared to other traits. Rafii et al. (2014) mentioned that the value of grain length and width determined the grain shape of rice. The results indicated that all parental lines and cross combinations showed grain shape more than 3 mm (>3.0 mm) that is categorized as slender grain shape. All crosses plants also differed significantly with parents Basmati 370 and MRQ50 that have grain shape values of 4.05 and 4.25, respectively. Grain size and shape are among the most important criteria in determining grain quality and yield (Wang et al., 2012). Moreover, the characteristics of long and slender grain as found in parents Basmati 370, MRQ50, MRQ74, MR219 and Mahsuri Mutan are the main focus on selecting breeding material (Rafii et al., 2014).

Besides quality traits, the observation of some characters contributed to grain yield performances can also be used in selection of promising high yield variety. The shortest heading days was found in variety Mahsuri Mutan 98 (79 days) and cross RU14705 (75 days), while Basmati 370 and cross RU14703 recorded the longest heading days, 97 days and 94 days, respectively. Among all yield traits studied, cross RU14387 showed significant differences and highest in plant height (120.2 cm), panicle length (30.4 cm), thousand grain weight (26.0 g), and flag leaves length (42.4 cm) compared to other crosses in plants. In contrary, cross RU14703 showed significant difference in panicle fertility (13.7) and tiller number (14.0) compared with its male parent, MRQ74. The yield and quality traits of the plant are related to each other. Grain filling of the plant is usually influenced by the photosynthesis process that occurred on uppermost leaf below the panicle, which is known as flag leaf. Flag leaf plays an important role in providing food for grain development (Rahman, Haque, Sikdar, Islam, & Matin, 2013) while grain filling contributes to grain weight (Wang et. al., 2008) and grain quality (Wei et al., 2017). Rahimi et al. (2010) reported that rice varieties with higher grain weight and more panicles per plant could be selected for the purpose of breeding for grain yield. Coefficient of variation was found to be 7.29% in 79% heading days, 10.4% in plant height, 8.5% in panicle length, 15.8% in tiller number, 18.0% in panicle fertility, 16.6% in flag leaf length, 13.78% in flag leaf width, and 7.43% in thousand grain weight. For grain quality traits, coefficient of variation was 3.19% in milled grain length, 7.82% in milled grain width, 10.45% in length/breadth ratio, and 20.55% in filled grain per panicle.

The analysis of correlation coefficients between all pairs of studied traits is presented in Table 3. Positive strong relationship and

Table 2
Genotypes mean for grain quality and selected yield traits of parental lines and F_1 generation

Parent / Cross	Traits											
	HD (70%)	PH (cm)	PL (cm)	TN	PF	FLL (cm)	FLW (cm)	TGW (g)	MGL (mm)	MGW (mm)	L/B ratio	FGP
Parents												
B370	97.0^{e}	127.6^{g}	26.7^{d}	12.0^{abcd}	10.0^{a}	27.5^{ab}	1.2^{a}	21.0^{b}	6.8^{fg}	1.7^{ab}	4.1^{f}	93.0^{b}
MRQ50	93.3^{cde}	93.7^{b}	24.4^{b}	13.0^{bcde}	12.7^{ab}	37.8^{f}	1.5^{cd}	20.2^{a}	6.9^{g}	1.6^{a}	4.3^{f}	77.7^{a}
MRQ74	88.0^{b}	85.0^{a}	25.0^{bc}	17.3^{f}	16.7^{d}	26.8^{a}	1.5^{cd}	21.4^{b}	6.7^{ef}	1.8^{c}	3.6^{d}	134.7^{de}
MRQ76	87.0^{b}	101.4^{c}	25.7^{c}	14.3^{e}	13.7^{c}	31.2^{d}	1.9^{g}	24.4^{f}	6.5^{bc}	2.0^{de}	3.3^{bc}	140.7^{e}
MR 219	86.3^{b}	100.5^{c}	27.7^{d}	12.0^{abcd}	12.0^{abc}	30.0^{cd}	1.6^{de}	25.8^{h}	6.7^{ef}	1.9^{de}	3.5^{cd}	138.7^{e}
MM	96.3^{e}	107.8^{d}	25.4^{bc}	11.7^{abc}	11.0^{ab}	28.5^{abc}	1.6^{de}	22.8^{c}	6.6^{def}	1.9^{cd}	3.5^{cd}	127.0^{d}
MM98	79.0^{a}	107.8^{d}	21.9^{a}	11.0^{ab}	10.0^{a}	27.0^{a}	1.8^{f}	25.0^{g}	6.6^{cde}	1.9^{cd}	3.5^{cd}	125.3^{d}
F_1 Generation												
RU14701	87.0^{b}	119.0^{ef}	27.5^{d}	11.7^{abc}	11.7^{abc}	40.3^{g}	1.6^{de}	24.1^{ef}	6.9^{g}	1.7^{b}	4.0^{e}	88.7^{b}
RU14702	91.0^{bcd}	118.6^{e}	29.3^{e}	12.3^{abcd}	11.0^{ab}	34.7^{e}	1.7^{ef}	23.5^{d}	6.4^{b}	2.0^{e}	4.0^{e}	110.3^{c}
RU14703	94.0^{de}	107.9^{d}	29.5^{ef}	14.0^{de}	13.7^{c}	35.8^{e}	1.3^{ab}	22.5^{c}	6.6^{cdef}	1.7^{ab}	3.2^{ab}	91.0^{b}
RU14387	89.0^{bc}	120.2^{f}	30.4^{f}	13.7^{cde}	12.0^{abc}	42.4^{h}	1.7^{ef}	26.0^{h}	6.8^{fg}	2.0^{de}	3.5^{cd}	136.0^{de}
RU14704	88.3^{b}	108.7^{d}	26.8^{d}	11.3^{ab}	10.3^{ab}	27.2^{a}	1.6^{cde}	21.0^{b}	6.1^{a}	2.0^{e}	3.0^{a}	93.3^{b}
RU14705	75.3^{a}	108.7^{d}	27.1^{d}	10.3^{a}	10.0^{a}	29.1^{bc}	1.4^{bc}	23.6^{de}	6.5^{bcd}	1.9^{cd}	3.4^{c}	91.3^{b}
CV (%)	7.3	10.4	8.5	15.8	18.0	16.6	13.8	7.4	3.2	7.8	10.5	20.6

Note: Mean in a column for each parent and crosses followed by the same uppercase letters are not significantly different at 0.05 level according to Duncan's New Multiple Range Test (DNMRT); HD: Heading days at stage 70%; PH: Plant height; PL: Panicle length; TN: Tiller number; PF: Panicle fertility; FLL: Flag leaf length; FLW:Flag leaf width and TGW: Thousand grain weight; MGL: Milled grain length; MGW: Milled grain width; L/B: Length to breadth ratio; FGP: Filled grain per panicle; CV %: Percentage of coefficient of variation, B370: Basmati 370, MM: Mahsuri Mutan, MM98: Mahsuri Mutan 98.

a significant association at $P < 0.05$ were found between traits panicle fertility and tiller number (0.843) and traits milled grain length and length/breadth ratio (0.768). Positive intermediate relationships with significant association at $P < 0.05$ were also found between panicle length and flag leaf length (0.538), thousand grain weight and flag leaf width (0.591), flag leaf width and milled grain width (0.643) include thousand grain weight and filled grain per panicle (0.587). Two of the lowest positive relationships were found between milled grain width and filled grain per panicle (0.413); and panicle fertility with filled grain per panicle (0.327) at significant association $P < 0.05$ and $P < 0.01$ respectively. In contrast, the highest negative relationship with the significant association at $P < 0.05$ was recorded between milled grain width and length/breadth ratio (−0.975). Milled grain length with milled grain width (−0.617), plant height and panicle fertility (−0.625) and traits length/breadth ratio and flag leaf width (−0.591) recorded intermediate negative relationship with significant associations at $P < 0.05$. The lowest negative relationship with significant association was recorded between heading days and flag leaf width (−0.338); and plant height with tiller number (−0.484) at significant association $P < 0.01$ and $P < 0.05$, respectively. Positive correlation among flag leaves and panicle length, panicle length and plant height, thousand grain weight and flag leaf width also have been reported in previous studies (Rahman et al., 2013; Ratna, Begum, Husna, Dey, & Hossain, 2015).

Table 4 presents the heterosis value of grain quality and selected yield traits in all six rice combinations. The results indicated

Table 3
Pearson correlation analysis of grain quality and selected yield traits of parental lines and F_1 generation

	HD	PH	PL	PF	TN	FLL	FLW	TGW	MGL	MGW	L/B	FGP
HD	1											
PH	.192	1										
PL	.200	.466**	1									
PF	.108	−.625**	-.053	1								
TN	.208	−.484**	.048	.843**	1							
FLL	.170	.278	.538**	.069	.077	1						
FLW	−.338*	−.198	−.183	−.014	−.066	.051	1					
TGW	.441**	.198	.237	−.222	−.162	.225	.604**	1				
MGL	.184	−.006	−.027	.127	.160	.458**	−.264	.053	1			
MGW	−.267	.002	.140	−.238	−.136	−.276	.643**	.483**	−.617**	1		
L/B	.286	.001	.110	.221	.150	.365*	−.591**	−.393*	.768**	-.975**	1	
FGP	−.116	−.308	.060	.327*	.400*	−.191	.433**	.587**	.010	.413**	−.357*	1

Note: *, ** Significant at the 0.01 level and 0.05 levels, respectively

Table 4
Heterosis value of grain quality traits and selected yield traits in F_1 generation among both parents, better parent and standard check variety

Crosses	Het	Traits											
		HD (70%)	PH (cm)	PL (cm)	TN	PF	FLL (cm)	FLW (cm)	TGW (g)	MGL (mm)	MGW (mm)	L/B ratio	FGP
RU14701	MPH	−41.25	−24.43	−27.06	−38.42	−16.31	−21.98	−26.18	−21.56	−33.11	−29.94	−36.41	−28.28
	BPH	−10.71	27.05	12.86	−10.00	22.92	6.38	3.08	19.13	−0.29	6.17	−6.12	14.16
	STH	−4.21	17.32	7.00	−18.18	−14.63	29.00	−17.89	−1.23	5.86	−13.13	21.65	−36.96
RU14702	MPH	−0.90	−33.49	−25.77	−26.89	−26.62	−19.54	−20.93	−29.24	−33.83	−37.97	−44.29	−44.29
12	BPH	4.17	16.92	14.14	−28.90	4.08	11.15	−10.53	−3.76	2.01	−16.67	−3.35	−35.32
	STH	5.36	39.47	17.18	−2.10	−34.00	29.19	15.65	9.60	2.01	−16.67	−3.35	−35.32
RU14703	MPH	−33.10	1.49	14.12	−16.04	7.92	31.66	−2.62	5.85	−4.70	14.53	5.22	−3.12
	BPH	8.05	26.89	17.84	−2.10	−2.68	33.29	−11.56	4.76	−4.05	9.24	11.63	−18.11
	STH	6.82	6.38	14.79	−13.99	0.00	14.64	−31.58	−8.01	−1.39	1.52	22.87	−21.61
RU14387	MPH	−2.91	5.44	11.78	14.17	2.08	47.48	20.24	11.14	0.45	8.03	−7.73	17.39
	BPH	3.09	19.67	9.76	14.17	−3.92	41.33	5.21	0.78	0.90	0.52	0.29	−1.95
	STH	2.30	18.53	18.16	−4.20	−12.20	35.90	−11.40	6.56	4.17	−1.52	5.49	−3.34
RU14704	MPH	−8.62	-7.61	2.88	−4.64	−0.52	−2.98	10.71	−3.94	−8.59	12.85	−19.52	−15.18
	BPH	−8.30	0.90	5.52	−3.42	−6.80	−4.68	−3.13	−7.74	−7.83	5.76	−12.93	−26.54
	STH	1.53	7.20	4.15	−20.98	−24.39	−12.93	−18.42	−13.84	−5.56	2.02	−7.62	−33.69
RU14705	MPH	−14.40	−7.65	11.46	−10.43	−4.71	6.91	−7.18	2.79	−2.33	7.00	−9.07	−16.35
	BPH	−4.65	0.83	23.59	−6.36	−9.90	7.9	−22.94	−5.42	−0.91	0.53	−1.16	−27.13
	STH	−13.41	7.16	5.32	−27.27	−26.83	−6.62	−26.32	−3.18	0.31	3.97	−6.65	−35.11

Note: Het: Heterosis; MPH: Mid parent heterosis; BPH: Better parent heterosis; STH: Standard heterosis

that crosses RU14701 and RU14702 posed negative values in all grain quality and yield traits studied when compared with both parents. However, when comparing with better parents, RU14387 showed a positive heterosis value in most of the traits studied except for traits panicle fertility and filled grain per panicle. The results indicated that crosses RU14701, RU14703, and RU14387 recorded positive heterosis over standard check variety in length/ breadth ratio. Positive heterosis values in milled grain length were also found in crosses RU14701, RU14702, RU14387, and RU14705 as compared to standard check variety, MRQ76. The positive or negative heterosis value gives significant information on preferred traits for the development of better rice quality and yield. Jelodar (2010) reported negative heterosis value in plant height and this information is desired for breeding of short stature hybrids and varieties. Rahimi et al. (2010) stated that plant height that posed negative heterosis value is used for the development of dwarf varieties. The same result is also reported by Kumar, Surendra and Sumer (2012) who agrees that short plant is needed to prevent lodging during planting and taller plants are usually vulnerable to disease infection that may then reduce quantity and quality of the grain. On the other hand, positive heterosis value also provides significant information for a variety of development. Nurruzzaman et al. (2010) have reported positive heterosis values in panicle length, grain length, and flag leaves of rice that are among the important traits for the development of high yield variety. Rahimi et al. (2010) also agrees that hybrids rice with more panicle and high grain weight is important for improving crop yield. The length of panicle is also one of the important traits in rice breeding as it is related with spikelets number that contributes to high yield production (Elixon, Asfaliza, Othman, & Mohd Solehin, 2015). Perera, Bentota, Ratnasekara and Senanayake (2013) mentioned that heterosis value was important in determining the successful production of hybrid varieties and both value, either negative or positive, were important in crop improvements.

CONCLUSION

The important outcome of this study is the knowledge and information on various genetic variations in grain quality and selected yield traits in parental lines and F_1 generations of rice that can be used for future rice breeding programs. It can be concluded that the characteristics of long and slender grain found in parents Basmati 370, MRQ50, MRQ74, MR219, and Mahsuri Mutan showed that they can be used in breeding rice for production of long grain rice. The study also observed good performances in grain shape of crosses RU14387, RU14701, and RU14702. Cross RU14387 also showed high performances in some yield traits and information on heterosis value can be further evaluated for the development of rice lines with good quality traits as well as yield related traits. In future, rice production

and quality improvements, evaluation of selected lines can be conducted followed by the implementation of molecular study to characterize specific desired genes for grain quality trait improvements.

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Effects of Drought Stress on Accumulation of Proline and Antioxidant Enzymes in the Different Varieties of Yardlong Beans

M. W. Lestari[1]*, Sugiarto[1] and Kuswanto[2]

[1]Agriculture Faculty, University of Islam Malang, 65144 Malang, East Java, Indonesia
[2]Faculty of Agriculture, Brawijaya University, 65145 East Jawa, Malang, Indonesia

ABSTRACT

The effect of drought stress on biochemical activities included changes in the concentrations of proline and the activity of antioxidant. This study aimed to determine the effect of drought stress on proline activity and antioxidant enzymes catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD) on varieties of yardlong beans. The first factor was the variety of yardlong beans, including Brawijaya Ungu-1 (BU-1), Brawijaya Ungu-2 (BU-2), Brawijaya Ungu-3 (BU-3), Brawijaya Ungu-4 (BU-4), Brawijaya Ungu-5 (BU-5), Brawijaya Ungu-6 (BU-6), Brawijaya-4 (Br-4), and Bagong-2 (Bg-2). The second factor was drought stress level consisting of 50% and 100% field capacity (FC) as a control. The results showed that the concentrations of proline and activity of antioxidant enzymes increased drought stress. BU-4 variety experienced the highest enhancement of proline, and BU-2 variety experienced the highest enhancement of catalase and superoxide dismutase while Br-4 variety experienced the highest enhancement of peroxidase. So BU-4, BU-2, and Br-4 varieties were said to be more tolerant to drought stress, based on proline and antioxidant enzymes accumulation.

Keywords: Antioxidants, drought stress, proline, yardlong beans

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E-mail addresses:
mwlestari@yahoo.com/mwlestari@unisma.ac.id (M . W. Lestari)
sugiarto_unisma@yahoo.co.id (Sugiarto)
kuswantoas@yahoo.com (Kuswanto)
* Corresponding author

INTRODUCTION

The yardlong bean is generally green, light green, or white green, all with different advantages, but a type of bean not widely known to the public is purple pods yardlong beans. Purple pod yardlong beans contain protein and anthocyanins substances, which are very beneficial to the body.

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The production of yardlong beans is currently low and insufficient for consumption (Kuswanto, 2002). This stems from the decreased production of yardlong beans from one year to another. Low production of yardlong stems from lack of area in which to cultivate plants. Therefore, it is necessary to use marginal lands, such as drylands, acid lands, and/or low fertility lands. The low productivity of yardlong beans also stems from a lack of availability of high yield, drought resistant, and pest resistant varieties.

Drought stress is one of the most endemic environmental factors for crop growth and production, and is the leading cause of crop losses in the world (Bray, 2002; Bruce, Edmeades, & Barker, 2002). The presence of yardlong bean varieties that are tolerant to drought stress and high yield results offer hope of developing yardlong bean crops on dry land.

Drought stress may affect the biochemical activity of the plant, including changes in hormone concentration for example, abscisic acid, proline content (Cárdenas-Avila et al., 2006; Vajrabhaya, Kumpun, & Chadchawan, 2001), and increased antioxidant enzymatic activity (Harinasut, Poonsopa, Roengmongkol, & Charoensataporn, 2003) to destroy reagent oxygen species (ROS).

Proline accumulation occurs in plants with drought stress or a deduction of soil moisture, so the level of tolerance of plants was often associated with a big accumulation of its proline. Mapegau (2010), Mathius *(*2004), Bandurska (2000), Kishor et al. (2005), Cárdenas-Avila et al. (2006), and Esfandiari (2008) research showed that the content of free proline in plants increased with the increasing levels of water stress.

Antioxidants played a significant role as a plant defence against oxidative stress. Oxidative stress, by increasing the level of ROS, can lead to growth inhibition. Plant cells require a mechanism to regulate the concentration of intracellular ROS by *scavenging* ROS (Bailey-Serres & Mittler, 2006). ROS *scavenging* mechanisms were done by plants through antioxidant enzyme catalase, peroxidase, and superoxide dismutase (Harinasut et al., 2003) and non-enzyme antioxidant (ascorbic, glutathione, α-tocopherol, and β-carotene). These compounds played a key role in changing the toxic oxygen compounds into non-toxic compounds. Bailey and Mittler (2006) showed that there was an enhancement in the activity of antioxidant enzymes in plants that experience abiotic stress, such as drought stress, because the plants employ defence mechanisms and increase the activity of antioxidant enzymes to destroy ROS.

This study aimed to determine the effect of drought stress on proline activity and antioxidant enzymes catalase, peroxidase, and superoxide dismutase on different varieties of yardlong beans, and to know which varieties are most tolerant of drought stress, based on the level of accumulation of proline and antioxidant enzymes.

MATERIALS AND METHODS

Time and Place

Planting was done in the greenhouse of Faculty of Agriculture, University of Islam Malang in dry season and aluvial land from March to June 2017, while the proline and antioxidant enzyme analysis was performed at the Laboratory of Science Faculty, Brawijaya University.

Research Methods

The design used a randomised block factorial design. The first factor was eight varieties of yardlong beans with unknown tolerance to drought stress, consisting of six varieties of purple yardlong beans, namely Brawijaya Ungu-1 (BU-1), Brawijaya Ungu-2 (BU-2), Brawijaya Ungu-3 (BU-3), Brawijaya Ungu-4 (BU-4), Brawijaya Ungu-5 (BU-5), Brawijaya Ungu-6 (BU-6), and two varieties of green yardlong beans, namely Brawijaya-4 (Br-4) and Bagong-2 (Bg-2). The second factor was the drought stress, which consisted of 100% and 50% field capacity (FC). Each treatment combination had three samples and was repeated three times. Watering was done every day, maintaining 100% FC until three weeks after planting. The drought test was done after this three week period, and the plant was given threats by reducing the water supply until it reached 50% FC, and this was carried out until its harvest time. Meanwhile, the water content was maintained at 100% FC for the control treatment. Watering occurred every other day, after finding out the water needs by measuring the capacity of the field.

Proline and Antioxidant Enzyme Activity Analysis

Leaf proline analysis was performed using the method of Bates et al. (1973). Extraction was done on every third leaf of all varieties studied. Measurement of proline accumulation and antioxidant enzyme was done on every third leaf , leaves that were not too young (shoots) or too old, and before the generative phase. Antioxidant enzyme activity was tested using three kinds of enzymes-catalase enzyme (CAT), peroxide enzyme (POX), and superoxide dismutase enzyme (SOD).

Estimation of Proline

Approximately 0.5 g of fresh or frozen plant material was homogenized in 10 mL of 3% aqueous sulfosalicyclic acid and filtered through Whatman's No. 2 filter paper. Two ml of filtrate was mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4 mL toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520 nm with a Bausch and Lomb Spectrometer 7 IO'. Appropriate proline standards were included for alculation of proline in the sample.

Estimation of Antioxidant Enzyme

Antioxidant enzyme activity was tested using three kinds of enzymes which were the enzyme catalase, peroxidase and superoxide dismutase enzyme.

Five hundred milligrams of frozen material was homogenized in 5 mL of ice-cold 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM PMSF. The extract was centrifuged at 4°C for 20 min at 12,500 rpm. The supernatant was used for enzyme assay.

The activity of enzyme catalase was measured using the method of Chandlee and Scandalios (1984), with modification. The assay mixture contained 2.6 mL of 50 mL of 50 mM potassium phosphate buffer (pH 7.0) 0.4 mL, 15 mM H_2O_2, and 0.04 mL of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in units 1 mM of H_2O_2 reduction minute^{-1} mg protein^{-1}.

Peroxidase was assayed by the method of Kumar and Khan (1982). Assay mixture of peroxidase contained 2 mL of 0.1 M phosphate buffer (pH 6.8), 1 mL of 0.01 M pyrogallol, 1 mL of 0.005 M H_2O_2, and 0.5 mL of enzyme extract. The solution was incubated for 5 min at 25°C, after which the reaction was terminated by adding 1 mL of 2.5 N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at zero time. The activity was expressed in unit mg^{-}1 protein. One unit is defined as the change in the absorbance by 0.1 min^{-1}mg^{-1} protein.

Crude enzyme extract was prepared, for the assay of superoxide dismutase by the method of Hwang et al. (1999). Extraction (1 g) of fresh tissue was homogenized with 10 mL of ice-cold 50 mM sodium phosphate buffer containing 1 mM PMSF. The extract was filtered through a double-layered cheese cloth. The extract was centrifuged at 12,500 rpm for 20 min at 4°C. The supernatant was saved and made up to 10 mL with the extraction buffer and used for estimation of the SOD enzyme activity. The enzyme protein was determined by the Bradford (1976) method.

Superoxide dismutase activity was assayed as described by Beauchamp and Fridovich (1971). The reaction medium was prepared and to 3 ml of reaction medium, 1 mL of enzyme extract was added. The reaction mixture contained 1.17×10^{-6} M riboflavin, 0.1 M methionine, 2×10^{-5} potassium cyanide, and 5.6×10^{-5} M nitroblue tetrasodium salt (NBT), dissolved in 0.05 M sodium phosphate buffer (pH 7.8). The mixture was illuminated in glass test tubes by two sets of Philips 40 W fluorescent tubes. Illumination started to initiate the reaction at 30°C for 1 h. Those without illumination were saved as blank and kept in the dark. The absorbance was read at 560 nm in the spectrophotometer against blank. Superoxide dismutase activity was expressed in units. One unit is defined as the amount of change in the absorbance by 0.1 h^{-1} mg protein^{-1} under the assay condition (Cherry, 1963).

Data Analysis

Observed quantitative data was statistically analysed using ANOVA with *SPSS software Release 15*. If significant treatment gave a real effect, it was continued by the

Duncan test 5%. Reduction percentage was calculated as follows: % reduction = (Yp –Ys)/Yp × 100 (Choukan, Taherkhani, Ghannadha, & Khodarahmi, 2006), where Yp is the yield under non-stress condition and Ys the yield under stress.

RESULTS AND DISCUSSION

Variations of response to drought stress can be determined by comparing the concentration of proline accumulation in the plants without drought stress to those experiencing drought stress treatment. Proline accumulation in the plants experiencing drought stress showed an increase compared with the controls.

All varieties of yardlong beans that were tested showed an increased accumulation of proline stemming from drought stress, but each variety had a different rate of proline accumulation (Figures 1 and 2). This showed that each variety had a different tolerance level. BU-4 experienced enhanced proline accumulation (166%), which was highly significant with other varieties, while BU-2 showed the lowest enhanced proline accumulation (53%).

Proline was the most stable and least inhibited amino acid when compared to the others which was synthesized in the phloem tissue of plants, roots, and seeds (Shimpson, 2001; Deivanai, 2010). The decrease of the water content induces the plants to produce proline to maintain cell turgor pressure (De Ronde, Van Der Mescht, & Steyn, 2000). In drought stress conditions and a variety of other osmotic stress, some plants had adaptation mechanisms that included the ability to synthesize the compound osmoprotectant, or a suitable solution. Osmoprotectant is a non-toxic solution that can be accumulated to a certain extent without disturbing the metabolism of plants, which usually consisted of several chains of amino acids. Proline accumulation served as a source of cytoplasmic osmoticum

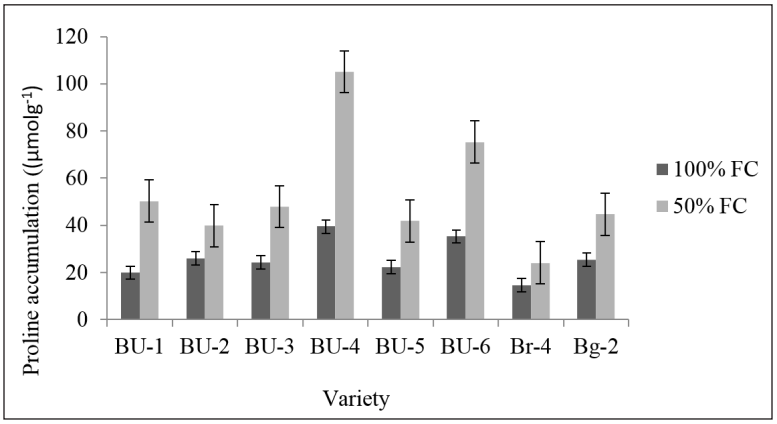


Figure 1. Proline accumulation (µmolg^{-1}) of yardlong beans stemming drought stress
Note: BU-1: Brawijaya Ungu-1, BU-2: Brawijaya Ungu-2, BU-3: Brawijaya Ungu-3, BU-4: Brawijaya Ungu-4, BU-5: Brawijaya Ungu-5, BU-6: Brawijaya Ungu-6, Br-4; Brawijaya-4, Bg-2: Bagong-2

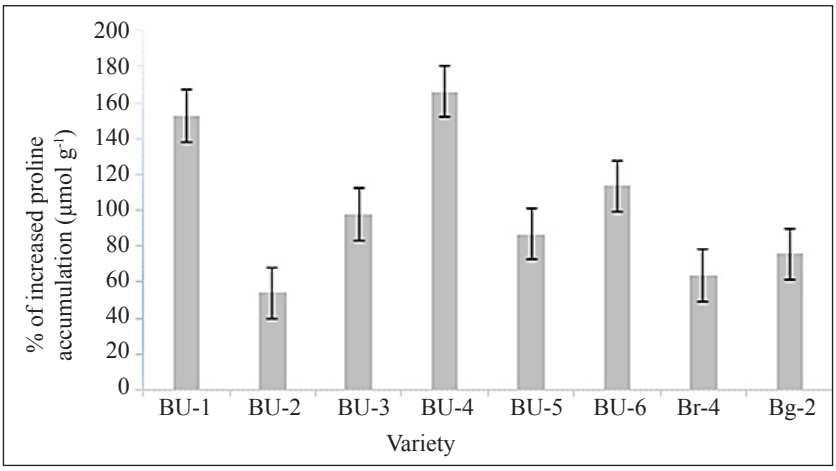


Figure 2. The increased level of proline accumulation of yardlong beans stemming from drought stress
Note: BU-1: Brawijaya Ungu-1, BU-2: Brawijaya Ungu-2, BU-3: Brawijaya Ungu-3, BU-4: Brawijaya Ungu-4, BU-5: Brawijaya Ungu-5, BU-6: Brawijaya Ungu-6, Br-4; Brawijaya-4, Bg-2: Bagong-2

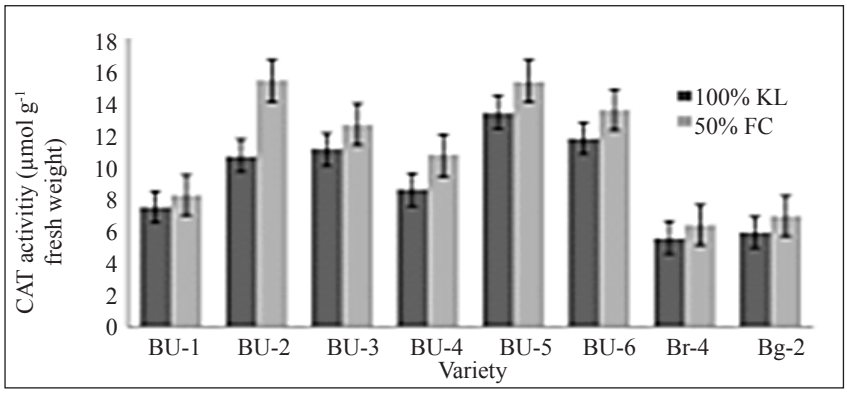
(Liu & Baird, 2003), as a patron and protector of the cytoplasmic enzyme cellular structure (Gibon, Sulpice, & Larher, 2000). Bandurska (2000) research showed that in wheat plants, proline increased ten-fold in plants that experienced drought stress. In a particular research on soybean plants, proline was more common in varieties that were drought-tolerant; produced in a plant's effort to increase the osmotic balance in drought conditions. However, some studies mentioned that the proline accumulation in response to various conditions of osmotic stress was very common in plants (Delauney & Verma, 1993).

The treatment of drought stress caused an increased activity of catalase, peroxidase, and superoxide dismutase enzymes, but the rate of enhancement of antioxidant enzyme activity of each variety was different (Figures 3 and 4).

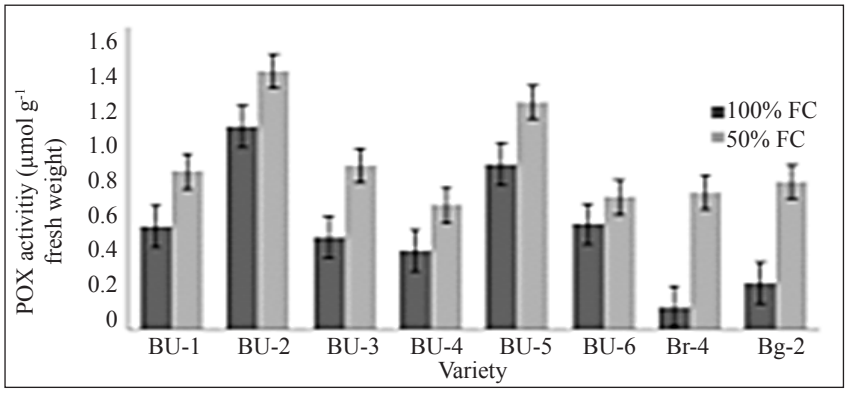
Antioxidant enzymes of catalase, peroxidase, and superoxide dismutase were supposed to play a vital role in the drought stress of the tolerance of pods yardlong beans. The increased activities of antioxidant enzymes in the pod's yardlong beans can inhibit tissue damage that survives in the drought stress condition.

Unyanyar and Cekic (2005) stated that the activity of antioxidant enzymes in plants under drought stress conditions was believed to be an indicator of its level of tolerance in facing drought stress conditions. Detoxification enzymes, which included catalase, peroxidase, and superoxide, were the enzymes that played a significant role in controlling the rate of oxidation and were associated with the tolerance of abiotic stresses.

The enhancement activity of catalase, peroxidase, and superoxide enzyme under the drought stress condition showed the



(a)



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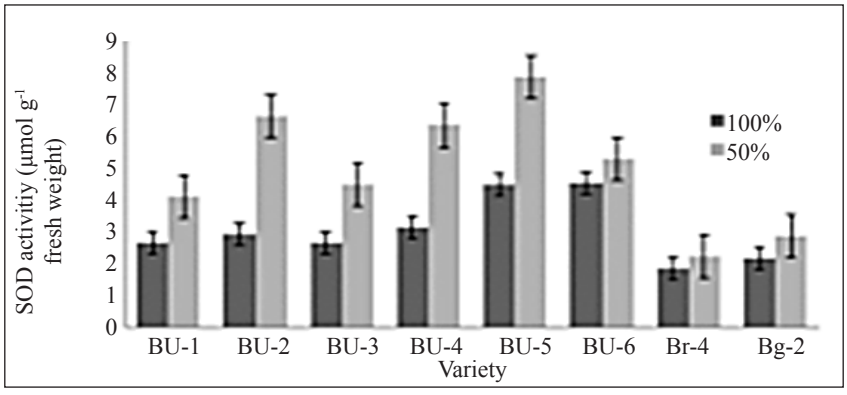
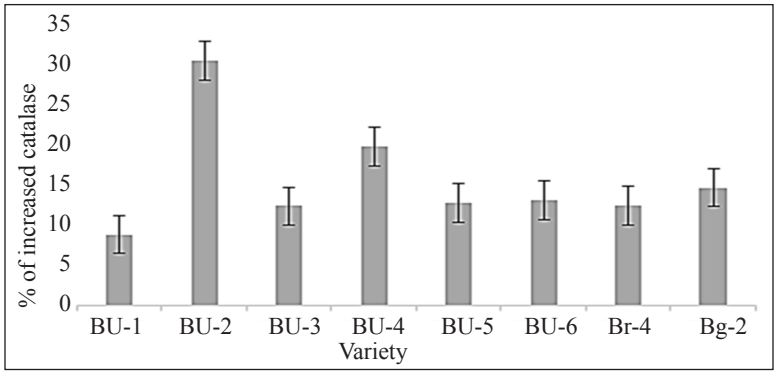
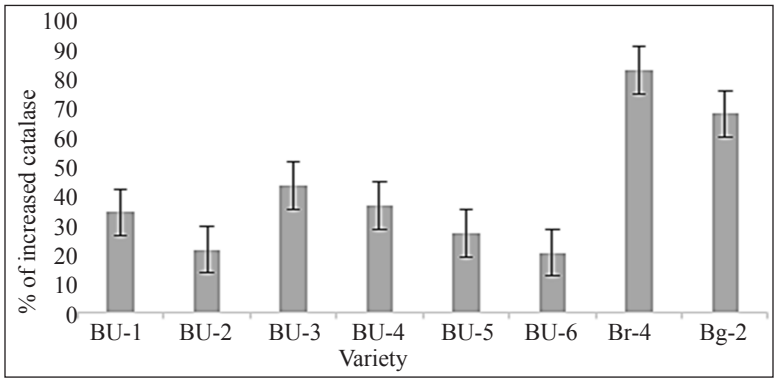


(c)

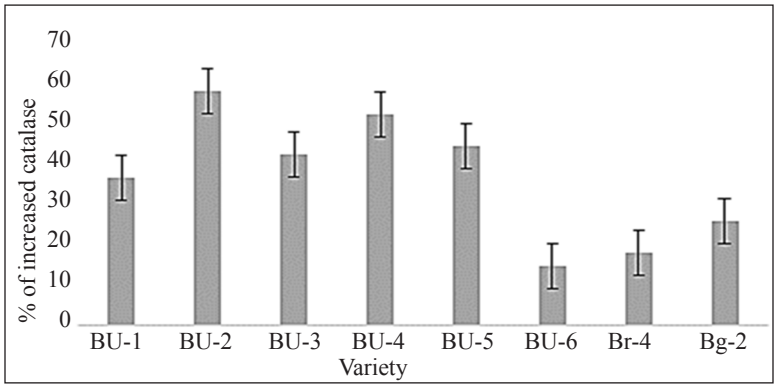
Figure 3. The enzyme activity of: (a) catalase; (b) peroxidase; (c) and superoxide dismutase enzymes, of yardlong beans in the control condition and experiencing drought stress
Note: BU-1: Brawijaya Ungu-1, BU-2: Brawijaya Ungu-2, BU-3: Brawijaya Ungu-3, BU-4: Brawijaya Ungu-4, BU-5: Brawijaya Ungu-5, BU-6: Brawijaya Ungu-6, Br-4; Brawijaya-4, Bg-2: Bagong-2



(a)



(b)



(c)

Figure 4. The enhancement level of: (a) enzyme catalase; (b) peroxidase; and (c) superoxide dismutase activity of yardlong beans stemming drought stress

Note: BU-1: Brawijaya Ungu-1, BU-2: Brawijaya Ungu -2, BU-3: Brawijaya Ungu-3, BU-4: Brawijaya Ungu -4, BU-5: Brawijaya Ungu -5, BU-6: Brawijaya Ungu -6, Br-4; Brawijaya-4, Bg-2: Bagong-2

plant adaptation in the face of increasing ROS stemming from the imposed drought stress conditions. The enhancement activity of those three enzymes showed that they played a key role in the detoxification process of hydrogen peroxide.

The highest enhancement activity of catalase and superoxide dismutase antioxidant enzymes stemming from drought stress was found in BU-2, (30% dan 56%). The highest enhancement activity of peroxidase antioxidant enzymes was in Br-4, i.e., 83%. The activity of catalase, peroxidase, and superoxide dismutase enzymes will be different, depending on tissues and species. This was proved by the drought stress, which increased the activity of peroxidase and superoxide in sprouts J. Oxycedrus (Alguacil, Caravaca, Díaz-Vivancos, Hernández, & Roldán, 2005).

The activity of enzymes catalase, peroxidase, and superoxide dismutase generally increased in plants under drought stress conditions, and, in some cases this activity gave a good indication of the level of tolerance (Criszar et al., 2007). Overexpression of superoxide dismutase increased the tolerance level of oxidative stress. Superoxide dismutase was an important antioxidant enzyme that functioned in cells to prevent the effects of ROS. Superoxide dismutase enzyme dismutated the destruction of ROS and formed H_2O_2, to be detoxified by catalase and peroxidase.

The difference of the enhancement activity of catalase, peroxidase, and superoxide in yardlong beans stemmed from the genetic diversity that also determined its adaptability and responses to the occurring environmental stresses. This indicated that the antioxidant system played a significant role in the tolerance of plants, when facing abiotic stress.

CONCLUSION

All varieties of yardlong beans that were tested showed an increased accumulation of proline and antioxidant stemming drought stress. BU-4 variety experienced the highest enhancement of proline, and BU-2 variety experienced the highest enhancement of catalase and superoxide dismutase while Br-4 variety experienced the highest enhancement of peroxidase. So BU-4, BU-2, and Br-4 varieties were said to be more tolerant to drought stress, based on proline and antioxidant enzymes accumulation.

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Intraspecific Morphological Variation of Crossbanded Barb, *Puntioplites Bulu* (Bleeker, 1851) From Selected River in Peninsular Malaysia Based On Truss Network Analysis

Intan Faraha A. Ghani[1,2]*, Aziz Arshad[1], Sharr Azni Harmin[2], Annie Christianus[1] and Muhammad Fadhil Syukri Ismail[1]

[1]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[2]Department of Science and Biotechnology, Faculty of Engineering and Life Sciences, Universiti Selangor (UNISEL), 45600 Bestari Jaya, Selangor, Malaysia

ABSTRACT

The study on intraspecific variation of wild crossbanded barb, *Puntioplites bulu* stock in Peninsular Malaysia was investigated based on truss network analysis constructed from the fish body. A total of 90 samples were collected from three different populations, namely Kelantan River, Perak River and Pahang River (n= 30/population). The 22 truss characters were standardised by an allometric formula and analysed by multi and uni-variate analysis. The results showed significant differences ($p< 0.05$) between mean of the three populations. The loadings of the first and second discriminant function accounted for 81.3% and 18.7% respectively in terms of group variability, and they explained 100% of the total among group variability. The results showed significant variation of *P. bulu* in morphology based on truss network caught from three different populations. The morphological differences were located mainly on the head region, body depth and median region. These findings indicate the presence of morphometric variations between three populations of *P. bulu* in Peninsular Malaysia based on their locations.

Keywords: Morphology, *Puntioplites bulu,* stock identification, truss network, variation

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E-mail addresses:
intanfaraha@unisel.edu.my (Intan Faraha A. Ghani)
azizarshad@upm.edu.my (Aziz Arshad)
sharrazny@unisel.edu.my (Sharr Azni Harmin)
annie@upm.edu.my (Annie Christianus)
fadhil@upm.edu.my (Muhammad Fadhil Syukri Ismail)
* Corresponding author

INTRODUCTION

Stock identification is essential in fish stock assessment and sequentially for effective fisheries management (Turan, 2004). Accuracy of stock has remained the core challenge for fisheries scientists, as

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it is not possible to directly map how far the larvae scatter. Thus, stock structure has been studied using a variety of techniques, mostly on genetic and phenotypic variations. The study on phenotypic variation between stocks can deliver as an indirect basis or initial step for stock structure. Although it does not provide direct evidence based on genetic variation, this method may be applicable for short term environmentally induced variation study (Begg, Friedland, & Pearce, 1999).

Morphometric characteristics is a quantitative description that have been effectively used for taxonomic inferences, based on a set of traditional measurements (Hubbs & Lagler, 1947). It can be defined as a technique for describing size and shape variations and has been commonly used in fisheries biology for measuring discreteness and relationship among taxonomic categories (Strauss & Bookstein, 1982). Although the traditional measurement has been criticised, it is still considered a useful tool for fish identification. Regardless, a new approach in morphometric measurement called a truss network analysis is being used, especially for stock differentiation since this system covers the entire fish in a uniform network, and theoretically would increase the probability in extracting morphometric differences within and between species or populations (Abdurahman et al., 2016; Hossain, Nahiduzzaman, Debasish, Khanam, & Alam, 2010; Muchlisin, 2013; Turan, 2004; Turan, Erguden, Gurlek, Basusta, & Turan, 2004;). This method is considered revolutionary to overcome weakness of traditional morphometric measurements, which were limited to certain body structures, such as fin and not being able to enumerate body shape (Mojekwu & Anumudu, 2015).

Crossbanded barb, *Puntioplites bulu*, or locally known as "Tenggalan", is a cyprinid fish of the genus *Puntioplites* which are naturally found throughout Southeast Asia including Indonesia (Kalimantan), Malaysia (Perak, Pahang, Kelantan, Johor, and Sarawak), Brunei, and peninsular Thailand (Ambak, Isa, Zakaria, & Ghaffar, 2010). This omnivorous species feeds mainly on submerged aquatic plants, algae, and benthic organisms often occurring in mid water benthic level that can normally be found in large lowland rivers and lakes including streams and coastal rivers (Allen, 2011). *Puntioplites bulu* is commercially important and sought after by anglers and fishermen (Ambak et al., 2010) for its high price of RM 30–50/kg live weight. It also has a good taste. Nevertheless, in recent years, this species is at risk from fishing pressure and habitat degradation, resulting from intensive development activities (Allen, 2011). In Malaysia, total landing production of *P. bulu* recorded a falling trend; shrinking from 93.68 tonnes in 2005 to only 43.44 tonnes in 2015 (Department of Fisheries Malaysia [DOF], 2005; 2015).

In order to prevent the decline in *P. bulu* landing in Malaysia, a study on the stock structure of this species is essential but it is yet to be done. Some studies examined the distribution status of *P. bulu* (Zarul et al., 2012) and phenotypic variation of two

different *Puntioplites* species in Peninsular Malaysia (Zakaria-Ismail, 1988). Therefore, in this study, examination of *P. bulu* stock structure in Peninsular Malaysia was carried out from phenotypic aspects based on truss network analysis, in order to determine its relationship between morphological variations and geographical with origins of individuals from different populations.

MATERIALS AND METHODS

All samples (n = 90) with 30 samples for every population were collected from three different sites (Kelantan River, Perak River and Pahang River) (Figure 1) between January 2016 and January 2017. Total landing of *P. bulu* from these three states (Perak, Pahang, Kelantan) was the highest in Peninsular Malaysia with 14.35 tonnes, 5.35 tonnes, 4.71 tonnes respectively (DOF, 2015). In this context, the evaluation of stock structure between these three important rivers would be important for management purposes of this species.

The sampling locations and certain biological aspects of the samples are presented in Table 1. The collected samples were systematically identified based on literature findings (Ambak et al., 2010). The main morphological feature that distinguishes this species from the others in the genus of *Puntioplites* is the patches of darkened scale which form oblique cross bands (Ambak et al., 2010). All fish were preserved with ice before transported to the research laboratory for further analysis.

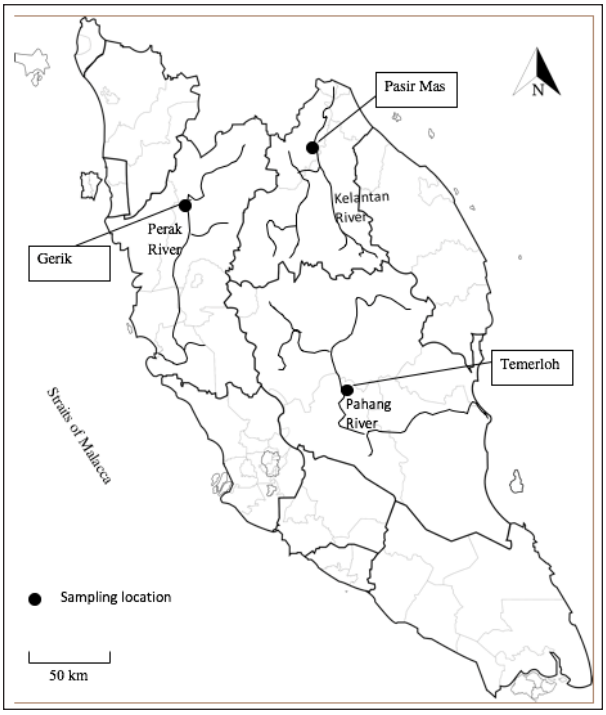

Figure 1. The map of the sampling sites of *P. bulu* in Kelantan River, Perak River and Pahang River

Table 1
Location, sampling sites and sample size of P. bulu *sample*

Sampling sites	Locations	Sample size	SL (cm) (min-max)	Mean SL (cm)	BW(g) (min-max)	Mean BW (g)
Kelantan River	6°02'N, 102°08'E (Pasir Mas Area)	30	12.3-24.5	16.17±3.1	59.9-172	144.7 ± 34.1
Perak River	5°24'N, 101°09'E (Gerik Area)	30	12.1-20.5	14.86±1.9	55-196.8	101.1 ± 36.1
Pahang River	3°31'N, 102°25'E (Temerloh Area)	30	9.7-14.5	11.53±3.1	30.4-104.9	46.9 ± 16.5

Note: SL: standard length (cm), BW: body weight (g) of samples

In the laboratory, body weight of the fish was recorded with a digital balance to the nearest 0.01g and internal gonad inspection for sex determination was done after obtaining the truss network analysis measurement. Sex of the samples were determined based on the gonad maturation stages as well as gonad external morphology appearance, such as colour, shape, size and degree of visualisation of the gonads (De Souza et al., 2011; Soetingya, Suryobroto, Kamal, & Boediono, 2017). Prior to analysis, the fish were defrosted and placed on a polystyrene board. The fish were then located on their left side on acetate sheets, with the body posture and fins placed into a natural position (Elliot, Haskard, & Koslow, 1995; Turan, 1999). Dissecting needles were used by piercing the acetate sheet in order to mark the 11 truss homologous landmark resulted in 22 liner measurements. The 11 landmarks produced for *P. bulu* are illustrated in Figure 2. All measurements were performed using a Mitutoyo digital calliper to the nearest 0.01 mm. All measurements were done four times in order to get the accuracy of the measurements.

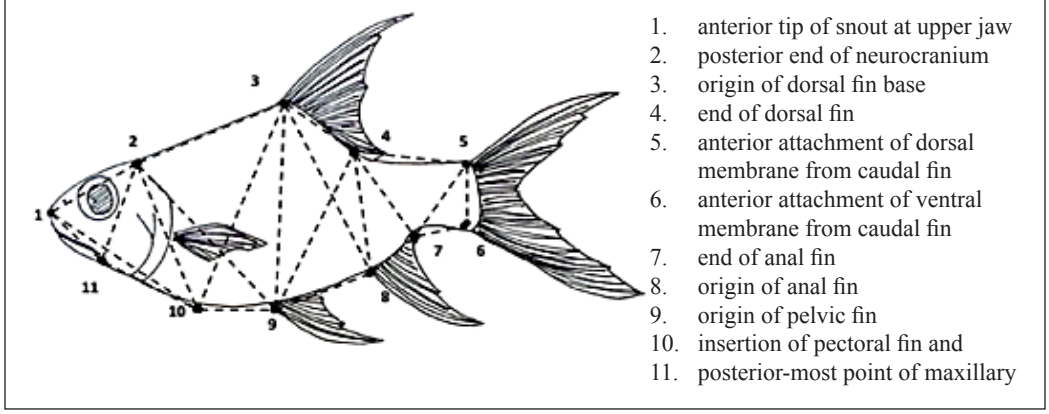

Figure 2. Locations of the 11 landmarks (illustrated as black dot) for constructing the truss network on *P. bulu* and distance measured between the dots as line

Prior to analysis, each measurement value was standardised according to the following formula to reduce the allometric effects on these value (Komiya, Fujita & Watanabe, 2011).

$$Yi = \log (Mi) - b \{\log (SLi) - \text{mean} (\log(SL)\}$$

Where Yi and Mi are the adjusted and original values for characters in individual i (i=1, …..N), SLi is the standard length and b is the slope regression coefficient of the logarithm M on the logarithm of SL using all fish in all groups.

The transformation will reduce the size effect on different specimen sizes as variation should be attributable to body shape differences and not associated to size of fish (Turan, 2004). Standard length (SL) was used as a common factor since it correlates strongly with other morphometric characters (Abdurahman et al., 2016). The size adjustment efficiency was assessed by testing the significance of the correlation and incomplete removal of size effects can be determined by a significant correlation between standard length and transformed variables (Turan, Oral, Ozturk, & Duzgunes, 2006).

All transformed data was evaluated using one-way ANOVA and multivariate analysis of discriminant function analysis (DFA) using Statistical Package for Social Science (SPSS) version 16.0 software for windows. One-way ANOVA was used to classify any significant variables among the truss measurement and a post-hoc test was carried out when significant results ($p < 0.05$) were obtained from the ANOVA result in order to investigate the groups that were significant to each other. The eigenvalues, cumulative percentage, percentage of total variances and canonical correlation were generated in this study. The DFA combines a selection of body shape measures into a linear mode to produce a mathematical function to be used to classify individuals into group. The group separation was is in a scatterplot of a function 1 versus function 2 (Figure 3).

RESULTS AND DISCUSSION

The descriptive data of SL and BW including mean values and standard deviation for each sample are presented in Table 1. None of the 22 transformed truss measurement showed a significant correlation with the standard of the fish indicating that the allometric formula has successfully removed the size effect from the data. Univariate statistics results in Table 2 showed that out of 22 truss measurements, 17 measurements were significantly different ($p < 0.05$) among three different populations except for measurements (1-2, 1-11, 9-10, 4-7, 6-5). The morphometric characters did not differ significantly ($p > 0.05$) between both sexes. Therefore, data for both sexes were pooled for subsequent analysis. According to Kocovsky, Adam & Bronte (2009), recommended ratio of the sample number relative to the landmark positions must be at least 3-3.5 times in order to avoid false conclusions on variations among groups. In

this analysis, 22 characters were used and the number of fish examined (N) relative to the number of truss elements (P), N:P ratio was 4.09 for all 22 truss measurements. Summary of the relevant statistics of the DFA for *P. bulu* from three populations are shown in Table 3.

The eigenvalue designates the percentage of variance explained, with a large eigenvalue is related to a strong

Table 2
Univariate statistics testing differences between samples from all truss measurements

Variables	Characters	F	Significance
V1	1-2	2.985	0.056
V2	2-10	19.049	0.000*
V3	11-10	7.362	0.001*
V4	1-11	1.468	0.236
V5	2-11	10.823	0.000*
V6	1-10	7.373	0.001*
V7	2-3	3.776	0.027*
V8	3-9	9.258	0.000*
V9	9-10	0.492	0.613
V10	2-9	5.425	0.006*
V11	3-10	4.549	0.013*
V12	3-4	8.380	0.000*
V13	4-8	7.917	0.001*
V14	8-9	6.956	0.002*
V15	4-9	6.654	0.002*
V16	3-8	7.197	0.001*
V17	4-5	4.672	0.012*
V18	5-7	4.710	0.011*
V19	7-8	5.570	0.005*
V20	4-7	2.997	0.055
V21	6-7	9.130	0.000*
V22	6-5	0.038	0.963

*Indicate significance level at $p<0.05$. Characters are defined in Figure 2.

Table 3
Summary of canonical discriminant for P. bulu *from three populations*

Function	Eigen-value	Variance (%)	Cumulative (%)	Canonical Correlation
1	1.514	81.3	81.3	0.776
2	0.394	18.7	100.00	0.508

First two canonical discriminant functions were used in the analysis.

function. The canonical correlation summarises the degree of relatedness between the groups (populations), where larger value indicates greater degree of association connection and 1.0 considered as their utmost value. A total of 17 significant variables explain 100% of total variability with the first function described 81.3% of discriminating power, while second function with 18.7% respectively (Table 4).

Table 4
Standardised canonical discriminant function coefficients

Variables	Characters	Function 1	Function 2
V2	2-10	-0.509*	0.360
V21	6-7	0.367*	0.127
V13	4-8	-0.329*	0.227
V6	1-10	-0.323*	0.178
V14	8-9	0.320*	-0.117
V3	1-11	-0.318*	0.216
V16	3-8	-0.185	0.571*
V15	4-9	-0.161	0.571*
V8	3-9	-0.267	0.549*
V10	2-9	-0.149	0.511*
V11	3-10	-0.100	0.506*
V18	5-7	0.131	0.486*
V19	7-8	-0.193	0.452*
V7	2-3	0.104	0.449*
V5	2-11	-0.347	0.436*
V12	3-4	-0.295	0.417*
V17	4-5	0.227	0.291*

Pooled within-groups correlations between discriminating variables and standardised canonical discriminant functions. Characters are defined in Figure 2.
* denotes the largest correlation between each variable and any discriminant function.

The truss distances with loadings on first factor (DF1) were 1-2, 6-7, 4-8, 1-10, 8-9, 1-11 while second factor (DF2) were loaded by characters 3-8, 4-9, 3-9, 2-9, 3-10, 5-7, 7-8, 2-3, 2-11, 3-4, 4-5. The characters which contributed to Function 1, were strongly correlated to head region whilst characters which contributed to Function 2, were strongly correlated to median region of the body implying that these characters are the most important in the description of population characteristics. The discrimination of *P. bulu* from three different populations based on truss network measurements was clearly illustrated in scatter plot as shown in Figure 3. The results showed that Function 1 successfully discriminated the individuals into three independent groups.

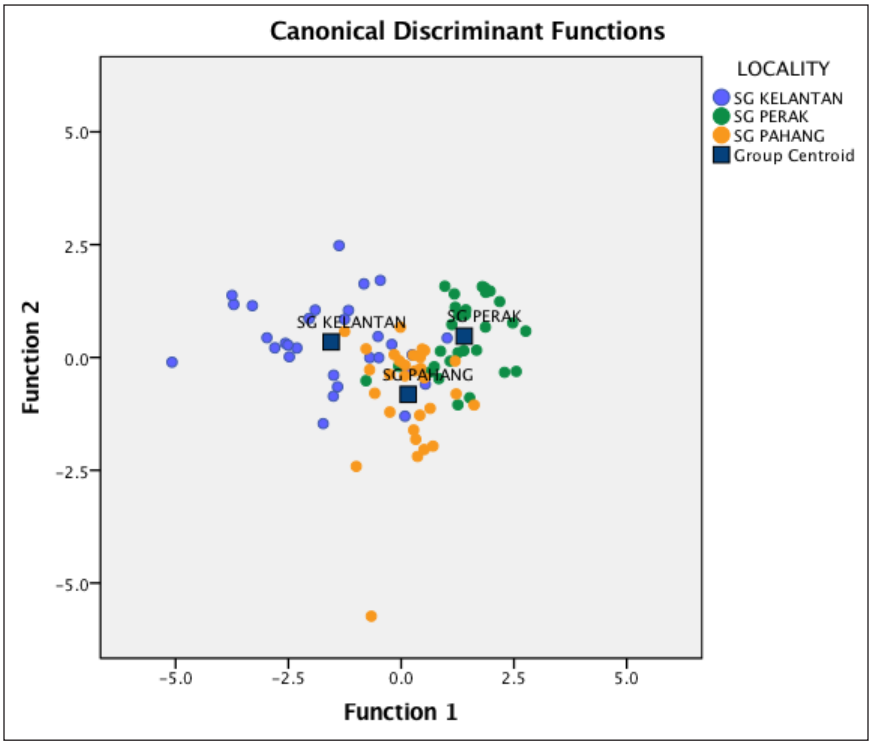

Figure 3. Discriminant scatter plot of *P. bulu* from Kelantan River, Perak River and Pahang River

The present study revealed evidence of significant morphometric heterogeneity among samples from three different rivers in Peninsular Malaysia. Findings generated from DFA showed that truss measurements that represent body depth and head region were the most discriminate characters among all the samples. A visual examination of the plots through F1 and F2 scores indicated that samples were grouped into three major areas with a certain overlap among them. Both higher mean values functions were recorded for *P. bulu* from the Perak River, in (V2-head region) and (V16-median region of the body). The main differences between these populations were observed in the head region area. The variation among the stocks could be due to environmental response, geographic position, ecological change and uncommon hydrological conditions (Khan, Miyan, & Khan, 2012; Mir, Sarkar, Dwiyedi, Gusain, & Jena, 2013; Siddik, Hanif, Chaklader, Nahar, & Fotedar, 2016). Decreasing food availability and heavy pollution load may lead to smaller body depth and head length (Khan et al., 2012). The Kelantan River population showed a comparatively smaller body depth which may be related to water quality and uncommon hydrological conditions in that area as argued by Radhi, Roshaliney and Zarul (2017). Mir et al. (2013) noted

similar observation in Roho lobeo, *Labeo rohita* from Ganga basin India, where the uncommon hydrological conditions such as differences in alkalinity, temperatures, current pattern and turbidity contribute to phenotypic plasticity. Komiya et al. (2011) studied the morphological differences of Japanese gudgeon, *Sarcocheilichthys* between two different habitats in Lake Biwa Japan, and found significant morphometric divergence where fish inhabiting pebbly zones had e streamlined body with a short round head compared with fish inhabit rocky zone found to have deep compressed body with a long head. According to Muchlisin (2013), the morphometric system generally showed that the head and caudal region were major characters in distinguishing the group and same similar findings have been observed in Ariid catfishes, *Plicofollis* spp. (Abdurahman et al., 2016) in Peninsular Malaysia as well as Pangasiids catfishes, *Pangasius* spp and Mahseers*, Tor* spp. in the Pahang River (Akhbar, Jalal, Nasuha, Faizul, & Ambak, 2015; Akhbar et al., 2016).

The variation among *P. bulu* from three different populations based on the truss network system suggests a direct relationship between the level of morphometric divergence and geographic separation. The detected variation may signify reproductive isolation among local *P. bulu* populations. It is quite difficult to explain the reasons for morphological variations between populations but it is expected that these differences may be genetically associated or might be related by phenotypic plasticity in response to different environmental factors (Murta, Pinto, & Abaunza, 2008). Fish are very subtle to environmental changes and demonstrate greater variances in morphology both within and between populations compared with other vertebrates for their phenotypic plasticity. This allows them to adapt to environmental changes, which modifies their behaviour and physiology, leading to changes in their morphology, reproduction, and survival that reduce effects of environmental changes (Hossain et al., 2010; Wimberger, 1992). Consequently, effects of some environmental factors, such as temperature, food availability, and migration distance may affect and determine the phenotypical discreteness of *P. bulu*. Phenotypic variation within the same species in different populations may have advantages in stock structure analysis, particularly when time is limited for significant genetic differentiation to accumulate among populations. This method has also been successfully used in stock identification and to differentiate stock of horse mackerel, *Trachurus trachurus* (Murta et al., 2008), Abu mullet, *Liza abu* from three different populations (Turan & Erguden, 2004), and anchovy, *Engraulis encrasicolus* in the Mediterranean seas (Turan et al., 2004). Thus, a morphological analysis could be the initial step in exploring large population size of species. The morphometric variations between stocks are predictable as they are geographically separate and may originate from different ancestors. The truss network system can

effectively be used to study separations of stock within a species as reported in other freshwater and marine environments (Mir et al., 2013).

CONCLUSION

These present findings reveal the potential power of the truss network method for identification of *P. bulu* stock in Peninsular Malaysia, suggesting a need for separate management approach to sustain the stock for future use. Environmental factors may be contribute to morphometric variations of *P. bulu* between the three populations. However, the results from this finding can be further confirmed based on biochemical and molecular procedures to support the morphometric data.

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Ornamental Carp Fish Cultured in Settling Pond after Revegetation of Ex-Silica Mining Area

Iis Diatin[1]*, Muhammad Mujahid[2], Ahmad Teduh[3] and Juang R. Matangaran[4]

[1]Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University (IPB), Dramaga, Bogor 16680, Indonesia
[2]Ocean Engineering, Faculty of Civil Engineering and Environmental, Bandung Institute of Technology, Bandung 40132, Indonesia
[3]Alumnus of Bogor Agricultural University (IPB), Bogor 16680, Indonesia
[4]Department of Forest Management, Faculty of Forestry, Bogor Agricultural University (IPB), Dramaga, Bogor 16680, Indonesia

ABSTRACT

This study looked at a settling pond at an ex-silica mine located in Sukabumi, Indonesia. The settling pond was built in order to reduce the effect of acid mine drainage. Acid mine drainage is the main pollutant at the open pit mining and it can contaminate aquatic biota. In order to utilise the settling pond, ornamental fish was cultured. The objective of this research was to analyse the possibility of aquaculture for ornamental fish in the settling pond ex-silica mining area. For this purpose, three species of ornamental fish of koi carp (*Cyprinus carpio*), gold fish (*Carassius auratus*) and comet carp (*Carassius auratus auratus*) at the settling pond were compared with control pond outside the mining area. We measured their specific growth rate and survival rate for 10 weeks. Results showed that specific growth rate indicated by length and weight for all species was higher at the settling pond compared with the control pond. In contrast, the survival rate percentage of all the three species was higher than those of the control pond, i.e. 48.5-93 % at the settling pond vs. 94.5-96% at the control pond. Meanwhile, the specific growth rate of weight was 0.92-1.88 % day^{-1} at the settling pond and 0.34-0.61 % day^{-1} at the control pond, while the length was 0.70-1.02 % day^{-1} at the settling pond and 0.31-0.63 % day^{-1} at the control pond. These indicated

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E-mail addresses:
iisdiatin@gmail.com (Iis Diatin)
muhammad.mujahid1605@gmail.com (Muhammad Mujahid)
teduhahmad@yahoo.com (Ahmad Teduh)
jrmatangaran@yahoo.com (Juang R. Matangaran)
* Corresponding author

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that the three species of ornamental fish can be cultured at the settling pond even at low survival rate condition.

Keywords: Acid mine drainage, ornamental fish, plankton, settling pond, specific growth rate, water quality

INTRODUCTION

Mining is an exploitation of non-renewable resources with significant impacts on the environment, such as alteration in landscapes, flora and fauna, soil structure, pattern of surface and deep soil water flow. An unfortunate outcome of this activity is acidic water. Due to its very low pH value, together with exposure to remaining metals and reactions with rain water, aquatic biota can be contaminated by high acidity which can also dissolve metal. The water becomes too toxic for aquatic organisms, and harm the ecosystem (Simate & Ndlovu, 2014). Vegetation alteration due to mining also affects metal, chemical and physical properties, which mobilise and transport them into soil and settling ponds (Huot et al., 2015). According to Brain (2017), mining operations change water quality and quantity in the Andes.

Fish farming is one of the efforts to make use of ex-mining area settling ponds. Several research has also confirmed the feasibility of fish farming in ex-mining ponds. For instance, Pangas catfish (*Pangasius pangasius*) and catfish (*Clarias batrachus*) are widely cultivated in ex-mining ponds in Bangka Belitung Islands, Indonesia (Bidayani, 2007) and coal ex-mining ponds in Kalimantan Timur, Indonesia (Pagoray & Ghitarina, 2016). Mozambique tilapia, known locally known as mujair (*Oreochromis mossambicus*), has also been farmed in lead-contained ponds with weight and length growth rates of 0.453% and 0.223% respectively (Yulaipi & Aunurohim, 2013). In a similar condition, daily growth rate of catfish (*Clarias gariepinus*) was 11.17% (Prasetiyono, 2015). Blue tilapia (*Oreochromis aerus)* was also reportedly grown in settled pond containing heavy metal copper (Straus, 2003), while rainbow trout (*Oncorhynchus mykiss*) can be grown in mining acidic water without any physiological stress symptoms (Viadero & Tierney, 2003). However, to the best of author's knowledge, there is still limited information on ornamental fish farming in the settling pond of ex silica mining area. Therefore, this study analyses the potential of ornamental fish farming in the settling pond of an ex-silica mine.

METHODS

The research was conducted at a settling pond of an ex-mine. One of the silica quarries managed by a mining company is located in Sukabumi District of West Java Province Indonesia with an area of approximately 69 hectares. This company stopped its mining activities in 2013. The company has since tried restoring the ecosystem post-mining through reclamation and revegetation of their ex-mining area and it was designed to become a plantation forest. After three years planting the area,

38, 900 trees have been grown there. Twenty species of trees have been planted with by Pinus merkusii as the dominant tree species. Some cover crops have also been planted to cover the soil surface. Some kind of fertiliser was utilised in order to supply nutrient that is essential for plantation growth. There is a settling pond located in the middle of the ex-mining area. In this settling pond three species fish of ornamental carp were cultured and the specific growth rate and survival rate of those fish observed.

Experiments were carried out for four months (from March to June, 2017) in 5000 m^2 settling pond of ex-silica mining in Sukabumi and the control pond of 500 m^2 fish farming in Bogor, Indonesia. Three kinds of ornamental fish used in the study were koi carp (*Cyprinus carpio*) with average length of 8.5 ± 0.32 cm and weight of 19.7 ± 1.07 g, 5.17 ± 0.40 cm long goldfish (*Carassius auratus*) weighed 6.71 ± 0.60 g, and 5.36 ± 0.36 cm long comet carp (*Carassius auratus auratus*) weighed 6.40 ± 0.70 g, respectively. Fish was maintained in 1.5 m x 1 m x 1 m hapa, 100 fish per hapa, for 10 weeks. Each hapa contained only one kind of ornamental species. The experiment was run in triplicates of each ornamental species, with a total of 9 hapas in each settling pond and control pond. The fish were fed daily, once every morning and afternoon, with commercial pellet Hi-Pro-Vite 788-2 (Indonesia) at 5% of biomass weight.

Fish sampling for length and weight measurement (30 number of fishes) as well as water quality, was done once every two weeks, while visual observation was done daily. Fish body weight was measured using electronic balance Kris Chef EK935OH (Indonesia) and fish body length was measured using Vernier caliper. Sampling for plankton abundance measurement was done by filtration 100 litres of surface water using 45 µm sized plankton net. Filtered water was put into 30 ml sample bottle before preservation using 3─5 drops of Lugol. Plankton abundance was expressed as individuals per litre. Temperature was measured using thermometer, while dissolved oxygen and pH were measured using DO-meter TOA-DO20 (Japan) and pH meter TOA-HM30 (Japan), respectively. Alkalinity, turbidity, TAN, nitrite, and nitrate were measured using Spectrophotometer Optima-SP300 (Japan) with regard to APHA (1989). Plankton microscopic observation was conducted in Laboratory of Aquaculture Environment of Bogor Agricultural Institute. Perimeters analysed were daily specific growth rate and survival rate, plankton abundance (phytoplankton and zooplankton), and water quality (temperature, dissolved oxygen (DO), pH, alkalinity, turbidity, total ammonia nitrogen (TAN), nitrite and nitrate).

The specific growth rate (SGR) and survival rate (SR) were calculated according to Huisman (1987):

$$SGR\ weight = [(\ln W_t - \ln W_0)/t] \times 100$$

Where W_0 and W_t are the initial and final weight of the fish (g), respectively, and t is the culture period in days.

SGR length = $[(\ln L_t - \ln L_0)/t] \times 100$

Where L_0 and L_t are the initial and final length of the fish (cm), respectively, and t is the culture period in days.

SR (%) = $N_t/N_0 \times 100$

Where N_0 and N_t are the initial and final number of fish, respectively.

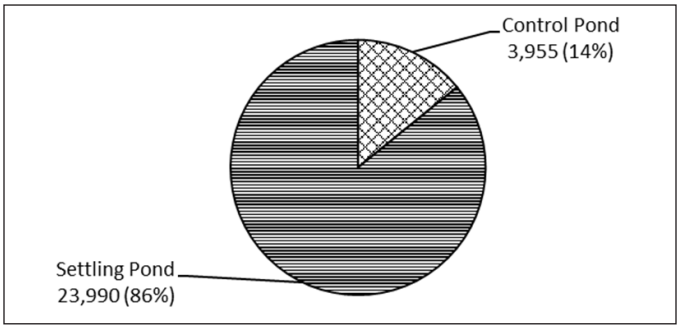
RESULTS

Daily weight and length growth rate of all fish were found to increase, both in the settling and control ponds, indicating that the settling pond aquatic environment can be utilised for ornamental fish farming. Specific growth rate (SGR) of ornamental fish, both in weight and length, in the settling pond was higher than that of the control. The specific growth rate of weight was 0.92-1.88 % day^{-1} at the settling pond and 0.34-0.61 % day^{-1} at the control pond respectively, while the length was 0.70-1.02 % day^{-1} at the settling pond and 0.31-0.63 % day^{-1} at the control pond respectively. Specific growth rate (SGR) and survival rate (SR) of ornamental fish in this study are shown in Table 1.

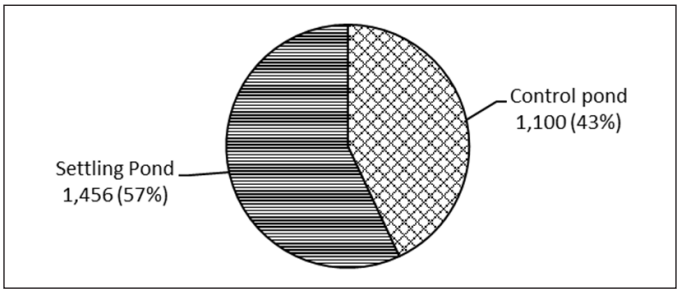
Table 1
Specific growth rate (SGR) and survival rate (SR) of ornamental fish

No	Location	Koi carp	Gold fish	Comet carp
		SGR weight (% day^{-1})		
1	Settling Pond	0.92 ± 0.05	1.61 ± 0.47	1.88 ± 0.15
	Control Pond	0.61 ± 0.37	0.34 ± 0.13	0.58 ± 0.07
		SGR length (% day^{-1})		
2	Settling Pond	0.70 ± 0.33	0.97 ± 0.4	1.02 ± 0.46
	Control Pond	0.63 ± 0.36	0.31 ± 0.17	0.42 ± 0.15
		SR (%)		
3	Settling Pond	93.00 ± 0.00	48.50 ± 0.71	61.00 ± 1.41
	Control Pond	94.50 ± 0 .71	95.00 ± 0.00	96.00 ± 0.71

Phytoplankton abundance and zooplankton in the ex-mining area settling pond and control pond can be seen in Figure 1, while dominant compositions of phytoplankton and zooplankton in the ex-mining settling pond and control pond are shown in Figure 2 and 3 respectively. Water quality is shown in Table 2.

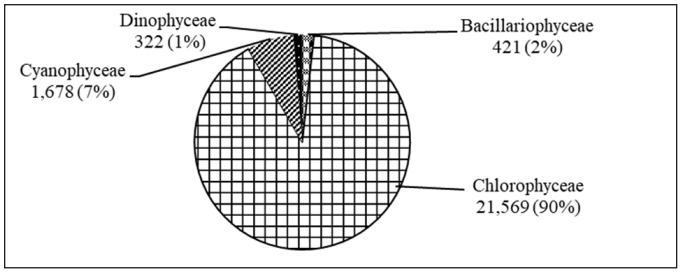


(a)

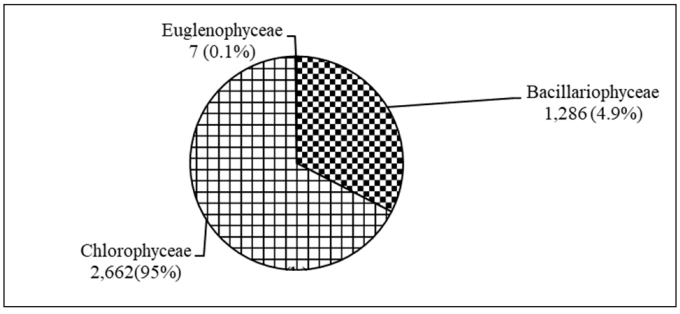


(b)

Figure 1. Phytoplankton (a) and zooplankton (b) abundance (individual L^{-1}) in the settling pond and control pond

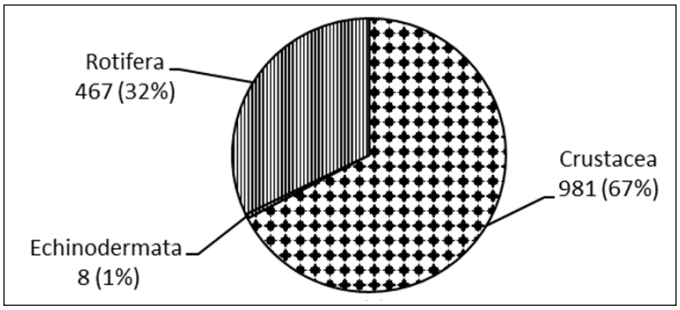


(a)

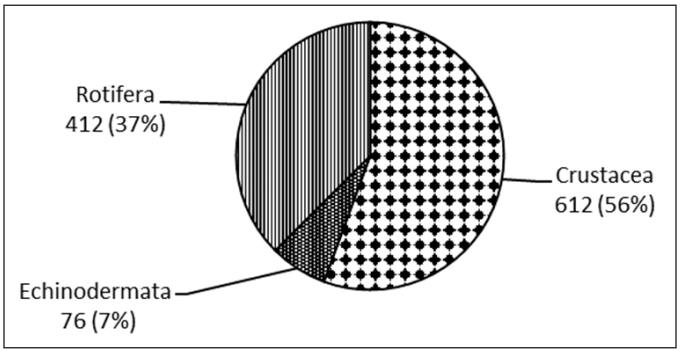


(b)

Figure 2. Composition and abundance of phytoplankton (individual L^{-1}) in the settling pond (a) and control pond (b)



(a)



(b)

Figure 3. Composition and abundance of zooplankton (individual L^{-1}) in the settling pond (a) and control pond (b)

Table 2
Water quality in the settling pond and control pond

	Settling pond	Control pond
Temperature (°C)	27.2-29.2	26.5-27.9
DO (mg L^{-1})	6.1-7.8	6.0-7.1
pH	7.08-7.93	6.80-7.67
Alkalinity (mg L^{-1})	28.83-104.16	22.42-70.47
Turbidity (NTU)	20.5-108.0	21.0-53.0
TAN (mg L^{-1})	0.003-0.642	0.001-0.620
Nitrite (mg L^{-1})	0.005-0.457	0.008-0.424
Nitrate (mg L^{-1})	0.167-1.434	0.117-0.670

DISCUSSION

The highest rate obtained by comet fish (*Carassius auratus auratus*) of settling pond SGR length and weight of 1.02 ± 0.86 % day^{-1} and 1.88 ± 0.15 % day^{-1}, respectively, while the lowest was obtained by control goldfish at 0.31 ± 0.17 % day^{-1} and 0.34 ± 0.13 % day^{-1} respectively (Table 1). Previous research on goldfish (*Carassius auratus*) maintained in controlled container

(tank, aquarium) fed with commercial feed resulted in the SGR of 1.56 ± 0.02 day^{-1} (Bandyopadhyay et al., 2005), whereas those of monoculture goldfish was 5.21 ± 0.31% day^{-1} (Jha et al., 2006b), those of 0.08 fish L^{-1} density was 1.97 ± 0.014% day^{-1} (Niazie et al., 2013), compared to the control of 1.07 ± 0.03% day^{-1} (Wang et al., 2015). The SGR of goldfish fed four times day^{-1} was 0.499 ± 0.025% day^{-1}, while those obtained by twice a day feeding was 0.472 ± 0.009% day^{-1} (Priestley et al., 2006). According to Popovic et al. (2016), prussian carp (*Carassius gibelio*) grows rapidly and it can tolerate water pollution. SGR of koi carp (*Cyprinus carpio* var *koi*) farmed in a density of 0.1 fish L^{-1} was 4.38 ± 0.03% day^{-1} (Jha & Barat, 2005a), whereas those of fish maintained without water circulation was 3.92 ± 0.03% day^{-1} (Jha & Barat, 2005b). The koi fish fed with commercial feed was 3.66 ± 0.05% day^{-1} lower than the natural feed of 5.09 ± 0.14% day^{-1} (Jha et al., 2006a), while those of monoculture was 5.45 ± 0.09% day^{-1} (Jha et al., 2006b). Based on the research, ornamental fish growth rate in this study was relatively low, which was probably caused by settling and control pond water content and quality difference to those of the controlled farming media such as in the aquarium or fish tank. Fish growth is affected by age of fish, adult fish grows slower than fry and juvenile fish (Rahardjo et al., 2011). In this study, we used adult fish, so its growth was relatively slower.

Meanwhile, the SGR difference of both length or weight in settling and control pond was probably caused by availability of natural feed in those ponds. Growth is correlated with the ability to obtain food and environmental adaptation, thus food as energy source becomes one of the main factors. Energy is mainly used for metabolism, the demand which needs to be fulfilled first, with the remaining used for growth (Goddard, 1995; Jobling, 1994). Farming performances in the form of growth and survival rate, biomass, and feed conversion ratio are highly dependent on feed (Martinez-Cordova et al., 2014). A research by Mladineo et al. (2010) on sea bass (*Dicentrarchus labrax*) showed that fish maintained in recirculated system with algal addition (RAS+HRAP) had a higher growth rate of 0.3 ± 0.1% day^{-1} than without algal of 0.23 ± 0.1% day^{-1}. Ornamental fish in this study was fed with commercial feed and supported by aquatic natural source, such as plankton. It was reported that plankton plays an important role in fish as feed source (Feuga, 2000).

It can be seen that settling pond had higher phytoplankton and zooplankton abundance of 23, 989 individual L^{-1} (86%) and 1456 individual L^{-1} (57%) than the control (Figure 1), thus fish in the settling pond had higher access to natural feed than those of the control. In the area around the settling pond there was revegetation activity which used fertiliser. The nitrogen and phosphorus of fertiliser drifted through the surface run off into the settling pond. The results of Jha et al. (2004) showed that plankton abundance increased 5 times in fertilised tanks compared with those not fertilised. In both the settling and

control ponds, phytoplankton abundance was dominated by Chlorophyceae (Figure 2) indicating healthy aquatic condition (Barinova & Krupu, 2017). According to Wu et al. (2014), chlorophyte index is usable for bio-assessment of water quality. Indicators of polluted water is usually dominated by Bacillariophyceae as in the waters of the ex-gold mining area (Priyono, 2012), in estuarine Creek Nigeria (Onyema, 2007) and in a Moroccan shallow reservoir (Belokda et al., 2017). Phytoplankton acts as a primary producer for zooplankton presence as a function of food source. It was also reported in other research that zooplankton abundance directly correlated to growth rate (de Souza, 2015). High-zooplankton aquatic region is able to produce better fish growth (Jha & Barat, 2005b; Jha et al., 2006b). The growth of koi carp increased by about 1.3 times on zooplankton abundance increased approximately 5 times (Jha et al., 2004). Zooplankton observed in this research was dominated by crustacean (Figure 3), good natural feed source for fish. A report noted that koi generally likes crustaceans, such as cladocera (daphnia, moina, bosmina) and copepoda (cyclops, diaptomus, nauplii), which were found in koi gut (98.02%) from its composition, while those of goldfish was 96.55% (Jha et al., 2006b). Koi fish culture in concrete tanks containing cladocera and copepods of 1286.63 numbers L^{-1} (79.29%) resulted in the highest SGR of 5,14% ± 0.03 % day^{-1} (Jha et al., 2004). Settling pond has an abundance crustacean higher at 67%, while 57% at control, supporting better growth in the settling pond. Cladocerans is usable for bio-indicator of water quality (Montemezzani et al., 2017).

Survival rate of ornamental fish in settling pond was lower than those of control, with the highest of settling pond obtained by koi carp at 93.0 ± 0.00% and the lowest by goldfish of at 48.5 ± 0.71%. In the control pond, the highest survival rate was obtained by comet carp 96.5 ± 0.71% and the lowest by koi carp of 94.5 ± 0.71%. The survival rate is determined by water quality as certain aquatic condition is needed for optimum fish growth. Jha and Barat (2005b) reported 95.21 ± 1.03% survival rate of koi maintained in daily water exchange, ensure better water quality, much higher than those of otherwise of 60.43 ± 2.39%. The SGR of corydoras (*Corydoras aeneous*) maintained in 30% day^{-1} water exchange had a survival rate of 87% (Diatin et al., 2014), while those of 50% day^{-1} water exchange had a higher rate of 97% (Diatin et al., 2015).

In this study, data for water quality are presented in Table 2. It can be seen that temperature, DO, and pH in the settling and control ponds were in feasible range for ornamental fish farming, as koi, goldfish, and comet need to live in neutral pH of 7.0 (Petrovicky, 1988) or 6.5-7.6 (Axelrod et al., 1988), temperature of 24-28^{o}C (Petrovicky, 1988) and minimum dissolved oxygen of 3 mg L^{-1} (Boyd, 2001). The low survival rate of ex-mining settling pond was probably caused by too high alkalinity rate and turbidity. Minimum alkalinity concentration for fish farming is 20 mg L^{-1} (Wedemeyer, 1996), while lower rate will cause fish

physiological stress (Bhatnagar & Devi, 2013). The most suitable alkalinity for fish is around 50 mg L^{-1} (Boyd, 2007), 100─150 mg L^{-1} (Wedemeyer, 1996) and 50-200 mg L^{-1} (Bhatnagar & Devi, 2013). In this study, the alkalinity rate of settling pond (28.83-104.16 mg L^{-1}) was relatively higher than the control pond (22.42-70.47 mg L^{-1}). Previous research conducted on koi fish farming indicated that the alkalinity rate of 35.14 ± 0.77 mg L^{-1} led to a survival rate of 93.363 ± 0.89% (Jha & Barat, 2005a), while lower survival rate of 60.43 ± 2.39 % was recorded at 146.25 ± 11.02 mg L^{-1} alkalinity rate (Jha & Barat, 2005b). Goldfish farming at 33.92 ± 1.40 mg L^{-1} alkalinity rate generated 91.41% survival rate (Jha et al., 2006b). Lime leaching can increase alkalinity (Bhatnagar & Devi, 2013). Revegetation activity around the settling pond uses ameliorant soil containing lime to increase soil pH. This material is supposed to come into the settling pond through surface run off, so the value of alkalinity in the settling pond has a wide range.

Turbidity represents organic and inorganic matters; both suspended, or dissolved. In this study, turbidity of ex-mining settling pond was at 20.5-108.0 NTU range, slightly lower than the control of 21.0-53.0 NTU. Previous research in tin ex-mining settling pond reported 300 NTU turbidity (Henny, 2010), higher than those of coal mining of 42-271 NTU (Pagoray & Ghitarina, 2016). Feasible turbidity concentration for fish farming ranged between 25 and 50 NTU (Ozbay & Boyd, 2003), less than 20 mg L^{-1} (Wedemeyer, 1996). Other studies mentioned 30-80 NTU (Bhatnagar & Devi, 2013), 0.9-132.3 NTU in pond (Xu & Boyd, 2016), and 50.7 ± 5,5 NTU for carp fish in freshwater reservoir (Akhurst et al., 2017). Turbidity is influenced by the resultant effect of several factors, such as suspended clay particles, dispersion of plankton organisms, and particulate organic matter (Bhatnagar & Devi, 2013). The high turbidity in the settling pond was due to the high plankton abundance, whereas in the control pond its abundance was relatively low. More specifically, the phytoplankton abundance in the settling pond was 6 times higher than that of the control pond (Figure 1). Relatively high alkalinity and turbidity rate presumably indicate lower survival rate in the settling pond.

Toxic parameter of water quality for fish is ammonia (Ebeling et al., 2006) that is harmful even in very low concentration (Crab et al., 2007; Hu et al., 2013), and thus generally recognised as pollutant (Yang et al., 2011). According to Lamoljo et al. (2009) there was a relationship between concentration of N and the nutrient from fertiliser. In this research location, the change of N concentration in water was associated with the applications of fertiliser. Research showed that the total ammonia nitrogen (TAN) value of settling pond was at 0.003-0.642 mg L^{-1}, and this was similar to that of the control at 0.003-0.620 mg L^{-1}. Ammonia toxicity level also depends on fish species and size (Stickney, 2005). According to Boyd (2008), TAN concentration above 2 mg L^{-1} is potentially dangerous at pH

above 8. Total nitrogen of carp (*Cyprinus carpio*) maintained in freshwater reservoir was 1.74 ± 0.26 mg L^{-1} (Akhurst et al., 2017). Acute ammonia concentration for common carp (*Cyprinus carpio*) was at LC_{50} 96 h, pH 7.46-7.53, was 0.76-1.26 mg L^{-1} (Abbas, 2006), at LC_{50} 96 h of crucian carp (*Carassius auratus*) was 0.511 ± 0.007 mg L^{-1} (Yang et al., 2010), while at EC_{50}s was 0.1997 mg L^{-1} (Yang et al., 2011).

The nitrite level of the settling pond was 0.005-0.457 mg L^{-1} lower than the control pond at 0.008-0.424 mg L^{-1}. Sensitivity against nitrite is determined by fish size and age (Kroupova et al., 2016; Zhang et al., 2012), species, sex (Zhang et al., 2012), pH, dissolved oxygen, and temperature (Kroupova et al., 2005). Bigger size or older fish is more tolerant to nitrite (Zhang et al., 2012). Nitrite concentration of 0.7 mg L^{-1} in common carp (*Cyprinus carpio*) induced retards growth (Kroupova et al., 2016). Nitrate concentrations of settling and control ponds were 0.167─1.434 mg L^{-1} and 0.117─0.670 mg L^{-1} respectively. Nitrate is relatively not toxic for fish, however the maximum nitrate concentration that is safe for fish farming is 50 mg L^{-1} (Kroupova et al., 2005; Yusoff et al., 2011). At 0.211 ± 0.048 mg L^{-1} nitrite concentration and nitrate at 0.608 ± 0.96 mg L^{-1}, approximately 40% of koi would not survive (Jha & Barat, 2005b) while mortality rate was around 32% at 0.014 ± 0.005 mg L^{-1} nitrite and 0.095 ± 0.012 mg L^{-1} nitrate (Jha et al., 2006a). The values of TAN, nitrite and nitrate in the settling ponds were still within tolerable limits for ornamental fish farming; however, if the concentration increased it could have inhibited the growth and survival of fish.

CONCLUSION

Ornamental fish maintained in the settling pond an ex-silica mine had higher specific growth rate of length and weight than those of the control pond, but lower survival rate. All the three ornamental fish types can be maintained in the ex-silica mining area settling pond. The settling pond of ex-silica mining which had been closed for mining activity, can be utilised for fish culture. It can become a model for utilisation of settling ponds of other ex-mining areas.

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Chemical Profiles of Methanolic Extracts from Two Species of Microalgae, *Nannochloropsis* sp. and *Spirulina* sp.

Haziq Ahmad Hazwan Zainoddin[1], Azhar Hamzah[2], Zainoddin Jamari[3] and Wan Adnan Wan Omar[1]*

[1]*Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia*
[2]*Crustacean Aquaculture Research Division, FRI Pulau Sayak, 08500 Kota Kuala Muda, Kedah, Malaysia*
[3]*Fisheries Research Institute (HQ), FRI Batu Maung, 11960 Batu Maung, Pulau Pinang, Malaysia*

ABSTRACT

Microalgae are potential sources of bioactive compounds that have health promoting effects due to the abundant presence of flavonoids and polyphenols. The goal of this study was to investigate the chemical profiles of microalgae extracts from *Nannochloropsis* sp. and *Spirulina* sp. Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (DPPH) were measured. *Nannochloropsis* sp. had a significantly higher TPC value than *Spirulina* sp. (58.43 ± 0.85 mg GAE/g DW vs. 19.64 ± 0.52 mg GAE/g DW, $p < 0.01$) and a significantly higher TFC value than *Spirulina* sp. (79.87 ± 0.12 mg QE/g DW vs. 11.19 ± 0.07 mg QE/g DW, $p < 0.01$). *Nannochloropsis* sp. also exhibited a greater percentage of DPPH inhibition compared to *Spirulina* sp. (EC_{50} 0.195 ± 0.007 mg/mL vs. 0.613 ± 0.003 mg/mL, $p < 0.01$). TPC and TFC were negatively correlated with the EC_{50} of DPPH antioxidant inhibition activity (–0.956, $p = 0.003$, and –0.899, $p = 0.015$, respectively). These negative correlations indicate that polyphenol compounds are the major contributors to the antioxidant activity observed in this study. Further analysis is needed to determine how to utilize the health benefits of both microalgae.

Keywords: Antioxidant, microalgae, *Nannochloropsis,* S*pirulina,* total flavonoid content (TFC), total phenolic content (TPC)

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E-mail addresses:
wanadnan@usm.my (Wan Adnan Wan Omar)
haziqhazwan92@gmail.com (Haziq Ahmad Hazwan Zainoddin)
azhhas@yahoo.com (Azhar Hamzah)
drzdin@gmail.com (Zainoddin Jamari)
* Corresponding author

INTRODUCTION

Microalgae are unicellular and multicellular microorganisms that belong to Kingdom Plantae. They are capable of undergoing photosynthesis due to the chlorophyll content in their cells and can be found in both terrestrial and aquatic ecosystems.

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Microalgae can be divided into two types, fresh water microalgae and marine microalgae (Chu, Yuen, Wong, Teoh, & Phang, 2002). They were a food source for ancient civilizations in some parts of the world, such as Asia, Africa, and South America (Santoyo et al., 2012). In addition, marine microalgae have been used as medicine to treat illness for centuries (Pooja, 2014).

Marine microalgae contain many bioactive compounds, including lipids that are useful in producing antimicrobial drugs (Lazarus & Bhimba, 2008). Priyadarshani and Rath (2012), and Volk and Furkert (2006) reported that marine microalgae can be a promising source of chemical compounds used in pharmaceutical, aquaculture, agrochemical, and biofuel industries. Microalgae extracts have been developed into cosmetics, anticancer agents, enzymes, pigments, poly-unsaturated fatty acids, and dietary supplements (Demirel, Yilmaz-Koz, Karabay-Yavasoglu, Ozdemir, & Sukatar, 2009; Guedes et al., 2011).

Microalgae are a good source of antioxidant compounds, which have health benefits and are useful in prevention of cardiovascular disorders, age-related diseases, cancer, and Alzheimer's disease (Goiris et al., 2012; Li et al., 2007). Antioxidant compounds, such as carotenoids that are commonly found in microalgae, play a major role in dealing with reactive oxygen species (ROS) and oxidative stress, which can cause multifactorial diseases such as cancer, cardiovascular diseases, and inflammatory disorders (Laguerre, Lecomte, & Villeneuve, 2007; Sachidanandam, Fagan, & Ergul, 2005).

Some classes of flavonoids, such isoflavones, flavanones, flavonols, and dihydrochalcones, are also common in microalgae (Klejdus, Lojková, Plaza, Šnóblová, & Štěrbová, 2010). Flavonoid compounds are strong antioxidants that inhibit lipid oxidation by scavenging directly on hydroxyl groups, singlet oxygen, and lipid peroxyl radicals. Flavonoids also act as metal chelators and inhibit the activity of lipoxygenase (Pietta, 2000). Inhibition of lipoxygenase activity is important to prevent inflammation-related diseases such as cancer (Wisastra & Dekker, 2014).

Spirulina is a blue green alga that has therapeutic effects such as anticancer properties and lowering blood cholesterol level (Kumar, Bhatnagar, & Srivastava, 2011). It has strong antioxidant activity due to the presence of phenolic compounds such as salicylic acid, chlorogenic acid, and caffeic acid (Pratt, 1992). Salicylic acid found in *Spirulina* was reported to have medicinal benefits that involve disrupting eicosanoic acid metabolism and altering levels of prostaglandins and leukotrienes (Mitchell, Akarasereenont, Thiemermann, Flower, & Vane, 1993). McCarty and Block (2006) showed that salicylic acid regulates molecular signaling through nuclear factor–KB (NF-KB), a transcription factor that plays a central role in immunity. Chlorogenic acid is a natural chemical compound formed from esterification of

caffeic acid and quinic acid. Chlorogenic acid has a significant effect on absorption and utilization of glucose from coffee, which may help reduce body mass and body weight in a healthy person (Thom, 2007). Caffeic acid, which is present in many fruits and plants, is well known to have strong antioxidant, anti-inflammatory, and immunomodulatory functions (de Oliveira et al., 2013; Murad, Soares, Brand, Monteiro, & Teodoro, 2015; Wang, Chu, Liang, Lin, & Chiang, 2010). Caffeic acid has been used to treat diseases such as cancers (Lewandowska, Kalinowska, Lewandowski, Stępkowski, & Brzóska, 2016) and upper respiratory tract infections in children (Yuksel & Akyol, 2016).

The green microalga *Nannochloropsis* produces high concentrations of important antioxidants such as zeaxanthin and canthaxanthin. Zeaxanthin and canthaxanthin are valuable nutraceuticals with medicinal applications in food and pharmaceutical industries. Asker, Tarek, Beppu and Ueda (2012) reported that consumption of zeaxhanthin helps lower the risk of age-related macular degeneration and prevent glaucoma and cataracts. Carpenter et al. (1997) showed that both zeaxanthin and canthaxanthin are useful in slowing the atherosclerosis process by inhibiting low-density lipoprotein oxidation. Chew, Park, Wong and Wong (1998) showed that canthaxanthin directly inhibits the growth of mammary tumors in mice.

The goal of this study was to determine the chemical properties of methanolic extracts from *Spirulina* sp. and *Nannochloropsis* sp. The presence of high phenolic and flavonoid contents that contribute to high antioxidant activity may have beneficial health effects that can be further studied.

MATERIALS AND METHODS

Microalgae Samples

Spirulina sp. and *Nannochloropsis* sp. were obtained from the Fisheries Research Institute in Pulau Sayak, Kedah. The microalgae (1000 mL) were cultured in three replicate flasks using sea water (10 ppt) containing Walne medium (Table 1). Media for stock cultures were replaced every 2 weeks, and the cells were maintained at 25 ± 1°C. The cells were cultured under continuous exposure to white fluorescent lamps (50.05 µmol photons m^{-2} s^{-1}) with aeration of normal air. The cells were harvested at the exponential phase of growth (i.e. 1.5 × 10^6 cells/mL) for the extraction.

Table 1
Ingredients used to prepare 1 L of Walne medium

Chemical	Weight
Potassium nitrate (KNO_3)	116 mg
Sodium nitrate ($NaNO_3$)	100 mg
Disodium salt (EDTA)	45 mg
Boric acid (H_3BO_3)	33.6 mg
Disodium phosphate (NaH_2PO_4)	20 mg
Ferric chloride ($FeCl_3$)	1.3 mg
Manganese chloride ($MnCl_2$)	0.36 mg
Zinc chloride ($ZnCl_2$)	21 mg
Cobalt chloride ($COCl_2$)	20 mg
Ammonium molybdate $(NH_4)_6$ Mo_7O_{24}	9 mg
Copper sulphate ($CuSO_4$)	20 mg
Thiamine.HCl (B_1)	10 µg
Cyanocobalamin (B12)	10 µg
Biotin (H)	0.2 µg

Microalgae Extracts

Harvested *Spirulina* sp. and *Nannochloropsis* sp. cells were extracted following the method of Pereira et al. (2015) with some modifications. Briefly, the cultured cells were freeze dried for 72 h. The dried microalgae were subjected to sonication to disrupt the cell wall and then extracted using 100% methanol at the ratio of one part algae to 40 parts methanol (w/v). The extraction was performed overnight at room temperature (20°C) under continuous stirring. The extracted biomass was centrifuged (10,000 × *g*, 10 min) and the supernatant removed. The supernatant was filtered and dried using a rotary evaporator at 45°C under vacuum. Dried extracts were resuspended in methanol to a final concentration of 20 mg/mL and stored at −20°C for further use.

Determination of Total Phenolic Content (TPC)

The TPC of extracted microalgae was analyzed according to Chua, Rahaman, Adnan, Tan and Tjih (2013) with some modifications. The extracts were prepared in methanol at three different concentrations (0.1, 0.2, and 0.3 mg/mL). A 10 µL aliquot of each concentration was mixed with 50 µL of Folin-Ciocalteu reagent and incubated for 5 min at room temperature. Next, 40 µL of Na_2CO_3 were added and the solution was incubated for 30 min. After the second incubation, the absorbance of the solution was read at 760 nm wavelength using a spectrophotometer. Gallic acid in a serial dilution from 10 to 0.3125 µg/mL was used as a standard chemical for calibration curve preparation. The TPC was expressed as gallic acid equivalent (GAE) per gram dry weight (DW) of extract (mg GAE/g DW).

Determination of Total Flavonoid Content (TFC)

The TFC of extracted microalgae was determined based on the method of Isla et al. (2011) with slight modification. Three extracts were prepared in methanol at three different concentrations (0.5, 1.0, and 1.5 mg/mL). A 50 µL aliquot of each concentration was mixed with 50 µL of 2% $AlCl_3$ and incubated for 10 minutes at room temperature. A flavonoid-aluminium complex was formed and measured using a spectrophotometer at 415 nm. Quercetin was used as the standard compound with a serial dilution ranging from 50 to 1.5625 µg/mL for the calibration curve. The TFC was expressed as quercetin equivalent (QE) per gram dry weight (DW) of extract (mg QE/g DW).

2,2-diphenyl-1-picrylhydrazyl (DPPH) - High performance Liquid Chromatography (HPLC) Antioxidant Assay

DPPH-HPLC analysis was used to measure the free radical scavenging activity of extracted microalgae following Nurdianah, Ahmad Firdaus, Eshaifol Azam and Wan Adnan (2016). Briefly, the radical scavenging activity of DPPH was determined by adding 500 µL of microalgae samples to 500 µL of DPPH (2.5 mM/mL) with the final volume of 1 mL. Next, 10 µL from all extracted samples was injected into the HPLC machine (Varian, Germany). Each extracted sample

was run in triplicate. Trolox was used as the standard, with the standard calibration curve ranging from 0.15625 - 5 µg/mL. Methanol and DPPH were used as a negative control. Analysis of separation was carried out using an Eclipse XDB-C18 4.6 mm × 250 mm, 5u C18 column (250 mm × 4 mm, 5 µM) (Agilent, Germany). Isocratic elution was carried out with an 80:20 ratio of methanol to water at a flow rate of 1.0 mL/min. The DPPH peaks were measured at 517 nm wavelength. Data analysis was conducted using Galaxie Workstation software. The reduction of DPPH peak area (PA) was used to determine the percentage of radical scavenging activity.

% Radical scavenging activity

$$= (PA_{control} - PA_{sample}) / (PA_{control}) \times 100$$

Statistical Analysis

All results are presented as mean ± standard deviation. Significant difference of the experimental results between the two strains was evaluated using Student's t-test, and $p < 0.05$ was considered to be statistically significant. Spearman correlation analysis was used to evaluate the relationship between TPC or TFC and antioxidant activity. All analyses were conducted using SPSS software version 22.

RESULTS AND DISCUSSION

Microalgae are known to produce bioactive compounds that contain high levels of antioxidants (Pumas & Pumas, 2014). Examples of these bioactive compounds are β-carotene, xanthophylls, polyphenols, and essential fatty acids (Talero et al., 2015). High levels of antioxidant compounds can prevent or slow down the unfavorable effects of free radicals (Halliwell & Gutteridge, 1999). Antioxidant activity in both microalgae extracts were measured using the DPPH assay. The DPPH radical ions are free radicals, which are reduced in the presence of antioxidants, changing the color of the solution. This change was measured at 517 nm wavelength, and the data is presented as EC_{50}, which is the efficient concentration required to decrease the initial DPPH concentration by 50% (Mishra et al., 2012). In this study, the EC_{50} value of *Nannochloropsis* sp. was 0.195 ± 0.007 mg/mL, which was significantly lower than that of *Spirulina* sp. (0.613 ± 0.003 mg/mL) ($p < 0.01$) (Table 2).

The antioxidant activity of methanolic extracts of *Nannochloropsis* sp. in our study was much higher than that reported by Safafar, Van Wagenen, Møller and Jacobsen (2015), and Mekdade et al. (2016). This difference was likely due to different methodologies, as we used DPPH- HPLC whereas the other studies used the DPPH assay and a spectrophotometer. Methanolic extracts were better for the DPPH antioxidant assay compared to hexane and water extracts. Kothari and Seshadri (2010) reported that polar extracts were better free radical scavengers than less polar extracts. Custódio et al. (2015) reported higher EC_{50} values for *Nannochloropsis oculata* hexane (4.93 ± 0.37 mg/mL) and water (7.31 ±

0.71 mg/mL) extracts compared to our methanolic extract. Shalaby and Shanab (2013) analyzed phenolic compounds using HPLC and found that the methanolic extract contained a higher level of phenolic compounds than the water extract, which contributes to its high antioxidant activity.

The antioxidant activity of the methanolic extract of *Spirulina* sp. in our study was slightly lower than that reported by Shalaby and Shanab (2013). They reported that at 0.2 mg/mL, the methanolic extract of *Spirulina platensis* inhibited 89.61% of DPPH, whereas we found that at 0.613 ± 0.003 mg/mL, *Spirulina* sp. showed only 50% inhibition of DPPH. The methanolic extract in our study had lower antioxidant activity than the ethanolic extract of *S. platensis* (EC_{50} 0.1011 mg/mL) studied by Sutanto and Suzery (2016).

Findings from our study and previous studies for *Nannochloropsis* sp. and *Spirulina* sp. extracts are summarized in Tables 2 to 4. The use of more polar solvents such as methanol and ethanol are important to extract antioxidant compounds from *Nannochloropsis* sp. and *Spirulina* sp., as they are generally good at extracting phenolic content, which may have a greater effect than other compounds on DPPH activity. Furthermore, Dai and Mumper (2010) reported that methanol was more effective at extracting higher amounts of polyphenols, compared to other solvents.

The TPC data are presented in units of mg GAE/g DW. Gallic acid is a trihydroxybenzoic acid that belongs to the phenolic acid family, and is used as a standard reference in the measurement of phenolic compounds. The TPC of *Nannochloropsis* sp. (58.43 ± 0.85 mg GAE/g DW) was significantly higher than that of *Spirulina* sp. (19.64 ± 0.52 mg GAE/g DW) ($p < 0.01$) (Table 3). Rai and Rajashekhar (2015)

Table 2
Antioxidant activity (DPPH inhibition) of Spirulina *sp. and* Nannochloropsis *sp.*

Study	Safafar et al. (2015)	Mekdade et al. (2016)	Shalaby & Shanab (2013)	Sutanto & Suzery (2016)	Custódio et al. (2015)	Custódio et al. (2015)	Our Study
Nannocholoropsis sp.	0.25 mg/mL (12.55% inhibition)	3.28 mg/mL (50% inhibition)	–	–	4.93 ± 0.37 mg/mL (50% inhibition)	7.31 ± 0.71 mg/mL (50% inhibition)	0.19475 ± 0.007 mg/mL (50% inhibition)
Spirulina sp.	–	–	0.2 mg/mL (89.61% inhibition)	0.1011 mg/mL (50% inhibition)	–	–	0.6125 ± 0.003 mg/mL (50% inhibition)
Solvent Used	Methanol	Methanol	Methanol	Ethanol	Hexane	Water	Methanol
Cultivation Medium	Industrial waste water	–	–	–	–	–	Walne Medium

Table 3
Comparison of TPC from Spirulina *sp. and* Nannochloropsis *sp. between our study and others (comparison based* on *similar standard used in the experiment*)

Study	Safafar et al. (2015)	Abd El-Baky et al. (2009)	Agustini et al. (2015)	Custódio et al. (2015)	Rai & Rajashekhar (2015)	This Study
Nannochloropsis sp.	6.45 ± 0.25 mg GAE/g DW	-	-	0.261 ± 0.06 mg GAE/g DW	17.01 ± 0.09 mg GAE/ g DW	58.43 ± 0.85 mg GAE/g DW
Spirulina sp.	–	4.51 ± 0.23 mg GAE/g DW	2.117 ± 0.99 mg GAE/g DW	–	7.15 ± 0.07 mg GAE/ g DW	19.64 ± 0.52 mg GAE/g DW
Solvent Used	Methanol	Ethanol	Ethanol	Diethyl ether	Methanol	Methanol
Cultivation Medium	Industrial waste water	Zarrouks Medium	–	–	F/2 and Walne Medium	Walne Medium

Table 4
Comparison of TFC from Spirulina *sp. and* Nannochloropsis *sp. between our study and others (comparison based* on *similar standard used in the experiment)*

Study	Safafar et al. (2015)	Abd El-Baky et al. (2009)	Agustini et al. (2015)	Rai & Rajashekhar (2015)	This Study
Nannochloropsis sp.	3.18 ± 0.59 mg QE/g DW	–	–	9.87 ± 0.25 mg QE/ g DW	79.87 ± 0.12 mg QE/g DW
Spirulina sp.	–	1.32 ± 0.03 mg QE/g DW	25.6615 ± 1.62 mg QE/ g DW	2.21 ± 0.17 mg QE/ g DW	11.19 ± 0.07 mg QE/g DW
Solvent Used	Methanol	Ethanol	Ethanol	Methanol	Methanol
Cultivation Medium	Industrial waste water	Zarrouks Medium	–	F/2 and Walne Medium	Walne Medium

reported a similar pattern for the methanolic extract of *Nannochloropsis* sp. (17.01 ± 0.09 mg GAE/g DW) versus *Spirulina* sp. (7.15 ± 0.07 mg GAE/g DW). However, the TPC value reported in our study was much higher than that found by Rai and Rajashekhar (2015). The differences in TPC values might be due to different media used to culture the microalgae (Walne medium versus F/2 medium, respectively). Safafar et al. (2015) and El-Baky, El Baz and El-Baroty (2009) also reported that the TPC level of *Nannochloropsis* sp. (6.45 ± 0.25 mg GAE/g DW) was higher than that of *Spirulina* sp. (4.51 ± 0.23 mg GAE/g DW). Natrah, Yusoff, Shariff, Abas and Mariana (2007) reported that higher pigment content in microalgae had been associated with higher production of phenolic compounds. Thus, our results suggest that *Nannochloropsis*

sp. may have higher pigment content than *Spirulina* sp. However, this observation needs to be confirmed by future studies.

However, Agustini, Suzery, Sutrisnanto and Ma'ruf (2015), and Custódio et al. (2015) found that *Nannochloropsis* sp. had a lower TPC value (0.261 ± 0.06 mg GAE/g DW) than *Spirulina* sp. (2.117 ± 0.99 mg GAE/g DW). This difference could be due to the different solvents used to extract the sample, as they used ethanol and diethyl ether, respectively, rather than methanol. Goiris et al. (2012) reported that polarity of the solvent affected the total amount of phenolic content extracted. A polar solvent with high specificity was preferred because it gave a higher yield of phenolic compounds. Thus, in our study, methanol gave a higher yield of TPC because it is a solvent with relatively higher polarity.

Flavonoids are the most abundant polyphenols found in antioxidant compounds (Baxter et al., 1998). Flavonoids found in microalgae are believed to be one of the highest natural phenolics found in natural products (Kumaran & Karunakaran, 2007). The TFC measured in *Nannochloropsis* sp. and *Spirulina* sp. was presented as mg QE/g DW, as quercetin was used as the standard compound in the measurement of TFC. In our study, *Nannochloropsis* sp. contained seven times more TFC than *Spirulina* sp. (79.87 ± 0.12 mg QE/g DW vs. 11.19 ± 0.07 mg QE/g DW, $p < 0.01$) (Table 4). Rai and Rajashekhar (2015) reported a similar trend for methanolic extracts, as the TFC of *Nannochloropsis* sp. was four times greater than that of *Spirulina* sp. (9.87 ± 0.25 mg QE/g DW vs. 2.21 ± 0.17 mg QE/g DW). The differences between the studies might be due to differences in the culture medium and the drying method used. The TFC values of *Nannochloropsis* sp. and *Spirulina* sp. measured in our study was much higher than those reported by Safafar et al. (2015) (3.18 ± 0.59 mg QE/g DW for *Nannochloropsis* sp.) and El-Baky et al. (2009) (1.32 ± 0.03 mg QE/g DW for *Spirulina maxima*). However, Agustini et al. (2015) reported a TFC value of 25.6615 ± 1.62 mg QE/g DW for the ethanolic extract of *Spirulina* sp. The huge differences in values among studies were probably due to different methods of cultivation and handling of materials. Furthermore, nutrients used in the cultivation of microalgae play an important role in producing flavonoids in both species of microalgae.

The study found a negative correlation between TPC vs. EC_{50} of DPPH antioxidant inhibition activity (–0.956, $p = 0.003$) and TFC vs. EC_{50} (–0.899, $p = 0.015$). Similarly, Farasat et al. (2014) reported that TPC and TFC in green seaweeds were negatively correlated with 50% inhibition of DPPH. These significant correlations indicate that polyphenol compounds were the major contributors to antioxidant activity in our methanolic microalgae extracts.

CONCLUSION

Methanolic extracts of *Nannochloropsis* sp. and *Spirulina* sp. possessed different antioxidant activities. The methanol extract from *Nannochloropsis* sp. had higher TPC and TFC compared to *Spirulina* sp. For

antioxidant activity, DPPH inhibition of *Nannochloropsis* sp. was greater than that of *Spirulina* sp. The health promoting effects of both microalgae, especially *Nannochloropsis* sp., should be further studied by evaluating the chemical compositions and biological activities of these species.

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Effect of Stage of Maturity and Frying Time on the Quality of Banana Springs

Rezaul S. M. Karim[1]*, Noorjanna Rahmatullah[1], Mariam Firdaus Mad Nordin[2] and S. M. Ataul Karim Rajin[3]

[1]Faculty of Agro Based Industry, Universiti Malaysia Kelantan (UMK), Jeli Campus, 17600 Jeli, Kelantan, Malaysia
[2]Department of Chemical Process Engineering, Universiti Teknologi Malaysia (UTM) Kuala Lumpur, Jalan Sultan Yahia Petra. 54100 Kuala Lumpur, Malaysia
[3]Digital Signal Processing Lab (DSP lab), Department of Electrical, Electronics & Systems Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia (UKM), 43600 Bangi, Selangor, Malaysia

ABSTRACT

Banana springs are a new snack food similar to banana chips. This study focuses on the quality improvement of banana springs. The effects of stage of maturity and time of frying on the quality of banana springs prepared from two varieties namely, *Musa acuminata* Colla cv. Berangan and *Musa paradisiaca* L. *cv.* Nangka, were evaluated. Five stages of maturity, i.e., i) mature, ii) early ripening, iii) intermediate ripening, iv) ripe, and v) overripe, were used in the trial. The total soluble solid (TSS) of two varieties at different maturity stages was measured. Banana springs were made by using spiral potato slicer. The slices of banana spring were 2±0.1 mm in thickness. The banana springs were deep fried in refined, bleached and deodorised (RBD) palm olein at a temperature of 170°C for 0, 3, 4, 5, and 6 minutes respectively. The fried banana springs were tested for their texture and crispiness. The samples were assessed for colour, flavour, texture, taste and overall acceptability by a 30-member taste panel. The TSS content of Nangka banana was greater than Berangan banana and the TSS content increased with maturity but it was a bit different between two varieties. Banana springs of Nangka variety had a higher value for texture (hardiness), especially at the early maturing stages in comparison to Berangan banana. Frying of banana springs for five and six minutes produced the same

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E-mail addresses:
rezaul@umk.edu.my (Rezaul S. M. Karim)
jannaheaven9104@gmail.com (Noorjanna Rahmatullah)
mariamfirdhaus@utm.my (Mariam Firdaus Mad Nordin)
rajin.karim@gmail.com (S. M. Ataul Karim Rajin)
* Corresponding author

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quality. An acceptable product with good taste, crispness, and the odour was obtained from both the varieties when fried at 170°C for 5 minutes. Nangka banana was better in respect of sweetness, odour, texture, and crispness vis-a-vis Berangan. Green matured banana (Maturity index I) of both the varieties was found suitable for the preparation of quality banana springs.

Keywords: Banana springs, banana variety, banana maturity index, berangan banana, frying time, nangka banana, sensory evaluation, total soluble solids

INTRODUCTION

Banana (*Musa sp.)* is an important source of carbohydrates, nutrients, minerals and fibre content (Robinson & Sauco, 2010). It is a low-cost food that serves as the primary food source in many developing countries including East African countries, Bangladesh and Malaysia (Mohapatra, Mishra, & Sutar, 2010). In Malaysia, banana covers more than 11% of the total area for fruit production and is a premier fruit, ranking second after durian. The annual production of banana in the 2014 was 303,107 tonnes and the area covered was 28,911 ha (FAOSTAT, 2017). But it is a perishable fruit, and it has a short lifespan between harvest and onset of deterioration. Therefore, it is essential to process the fruit into different downstream products to maximise its use. Usually, banana is processed into banana chips, French fries, banana powder and flour, banana cocoa, and coffee, alcohol, wine, and vinegar worldwide. There are many ways of processing and consuming banana in Malaysia, such as banana chips, which is one of the favourite snacks in the country. Borah and Nayak (2013) studied the drying behavior and quality of dried and fried banana chips in India. They observed that the effects of drying temperature, moisture content, and frying time on the hardness of chips were significant. Chips' thickness, frying temperature and frying time influenced the crispness, colour development and overall acceptance of banana chips as well (Wani, Sharma, & Kumar, 2017). They noted greater acceptance of banana chips with increased frying time. Banana springs are a similar kind of product made just like spiral springs using a slicer and then fried in hot oil at 130°C to 180°C. The demand for snack food is increasing in Malaysia since the number of working women who spend less time cooking is increasing (Norrita, 2015). Banana springs can be a rich source of nutrition though it is a snack.

Some studies have examined the effects of temperature, drying, breading of fried banana products and oil deterioration (Borah & Nayak, 2013; Wani et al. 2017; Zainun, 2008). However, very little attention has been given to the effects of the variety of banana and their stages of ripening on the quality of fried banana springs. The impacts of frying time on the quality of banana springs are also not well explored. Therefore, this investigation was done to study the effects of stages of ripening and frying time on crispness and taste of banana springs made from two banana varieties. It also assessed the overall acceptability of the deep-fat fried product.

MATERIALS AND METHODS

Fifty matured banana fingers (unripen green) of equal size (Berangan = 120 g ± 0.95; Nangka = 128.5 g ± 0.26) of two varieties, namely Berangan banana (*Musa acuminata* Colla cv. Berangan) and Nangka banana (*Musa paradisiaca* L. cv. Nangka) were bought from a local market. The banana varieties were identified based on their physical appearance. Berangan banana (Chestnut banana) has medium-long fingers, rounded in cross-section, parallel sided with apex usually rounded, and flesh is sweet; whereas Nangka banana (Jackfruit banana) are medium-long, rounded to slightly angular in cross-section, with apical teats and pulps are sour-sweet (Figure 1) (Casey, 2015). Refined, bleached and deodorised (RBD) palm olein (Brand: Saji) was obtained from the "Pantai Timur" supermarket, Jeli, Kelantan, Malaysia. Bamboo sticks (15 cm long and 2.5 cm diameter) were purchased from a local market.

Determination of Ripening Stage of Banana

The banana fingers were kept in a store room after wrapping them with plastic. They were allowed to ripen slowly under a room temperature of 25°C, relative humidity of 80%-85% until they reached the desired different stages of ripeness. No ripening agent was used. The stage of ripening of fresh banana was determined according to Caussiol and Joyce (2001) and Mba, Rahimi and Ngadi (2013) by using a colour chart and other physical observation. The ripeness of banana was assessed by comparing the colour of their peel and hardness with standardised banana maturity index as described in Table 1.

Determination of Total Soluble Solids (TSS)

Banana solutions were prepared by grinding 10 g of peeled banana sample with 50 ml of distilled water in a blender for 5 minutes and was filtrated to remove the solid particles. The TSS (Brix) was measured on the filtrate using the Atago Master-Alpha Refractometer (Agriculture Solutions LLC, Strong, ME, USA) at room temperature.

(a)

(b)

Figure 1. Photographs of: (a) Berangan banana; and (b) Nangka banana at early maturity stage (II)
Source: Casey, 2015

Table 1

Description of ripening stages and maturity index of banana

Maturity index	Storage day	Ripening stage	Colour of the peel
I	1	Mature	All peels are green
II	3	Early ripening	Green peel with a trace of yellow
III	7	Intermediate ripening	More green area than yellow
IV	9	Ripe	All yellow colour
V	11	Overripe	All yellow with brown speckles

Modified from Caussiol and Joyce (2001), and Mba et al. (2013)

Preparation of Banana Springs before Frying

The banana samples of two varieties were washed and manually peeled. The peeled banana (banana pulp) was sliced to a thickness of 2 ± 0.1 mm by using a Spiral Potato Slicer (MyDeal.lk Pvt. Ltd., Galle Road, Colombo 04, Sri Lanka). First, a bamboo stick was pierced through the peeled banana. It was placed on the slicer and by moving the handle of the slicer the banana springs were made. Twenty springs were pre-treated by soaking them in acidified water (a 1% solution of ascorbic acid) to prevent them from browning. The treated banana springs were blotted with blotting paper to dry out excess surface water from banana springs before frying. They were partially dried in a drying cabinet at 60°C for 15 minutes when the moisture content was reduced from 70.0% ± 2.1 (initial moisture) to 36.0% ±1.2, since dehydration of banana slices before frying improves the quality of banana chips (Agunbiade, Olanlokun, & Olanofe, 2006). The banana springs were fried in a deep-fat fryer (HKH-JB-ZL74 5.5L, HIKITCH Kitchen Equipment Co. Ltd., China) using palm olein at a temperature of 170°C ± 2°C. The RBD palm olein has essential characteristics of industrial frying oils such as high oxidative stability, high smoke point, low foaming, low melting point, bland flavour and nutritionally desirable attributes (Kochhar, 2000). The fryer had temperature control (range: 0°C to 200°C) and a perforated frying container. The deep-fat-fryer was set to the target temperature and was pre-heated to 170°C ± 2°C for one hour before the frying started. Four litres of fresh vegetable oil, RBD palm olein, (Free Fatty Acid (FFA) = 0.15% and Peroxide Value (PV) = 1.2 meq H_2O_2/kg oil, smoking point = 220°C) were used in all experiments. As per Kochhar (2000) good industrial frying oil should have less percentage of FFA, PV, and high smoke point (above 180°C) with a bland flavour. Oil temperature was measured by a thermometer held in a position 5 mm below the spring edge. The banana springs were divided into five different portions and were fried for 0 (control), 3, 4, 5 and 6 minutes. Five springs were put into sample holders, which were immersed completely in the hot oil. Fresh oil was used every time for

frying banana springs of both the varieties, Berangan and Nangka. After each frying, the banana springs were shaken well to drain out surface oil adhering to the surface of banana springs and lastly was blotted with a paper towel. The fried banana springs were allowed to be cooled for 10 minutes at room temperature before being tested for crispness and sensory evaluation. The fried samples were stored in an air-tight container before texture testing. The experiments were carried out in triplicates.

Texture Test

Compression/puncture test using a Testometric Universal Testing Machine (Testometric Company Ltd., Rochdale, UK) was done at room temperature immediately after frying to know the textural hardness and crispness of the fried banana springs. The puncture test was done by mounting the sample (a single spiral piece just like a banana chip) on a flat rigid support where the distance between the support and a cylindrical punch was 15mm. The punch diameter was 5.3 mm, and the crosshead speed was 25 mm min^{-1}. The force was applied to the fried banana springs until those were broken. Maximum breaking force (mm/minute) and deformation (mm) were measured. Normalised Maximum Force, measured in Newton (N) was the parameter used to measure the textural hardness (equals maximum force/maximum deformation) (Mba et al., 2013).

Sensory Evaluation

Sensory evaluation was done following the method of Ali, Muhammad, Sijam and Siddiqui (2011). The samples were assessed for colour, flavour, texture, taste and overall acceptability by a 30-member taste panel from the Universiti Malaysia Kelantan's lecturers, staff and students using a 9-point hedonic rating scale, where 1= dislike extremely and 9 = like extremely. Before the actual sensory evaluation, the panel was informed and instructed for the sensory attributes in the assessment to familiarise with the sensory procedure. The products were presented to the panellists after frying in refined palm olein for different periods as per treatment specification. The panellists were provided with a form which had the columns of all the attributes along with the column of overall acceptability for putting the sensory scores against different quality attributes of the banana springs. Data were tabulated properly for statistical analysis.

Experimental Design and Statistical Analysis

The effects of banana variety, stage of ripening, and frying time on the colour, odour, taste, texture, crispness of the banana springs were evaluated using a factorial design, with 2 levels for variety, five levels for the stage of maturation, and four levels for frying time. The effects of variety and ripening stages were analysed first. After selecting appropriate maturity based on brix% and texture, the effects of frying time were tested on two varieties. In case of evaluation of TSS contents, only the

effects of variety and stage of ripening were considered and therefore two-factor design was used, and data was analysed using two-way analysis of variance (ANOVA). The regression analysis between TSS content and stage of maturity (over time) has been done. Sensory data presented here are the means of three replicates. Data was subjected to descriptive analysis, where maximum, minimum and mean values were determined. One-way ANOVA was also done to determine if the samples were significantly different (Fetuga, Ajayi, & Karim, 2014). All the experiments were carried out in triplicates following the Completely Randomised Design. Data was analysed statistically using SPSS software. Duncan's multiple range tests were used to indicate the place of significant differences among the means at 5% probability level.

RESULTS AND DISCUSSION

Effect on Total Soluble Solids (TSS)

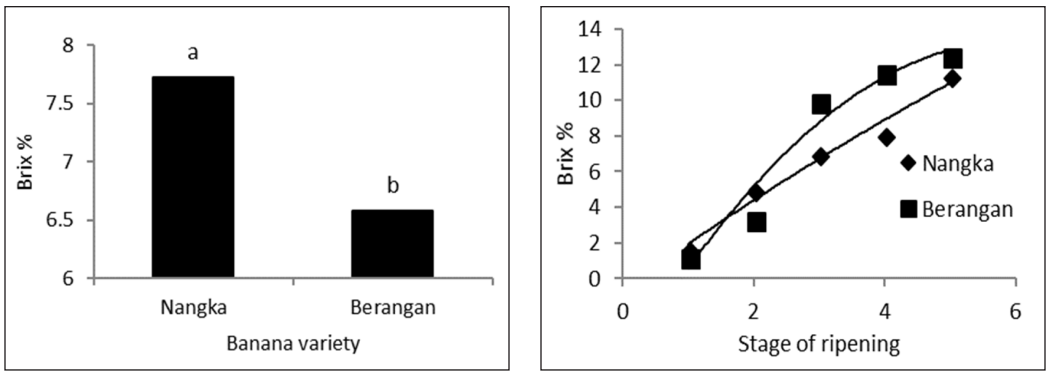
The results shown in Table 2 indicate the range of TSS and the effects of variety and stages of ripeness on the total soluble solid (TSS) value of fresh banana, while Figures 3 and 4 indicate the effects of variety and stage of ripening on texture/crispness of banana springs. Although the TSS usually increases with the maturity of banana (Wanna, Yaakob, Salmah, & Russly, 2001) a comparison was made between two varieties to see the trend and level of increase in TSS which might affect the quality of banana springs.

Effect of Variety. Figure 2A shows the mean Brix% for Nangka banana and Berangan banana. The results indicate there is a significant ($P < 0.05$) difference in TSS contents of the fresh banana of two varieties, and Nangka banana contained higher TSS than Berangan banana.

It is evident that on average, the Nangka banana contained about 15% more TSS than the Berangan banana, which might be due to their differences in plant genetics. Wanna et al. (2001) compared the physicochemical properties of Nangka banana with Abbu banana and found that Abbu banana had greater carbohydrate content than Nangka banana. However, Zainun (2008) noted the higher amount of TSS and total sugar contents in Nangka compared with Berangan, Raja and Mas varieties. The sugar content of banana varieties may vary due to edaphic and climatic differences as well (Rajkumar, Wang, EImasry, Raghavan, & Gariepy, 2012). The upward direction of successive data points indicates that the TSS increased with maturity. However, the regression equation is more fit to polygonal. The regression equation for Berangan banana is $Y = -0.517X^2 + 6.18X – 5.118$; $R^2 = 0.947$ and that of Nangka banana is $Y = - 0.063X^2 + 2.635X -0.63$; $R^2 = 0.974$. The overall increase of TSS was greater in Berangan banana than that of Nangka banana (Figure 2(b)).

Effect of Ripening Stage. Table 2 shows that there is significant ($P < 0.05$) difference in total soluble solid contents among the maturity stages of banana in both

the varieties. The TSS contents of both the varieties increased linearly with the advancement of ripening stages. However, the trend of increase with maturity was different in two varieties (Figure 2(b)). The increase in total soluble solid is related to fruit ripening which involves the conversion of starch to sugar in ripen banana. During the ripening process, hydrolysis of starch takes place, and it causes the accumulation of sugar. Other changes that happen during ripening include alteration of cell structure, changes in cell wall thickness, the permeability of the plasma membrane, hydration of the cell wall, decrease in structural integrity and increases in intracellular spaces. Rajkumar et al. (2012) observed polynomial relationship (i.e., first increased and then reduced) between maturity stages and TSS content of banana, which was modified due to temperature differences.



(a) (b)

Figure 2. (a) Effects of banana variety on the total soluble solid content (Dissimilar letters on the histograms indicate significant difference); (b) Effects of stage of maturity on the total soluble content of two banana varieties. The continuous thin lines indicate the polynomial relationship.

Interaction Effect of Variety and Stage of Ripening. Table 2 shows the values of TSS under different stages of ripeness of two banana varieties. However, the rate of increase was unusual in two varieties. For example, the rate of TSS increase in Berangan banana was low initially, but it increased at the later stage even more noticeably than the Nangka variant. In case of Nangka banana, the rate of increase was more or less linear maturity of banana (Figure 2(b)). There are differences between banana and plantains in starch contents. Plantains are the members of the banana family, but these fruits have more starch and less sugar than the banana and usually are cooked before serving. Marriott, Robinson and Karikari (1981) stated that fully ripe bananas have 1% starch but it contained 0% starch when overripe. In case of plantains, it contains 9% starch at the fully ripened stage and 3% starch at the over ripen stage

of maturity. In an investigation, Marriot et al. (1981) observed that bananas had 23% sugar at ripening or over ripen stage, but the plantain had 20% sugar at ripe and 27% at the overripe stage of ripening.

Effect on Texture

It is evident that the formation of crispness in potato and banana chips occurs at the end of frying (Wanna et al., 2001). Therefore, texture analysis as a function of frying time was done at the end of frying. The maximum breaking forces for banana springs fried at four different frying times (3, 4, 5 and 6 minutes) were compared using the two banana varieties and five maturity stages separately. The initial moisture of banana springs (70.0%) was reduced to 36.0% by drying at 60^{o}C for 15 minutes.

Effect of Variety. Table 2 and Figure 3 indicate that there is significant ($P < 0.05$) variation in the texture of banana springs of the test varieties of banana. A clear difference between maximum braking forces of banana springs was observed between Nangka and Berangan banana.

It is obvious that the banana springs of Nangka variety had a higher value for texture (hardiness), especially at the early maturing stages in comparison to Berangan banana (Figure 3(a)). The main difference in hardiness between the varieties is due to the differences in carbohydrate contents. Therefore, Nangka banana springs were crispier than Berangan banana. Elfnesh, Tekalign and Solomon (2009) noted a negative relationship between crispness of potato chips and dry matter contents of the potato cultivar.

Effect of Ripening Stage. There was a significant ($P < 0.05$) linear decrease in texture value (N) with the advance of maturity stages in both the varieties (Table 2). Negative correlations were noticed between the maximum breaking force and the stage of maturation of banana. As the starch content is converted to sugar and hydrolysis take place in the advanced maturity stage of banana, hardiness of banana springs was reduced at later maturity stages (Elfnesh et al., 2011). It might be the result of the breakdown of carbohydrates during respirations, and the hydrolysis of starch which leads to transfer of moisture from peel to pulp (Cano et al., 1997).

An optimal maturity stage was recognised when the maximum breaking force (1.8 N) of the springs was the highest. This point corresponded to maturity index of I – Green matured stage (Table 2). After this ripening stage, the carbohydrate was converted to sucrose and flesh of banana become soft which lead to poor quality of springs. Ammawath, Yaakob, Yusof and Rahman (2001) also marked that the crispness of banana chips was reduced with the ripeness of the Abu banana.

Effect on Crispness

Effect of Variety. The crispness is one of the textural qualities of fried food products. Loudness, snap, crackly, firmness, etc. are the examples of other texture qualities (Vickers & Christensen, 1980). During

frying process, the banana chips or springs lose the moisture from about 90% to 5%, and it absorbs the oil in the pores due to evaporation of moisture. Finally, these chips become crispy (Borah & Nayak, 2013). In this study, texture and crispness were measured following the same procedure. The statistical analysis of the results showed that banana springs produced from Nangka banana were significantly crispier ($P<0.05$) than the banana springs produced from Berangan banana (Figure 4 (a)). It might be because Nangka fresh banana had a higher total soluble solid content and was harder compared with Berangan banana. It was supported by Ammawath et al. (2001) who reported that fresh banana with higher carbohydrates content and fruit firmness gave chips with greater crispness.

Table 2
Interaction effects of banana variety and the stage of maturity on texture of banana springs and total soluble solid

Variety	Stage of Ripeness	Texture (N)	Total Soluble Solid (Brix %)
Berangan	Matured	1.74^{a}	1.63^{e}
	Initial Ripening	1.56^{b}	4.93^{d}
	Intermediate Ripening	1.45^{c}	6.96^{c}
	Ripe	1.34^{d}	8.03^{b}
	Overripe	1.13^{e}	11.37^{a}
Nangka	Matured	1.77^{a}	1.27^{e}
	Initial Ripening	1.76^{b}	3.33^{d}
	Intermediate Ripening	1.44^{c}	9.93^{c}
	Ripe	1.12^{d}	11.60^{b}
	Overripe	0.71^{e}	12.50^{a}
Min		0.71	1.27
Maximum		1.77	12.50
Mean		1.168	5.962
SD		± 0.25	± 2.53

Values in columns having dissimilar letter indicate significant differences

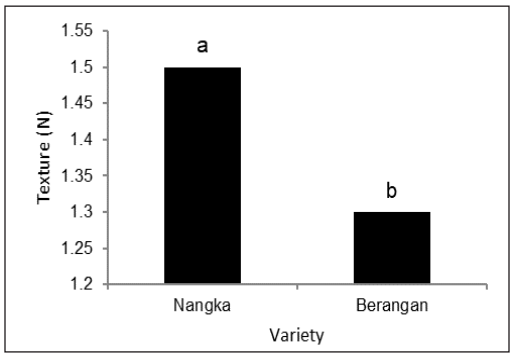


Figure 3. Effects of banana varieties on the texture (measured in Newtons) of banana springs (The dissimilar letters on the histograms indicate significant difference)

Effect on Frying Time. The results in Figure 4 show that there was a significant increase in crispness at every increase during the time of frying. The effects on two varieties are more or less similar. The regression equation for Berangan banana is $Y = 0.331x + 3.43$, $R^2 = 0.989$ and for Nangka banana it is $Y = 0.301x + 3.365$, $R^2 = 0.965$. The crispness score was the highest under 6 minutes frying time. However, no significant difference is noted in crispness between banana springs fried for 5 and 6 minutes respectively (Figure 4(b)). The crispness or hardiness increased during frying due to the crust development.

The frying process results in unique flavour, colour, and texture which affect consumer acceptability. The texture of the banana spring is an essential sensory parameter that determines the sensory acceptability and shelf stability. Since the same kind of quality found under frying for 5 and 6 minutes, 5 minutes frying is recommended for saving time, cost and energy.

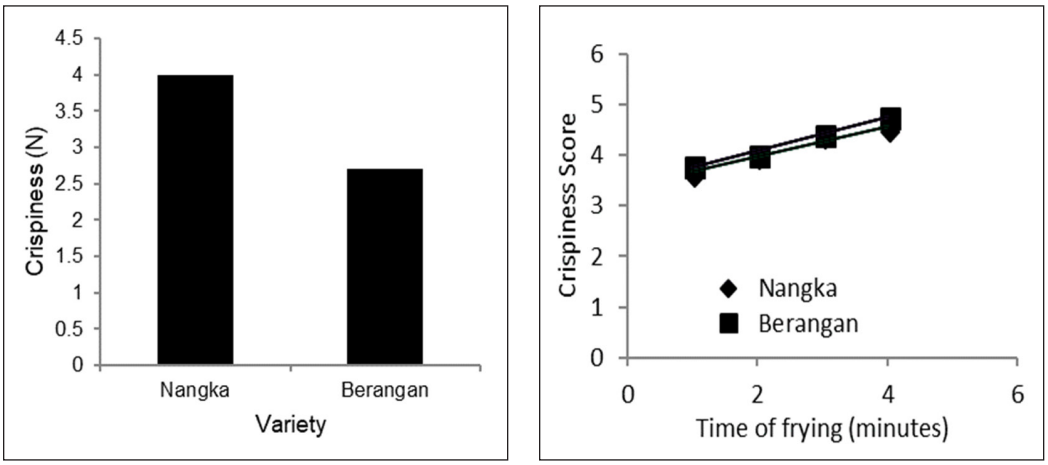
Acceptance by Consumers

The overall acceptability scores of fried banana springs of two varieties are shown in Figure 5. Results in Table 3 show the information on the sensory evaluation of different sensory attributes and overall acceptability of banana spring produced by deep-fat frying for varying periods of time.

For sensory evaluation, only banana at green mature stage (Maturity index I) was used to make banana springs. It is because in texture test, it proved that ripe green banana (Maturity index I) is the best stage for preparation of banana springs. The quality attributes of banana springs, especially its odour, texture, crispness, etc. produced after frying for different periods of time are reflected on the overall acceptability of products by the panellists. The panellist's scores for total acceptance for banana springs ranged from good to very good scale. None of the samples was rated as poor quality. It indicates that consumers may not reject banana springs though they are accustomed to banana chip or potato chip. There were significant differences ($P < 0.05$) between Berangan banana springs and Nangka banana springs fried at different times of frying in overall acceptability. However, no significant differences were noticed between two varieties in sweetness and odour of banana springs (Table 3). Moreover, Nangka banana springs fried for 5 minutes had a higher acceptability score (although non-significant) than frying for 6 minutes. In case of Berangan banana springs, the acceptability score when fried for 6 minutes was higher, but there was no significant difference between 5 and 6 minutes drying (Table 3).

Therefore, frying banana springs of both the varieties for 5 minutes (Figure 6) is recommended to save time and energy. Aurore, Purfait and Fahrasmane (2008) commented that it is important to generate new knowledge about bananas and banana products to improve their consumption and to explore the possibility of more agri-

businesses with banana products. This result provides information on the possibility of commercial production of banana springs to add new banana products in the food market. However, no comparison was done with other similar products in this study, which could be done in future.



(a) (b)

Figure 4. (a) Effects of banana variety on crispness of banana springs (The dissimilar letters on the histograms indicate significant difference). (b) Effects of time of frying on the crispness of banana springs of two banana varieties. Note that the effects are more or less similar on both the varieties. The continuous thin lines indicate the linear relationship.

Table 3
Interaction effects of banana variety and time of frying on the sensory evaluation (Sweetness, Crispness and Odour) and overall acceptability of banana springs by panellists

Banana spring	Time of frying	Sweetness	Crispness	Odour	Overall acceptability
Nangka	3	1.83[a]	3.67[d]	3.93[a]	2.83[b]
	4	1.83[a]	3.97[c]	4.13[a]	2.86[b]
	5	1.90[a]	4.37[b]	4.20[a]	4.63[a]
	6	2.17[a]	4.50[a]	4.27[a]	3.27[a]
Berangan	3	1.37[a]	3.80[a]	4.03[a]	2.37[b]
	4	1.40[a]	4.03[a]	4.07[a]	2.37[b]
	5	1.43[a]	4.43[b]	4.10[a]	2.93[a]
	6	1.57[a]	4.77[c]	4.27[a]	3.90[a]
Maximum		2.17	4.77	4.27	4.63
Minimum		1.40	3.67	3.93	2.37
Mean		1.687	3.817	4.125	3.145
SD		±0.89	±0.95	±0.92	±0.76

Values in columns having dissimilar letters indicate significant difference

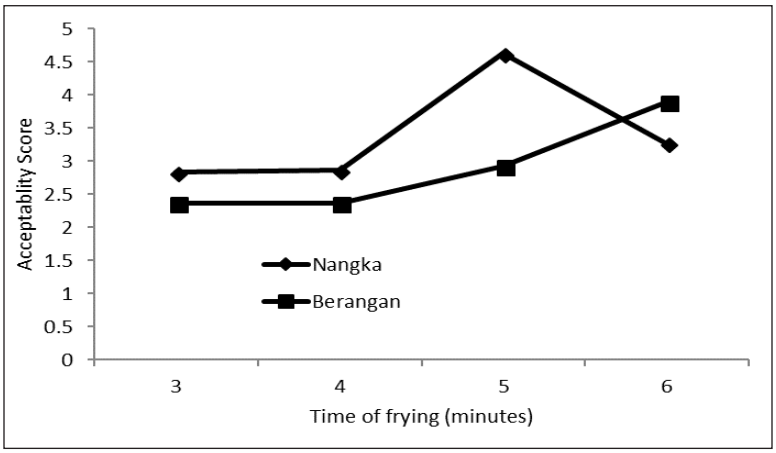


Figure 5. Effects of time of frying on overall acceptability of banana spring

(a) (b)

Figure 6. Fried banana springs of: (a) Berangan; and (b) Nangka varieties

CONCLUSION

The banana springs are a new addition to dried banana food products. Nangka banana at green matured stage can be used for making good quality banana springs. Frying the banana springs in a deep-fat fryer at 170°C for 5 minutes is enough to produce appropriate hardiness and crispness of the product. The observed acceptance score is support marketing prospects of banana springs in comparison to perishable raw fruit. Therefore, banana springs of both the varieties fried in a deep-fat-fryer at 170°C for 5 minutes may be promoted in food markets after comparing them with similar snack foods (e.g., potato chips, banana chips, etc.). This research also gives some impetus to study in-depth the physio-chemical properties of banana springs, effects of moisture content before frying, the shelf-life of banana springs under different packaging conditions, and effect of using crisping powder before frying to enhance the taste and quality of banana springs.

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Land Use Changes in Dharmasraya District, West Sumatra, Indonesia

Yurike[1], Yonariza[2*], Rudi Febriamansyah[2] and Syafruddin Karimi[3]

[1]Department of Agricultural Sciences, Graduate Program, Universitas Andalas, Padang, 25163 West Sumatra, Indonesia
[2]Department of Agricultural Socio Economic, Faculty of Agriculture, Universitas Andalas, Padang, 25163 West Sumatra, Indonesia
[3]Department of Economics Studies, Faculty of Economic, Universitas Andalas, Padang, 25163 West Sumatra, Indonesia

ABSTRACT

Deforestation in the tropics is having significant ecological, economic and social impacts (global warming, biodiversity loss, respiratory problems, flooding). Previous research has indicated a close relationship between poverty and deforestation. This study was motivated by high deforestation rates in Dharmasraya, West Sumatra, Indonesia and a desire to test the assumption that poverty was driving this. Using a mixture of primary and secondary data and drawing heavily on 250 interviews with households living in and around the forest in Dharmasraya, this study describes the context, the forest clearing technique used by households, and analyses what drives deforestation in Dharmasraya. The findings showed that people had cleared as much as 80% of the forest using slash-and-burn techniques. Hotspots were observed from the NOAA satellite. It was found that deforestation in Dharmasraya was driven by middle-class households who control land with an average of 14.97 ha. This finding is significant because it suggests that a forest-related development programme should benefit society economically and socially, the latter in terms of awareness of the importance of protecting the forest because of its ecological functions.

Keywords: Development programme, hotspots, slash and burn technique, society

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E-mail addresses:
ryurike@yahoo.com (Yurike)
yonariza@agr.unand.ac.id (Yonariza)
r_febriamansyah@agr.unand.ac.id (Rudi Febriamansyah)
syafruddinkarimi@gmail.com (Syafruddin Karimi)
* Corresponding author

INTRODUCTION

Tropical deforestation can have a major impact on global warming, water cycle, biodiversity (Geist & Lambin, 2002), carbon emissions, and species lost (Rosa, Smith, Wearn, Purves, & Ewers, 2016). Deforestation, the change of land coverage from forest to non-forest, can have natural

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or anthropogenic causes, temporary or permanent, and either legal or illegal conversion of land (Forest Watch Indonesia, 2014). Although tropical deforestation continues to the present day, the protagonists are changing. Previously the state and private sectors were dominant actors, whereas now households are increasingly playing an active role.

Most Asian countries have seen mass-deforestation (> 70%), Indonesia and Malaysia in particular, have seen rapid deforestation (Laurance, 2007). Deforestation in Indonesia is partly caused by widespread poverty and the conversion of the forest by the small-scale farmers. However, this is inversely proportional to deforestation in this case study at Dharmasraya.

Dharmasraya district in West Sumatra, Indonesia has 302,958 ha of forest or 31.12% of total forest cover in Indonesia. The Ministry of Forestry Decree No. SK.695 / Menhut-II / 2013 dated 21st October 2013 established the Production Forest Management Unit (PFMU) in Dharmasraya covering an area of 33,550 ha. Forest coverage within this PFMU has declined by 27,216.57 ha in recent years. Administratively, this PFMU area is the state forest which was awarded a timber concession licence by the government from 1972 to 2002. The high rate of deforestation occurred after the license ended.

There is a forest tenurial conflict where the local community claimed this land as their communal land while in the other FMU areas in Indonesia, land occupation occurred due to weak forest management and people's need to earn a living. In the Dharmasraya PFMU the land has been occupied and traded for investment. However, forest exploitation in FMU Dharmasraya did not occur spontaneously. It is because in PFMU areas, customary regulations still apply, requiring all communities, and others wishing to manage forests to seek permission from their customary law functionaries. There is a conflict in terms of forest land ownership (Mutolib, Yonariza, Mahdi, & Ismono, 2017) and a new approach of forest encroachment occurs systematically and structurally where formal power holders seek to gain benefits from a vacuum in forest management.

Since 2000, these local communities have gradually converted forest land into oil palm and rubber plantations. Although according to the Government Regulation No.6 of 2007, converting forest land into rubber plantation is still allowed because rubber trees can be categorised as forest plants (Ministry of Forestry, 2007), but not those of oil palm. Based on that regulation, the unproductive forest in this study site has been transformed by local communities into productive rubber plantations.

The official rights to the PFMU land were actually given to the government-owned forest company (called INHUTANI) to convert that forest into industrial forest by enriching a specific kind of plant that is *Dipterocarpaceae* trees. Unfortunately, the local community occupied the forest because INHUTANI failed to develop this industrial forest and left the forest land unutilised

for a long period. However, the local communities have replaced rubber trees with oil palm which is not included in the category of forest plants. This was mainly due to the steady decline in rubber prices, while the price of oil palm is promising.

Forest and land rehabilitation is an effort to restore, maintain and improve the function of forests and land so that the carrying capacity, productivity and its role in life supporting system are maintained (Ministry of Forestry, 2008). Rehabilitation must work in tandem with changing the local community's behaviour. Yet, the factors influencing this behavior are not well understood and many forest rehabilitation programmes failed. It is clear that the factors contributing to this land use change are complex. The relationship between forest clearing and household variable are varied and depends on its management (VanWey, Ostrom, & Meretsky, 2005).

Moonen et al. (2016) concluded only few studies examined the motivation for deforestation due to lack of consistent datasets, especially in the local context. Kaimowitz and Angelsen (1998) reviewed more than 150 cases of tropical deforestation using an economic model. They criticised the multi-nation research due to its low data quality (Moonen et al., 2016). They also found a variety of reasons for deforestation and suggested the need to carry out research at the local level. In addition, Kaimowitz and Angelsen (1998) found that the land market was one of the main uncertainties that could be solved only by further research. This study helps fill this gap.

The paper argues the deforestation in Dharmasraya is strongly related to the role of the households in the forest. Therefore, it is important to find out the characteristics of households contributing to high deforestation rates. The present research examines the forest condition in Dharmasraya, clearing techniques used by the people, and analyses the drivers of deforestation drivers.

MATERIALS AND METHODS

This research was conducted from April to October 2016 in the Production Forest Management Unit (PFMU) Dharmasraya in West Sumatra Province, Indonesia. The study used descriptive method based on a survey. It used various data collection techniques, such as observation, secondary data collection, documentary, historical data, key informants, and household survey. Figure 1 indicates the research site.

Respondents were the households encroaching on the forest land. There are no accurate data about how many encroachers are in the PFMU are, either locals or those from other surrounding villages. This makes it impossible to determine the population of the encroacher. Therefore, this study used a snowball sampling technique (Thompson, 2002). Preliminary visits were made to areas within PFMU Dharmasraya to identify locations where local people clear the forest. We identified encroachers in the following locations; *Bulangan, Sungai Jernih, Bukik Gadang, Sungai Likian, km 25, Sakaladi, Bukik Batu Basalai, Sungai Siek*. The encroachers were identified by asking the

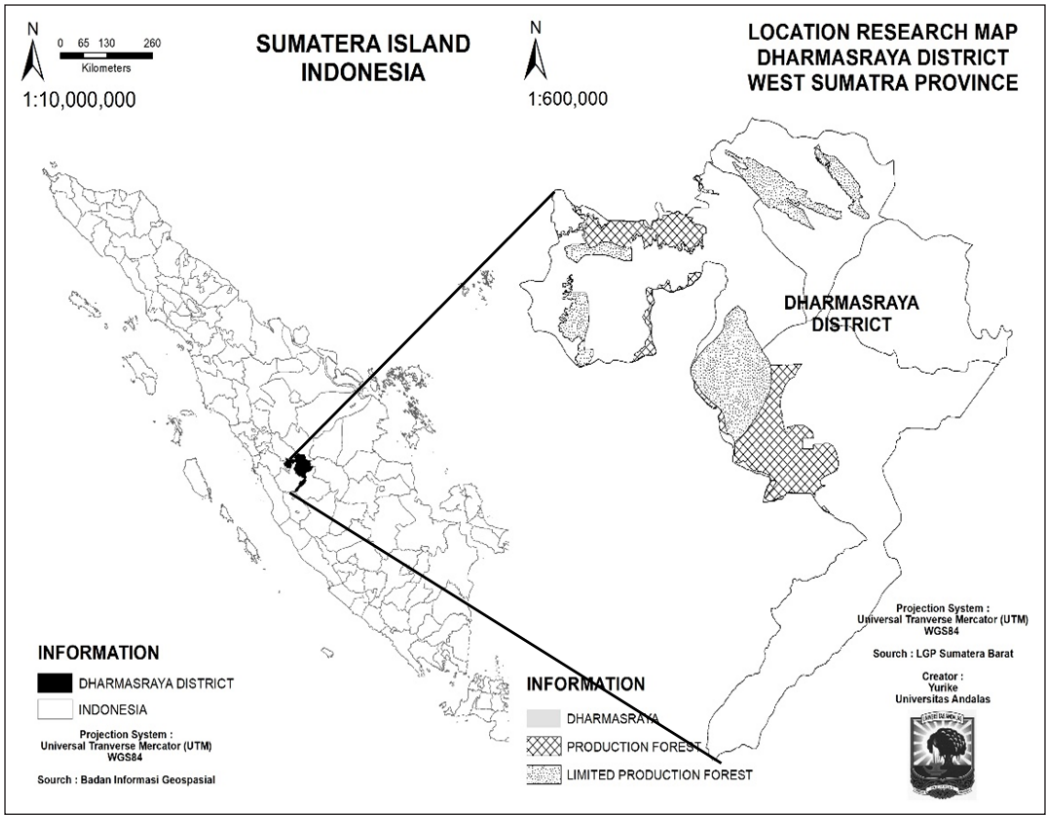

Figure 1. Location of the Production Forest Management Unit (PFMU) Dharmasraya, West Sumatra Province

owners of plot near their land. Overall, there were 250 respondents interviewed from various villages who occupied forest land.

Research variables were livelihood of respondents, plant type, road access condition, land cover change, forest clearing techniques used by households, asset ownership, forest fire hotspots, farmer's land occupation history (year), and size of land (ha). The data were analysed using descriptive quantitative statistics (Williams, 2007) by describing the collected data without generalising the findings (Sugiono, 2012).

RESULTS

Salient Features of Dharmasraya District

Dharmasraya is a district level administrative unit in West Sumatra Province, established in 2004. The current Dharmasraya District Government forest management strategy is a legacy of the previous administration. However, deforestation has increased during the current administration.

The trans-Sumatra highway passes through Dharmasraya and this district is located at the intersection of roads linking various cities in Sumatra Island, i.e., Padang the capital of West Sumatra,

Pekanbaru capital of Riau Province, and Jambi Province. One third of the district's population are migrants from various regions, who resettled in this area and opened new businesses. Previously, this area was still largely covered by forest. In 1979, the government sponsored a resettlement programme known as transmigration. In the first year, immigrants initially only relied on government allocations. After the aid ended people began to move to the plantation.

Most of the land use in Dharmasraya District is for agricultural purposes (89.98%). The plantation area is the largest with an area of 153,822 ha or 51.95% of the total area of Dharmasraya District, while land for rice field is 6,666.8 ha or 2.25% and community forest area is 61,274.8 ha or 20.69%.

Based on the research results, the occupation of forest encroachers are farmers (55.6%), entrepreneurs (18.8%), military personnel (2.8%), employees (20.0%), or have other occupations (2.8%). This showed that for 47.2 % of the respondents were not farmers, where gardening is an investment for them and not their main source of income. Those who have more financial capital tend to outsource the cultivation of their land.

Condition of the Forest

Dharmasraya forest is dry land forest with wet tropical rain forest on undulating areas. The Dharmasraya PFMU is home for about 80 species of plants. There is no competition among the species. The dominant species are Meranti (*Shorea leprosula*), Medang (*Litsia firma*), Sikubung (*Litsea cubeba*), Kelat (*Xylopia altissima*), Balam (*Palaquium walsurifolin*), Mempening (*Quercus lucida*), Arang-arang (*Diospyros puncticulosa*), Mahang (*Macaranga hypoleuca*), and etc. The tree species in this forest include rare species with small growing potency (Production Forest Management Unit, 2014).

The forest in Dharmasraya is relatively flat lowland forest (steep areas account for 11.8% of the forest, flat areas for 48.2%, and sloping areas for 40.1%). The area is easily accessible and only requires a low investment cost. Undulating parts of the PFMU are located near settlements, and it is easy to access the forest to harvest timber. More than half of the areas have been converted into plantation, the rest is secondary forest (logged over forest) and newly cleared areas.

The forest is easily accessible. A high demand for land, especially from newcomers, has intensified people's interaction with the forest. The economic activities within the PFMU areas are rubber and oil palm plantations. During the early stage of the rubber planting, the farmers also grow chilli and peanuts. The high interaction between people and forest has caused massive encroachment in PFMU areas (Figure 2 and Figure 3).

The ex-forest concession roads ease access to the forest and results in land conversion by small-holders. This also encourages the community members to clear the land. The local government also facilitated by constructing infrastructure

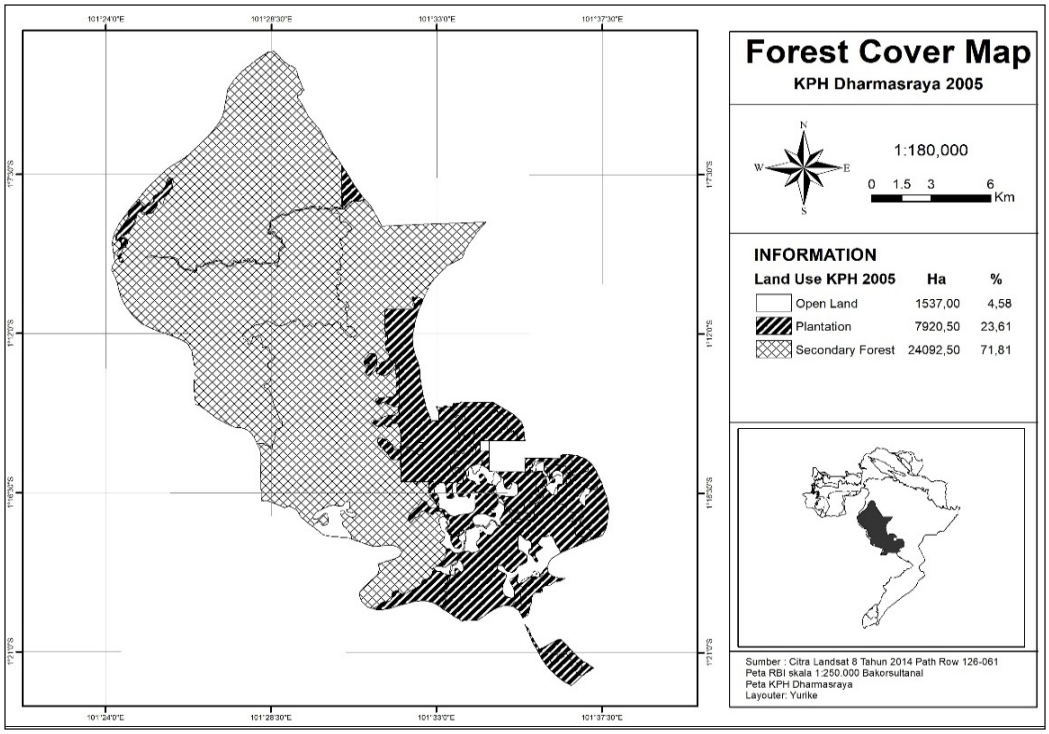

Figure 2. The extend of various forest cover in 2005

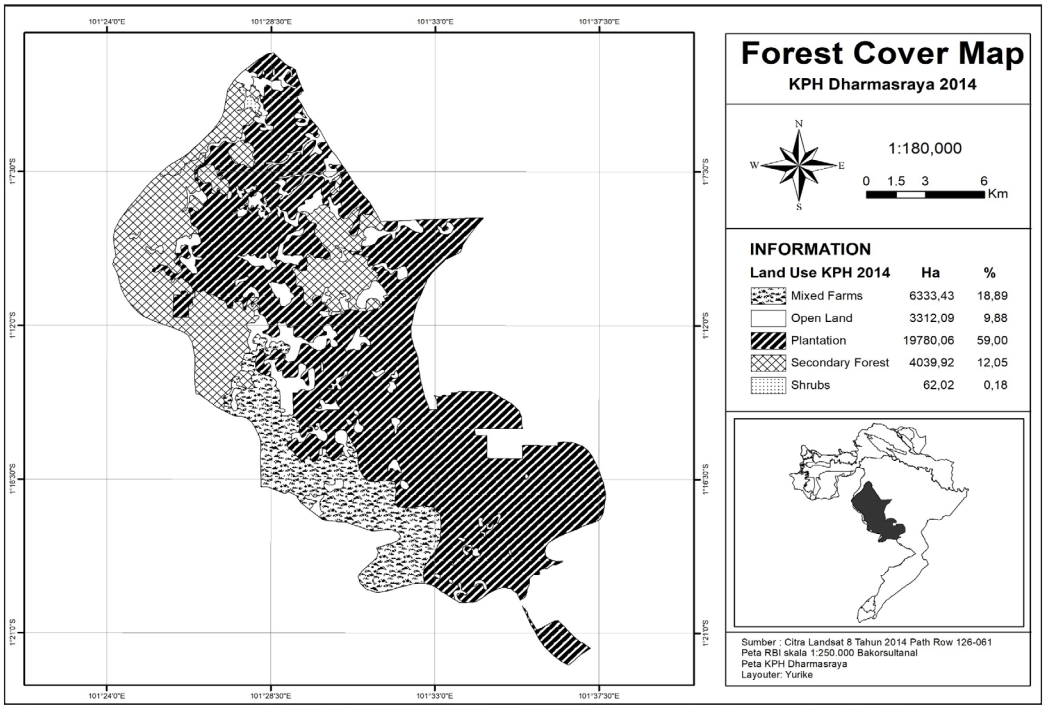

Figure 3. The extend of various forest cover in 2014

such as bridges and road to ease access to plantations in designated forest areas. The easy access obviously influences the forest cover. The negative effect is the loss of forest areas (Caliskan, 2013). This forest conversion, in turn, also affects the environmental and water management in Dharmasraya. It may cause Dharmasraya to be at high risk of environmental hazards, such as floods and soil erosion.

Land Clearing Technique

Communities surrounding the forest land are closely related to the utilisation of forest sources. This association can be seen from their existing assets (Table 1). Household assets that facilitate the plantation in the forest areas, including the possession of working tools, are shown in Table 1.

All farming households have, at least, one motorcycle, giving them easy access even though the distance to the forest can be more than 20 km from their settlement. They also have machinery for forest clearing such as chainsaw. For the rubber farmers, each household has supporting tools such as taping knives. These assets are used to clear forest land for plantations.

Encroachers who have more financial capital tend to outsource work force to cultivate their land. Despite 47.2% of them not being farmers, the encroachers have agriculture tools and machinery needed to support their own plantations. The plantation is also managed by members of their family as well as by outside workers.

In regard to forest land clearing techniques, the local community in

Table 1
Household farm assets in Dharmasraya District

Farm related asset		Number (and percentage) of asset unit by number of household (n=250)								
		0	1	2	3	4	5	6	7	8
a. Hoe	n	2	235	13	0	0	0	0	0	0
	%	0.8	94.0	5.2	0	0	0	0	0	0
b. Oil palm	n	0	76	26	9	7	0	2	0	1
Harvester	%	0	30.4	10.4	3.6	2.8	0	0.8		0.4
c. Chainsaw	n	53	145	49	1	1	1	0	0	0
	%	21.2	58.0	19.6	0.4	0.4	0.4	0	0	0
d. Rubber	n	53	6	93	61	31	3	3	0	0
taping knife	%	21.2	2.4	37.2	24.4	12.4	1.2	1.2	0	0
e. Grass cutter	n	102	143	5	0	0	0	0	0	0
	%	40.8	57.2	2.0	0	0	0	0	0	0
f. Manual saw	n	9	215	26	0	0	0	0	0	0
	%	3.6	86.0	10.4	0	0	0	0	0	0
g. Axe	n	13	220	17	0	0	0	0	0	0
	%	5.2	88.0	6.8	0	0	0	0	0	0
h. Motor bike	n	0	113	123	12	2	0	0	0	0
	%	0	45.2	49.2	4.8	0.8	0	0	0	0

Dharmasraya usually use the slash and burn technique. This method has been used for agricultural purposes, especially in plantation development (Yukili, Nuruddin, Adnan, Malek, & Razali, 2016). Initially, the big tree stands were cut and the timber sold. The remaining tree branches were cleared or left to dry naturally. The slashing uses axes, choppers and chainsaws. Vegetation drying rates depend on the season. They usually clear the land during the dry season when the remaining tree branches can be left to dry for a few days and then burned. The remaining biomass is then cleared after the fire is out. The remaining tree branches are also used as a hedge or for firewood.

The local communities in Dharmasraya have been using this technique for a long time. The main reason for using the slash-and-burn technique is because it is simple, fast and practical. Forest fires are observed through the NOAA satellite and recorded by the West Sumatra Forestry Department. For example, the high number of forest fires spots from 2014-2016 is shown in Figure 4.

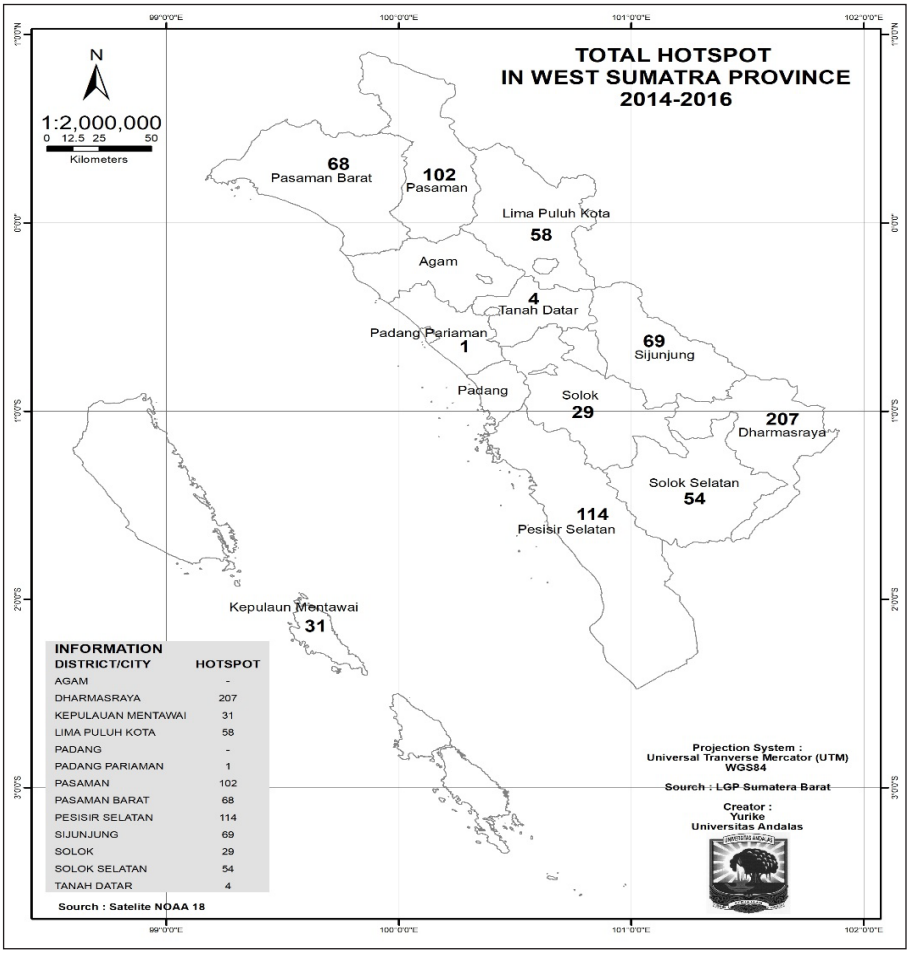

Figure 4. Occurrence of hotspots in West Sumatra 2014-2016

Forest fires are common in Dharmasraya in order to make way for plantations. The slash and burn technique has negative effects, such as environmental impacts on water supply, flooding, biodiversity loss and social effects. There are some negative effects of slash and burn including ecological (Goswami, Choudhury, & Saikia, 2012), land structural damage (Thomaz, 2017), and sedimentation risk (Wagenbrenner, Robichaud, & Brown, 2016). It also decreases soil quality and changes the soil structure in ways that lead to lower agricultural productivity (Tabi, Mvondo, Boukong, Mvondo, & Nkoum, 2013). N and P concentrations decrease after 3 years of slash and burn cultivation (Gay-des-Combes et al., 2017).

Deforestation Drivers at the Household Level

Forest clearing has increased over the last 10 years as shown in Table 2. There is a connection between the closure of timber concession and the time when local people begin clearing the forest for the small holder plantation. It was the time when the last forest management ended.

Table 2
Number of respondents by history of Smallholders' establishment Inside Forest Land

Year of plot clearance	Frequency (n)	Percentage (%)
before 2000	10	4.0
2001 – 2005	16	6.0
2006 – 2010	93	37
2011 – 2016	131	52
Total	250	100

According to local land tenure system, the forest is a communal property or *ulayat* land that is passed from one generation to the next in the Dharmasraya community. However, in 1972, the government granted timber concession to Ragusa Ltd covering an area of ± 66,000 ha. Then in 2002, the license to this company was revoked by the government because they did not perform as expected. After 2002, a portion of this concession area was given to three plantation companies, namely PT. Incasi Raya, PT. SMP dan PT. AWB, to convert half of this forest land into oil palm plantations. Another portion of the forest concession area was given to three other forest companies (PT. Inhutani, PT. Dara Silva, and PT. Bukit Raya Mudisa) for industrial forestry uses.

PT. Ragusa actually left their forest concession area unmanaged prior to revoking of timber concession in 2002, and the local communities started claiming their traditional rights over forest land for their own benefits. They used the stub roads built by PT Ragusa to go deeply to the forest area for their own purposes. Table 2 shows the increase in local communities who taken over the forests use in this region since before 2000 up to 2016. They claimed these forest areas are their *ulayat* (indigenous customary rights). Currently, those local communities have planted most of these forest areas with oil palm and rubber.

Based on the state administrative regulation, these forest areas are state property but in fact, the local communities recognise it as *ulayat* land. If any investors were granted the right to exploit the

forest from the government, they still need to seek permission from the *ulayat* leader. This caused conflict between the investors and *ulayat* leaders that resulted in ineffective work by the company which has a government licence (Mutolib, Yonariza, Mahdi, & Ismono, 2016; Yonariza, Mutholib, & Yurike, 2015; Yurike, Yonariza, Febriamansyah, & Karimi, 2015). The more local communities occupied the forest for their own purposes, especially small-holders, the more difficult for the licensed companies to use their right to develop plantations. Table 3 shows the size of smallholder plantations in the forests that have already have government licence. This is the main factor causing forest degradation in this study site.

Table 3
Size of smallholder plantation area

Area (Ha)	Number of plantations	Percentage (%)
<10	184	74
11-20	35	14
21-30	15	6
31-40	4	2
> 40	12	5
Total	250	100

Mostly the respondents (74%) have plantations of less than 10 ha but some of them occupy more than > 11 ha (Table 3). The average land occupation is about 14.97 ha. For some households, the main purpose is to have a plantation to increase their level of income. Other reasons include selling land, clearing an area of 10 ha with the intention of selling half of it to establish smallholder plantation. This clearly shows that plantation development is motivated by monetary factor although its economic value is much lower than that of conserving the forest (Ghani, Haron, Nor, Nuruddin, & Ahmad, 2007) if assessed from the overall economic value of the forest.

DISCUSSION

The findings of this study are consistent with others on the causes of deforestation. As suggested by Wunder (2000) and Kanninen et al. (2007), the main cause of deforestation is open access to forests. Chomitz, Buys, De Luca, Thomas and Wertz-Kanounnikoff (2007) added that road access is a major factor in deforestation. This is the situation in Dharmasraya where access road was provided by the former concession companies, making it easier for people to access the forest.

The finding of this study is also similar to that reported by Medrilzam, Dargusch, Herbohn and Smith (2014) who found that forest degradation in tropical peat land in Central Kalimantan, Indonesia was mainly triggered by community livelihood activity. However, this study also found another driver of change including uncertainty of land tenure, such as land right claims by the community based on customary law. Overlapping land right claims, like in Dharmasraya, reflect the weakness of existing law enforcement. This promotes the community to clear the land which leads to a significant decrease of forest cover.

Findings of the present study on the cause of deforestation at the household level are different from those reported by Zwane (2007) who studied the relationship between poverty and forest clearing in Peru. Zwane (2007) concluded that a small increase to household income will reduce deforestation. The government believes that it is important to increase household income to slow down the deforestation rate (Chomitz & Kumari, 1998). A similar case was reported by Miyamoto, Parid, Aini and Michinaka (2014), who look at poverty in Peninsular Malaysia poverty as a major factor leading to deforestation. The Dharmasraya case is different because many of the encroachers are middle-class people. This can be seen from the ownership of assets and the area of land cleared by households. The middle-class households are active in forest clearing.

These findings filled the research gap (Kaimowitz & Angelsen, 1998) but there is a need for more research at the local level. Moneen et al. (2016) stated relevant local context-specific information is needed for policy makers to design efficient, effective and equitable policies. This research helps fill the gap in information at the local level in Dharmasraya.

CONCLUSION

The physical changes in the forest area of an ex-forest concession in PFMU Dharmasraya areas contributed to a significant decrease in forest coverage. The forest encroachers in Dharmasraya are middle class people who use a slash and burn approach for clearing the forest. There is a need for forest development programmes that not only improve income of the local community but also people's awareness of the importance of protecting the forest to improve its ecological function throughout the tropical region.

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Anticancer and Antioxidant Activities from Sea Cucumber *(Stichopus variegatus)* Flour Dried Vacuum Oven

Ridhowati, S.[1]*, Zakaria, F. R.[2], Syah, D.[2] and Chasanah, E.[3]

[1]Department of Fiseheries Product Technology, Faculty of Agricultural, Sriwijaya University, 30862 Indralaya, South Sumatera Indonesia
[2]Department of Food Science, Bogor Agricultural University, 16680 West Jawa, Bogor, Indonesia
[3]Research and Development Center for Marine and Fishery Product Processing and Biotechnology, 10620 Jakarta Pusat, Indonesia

ABSTRACT

The anticancer and antioxidant activities from sea cucumber (*Stichopus variegatus)* flour dried vacuum oven have not been widely reported. This study aimed to determine *in vitro* inhibition of enzyme and water extracts from sea cucumber flour against colon cancer cells WiDr, breast cancer cells T47D, and normal cells Vero, then the apoptotic induction. Sea cucumber flour was made by vacuum oven. It was water extracted and hydrolyzed by digestive enzyme gradually. The water extract (SV-WE) and enzyme hydrolysis (SV-EE) were tested for their antioxidant activities using DPPH method. Samples of SV-WE and SV-EE were tested for their anticancer activities using MTT assays. Antioxidant activity of SV-EE at 1.67 ± 0.05 mg/mL was significantly different from SV-WE at 2.30 ± 0.30 mg/mL. SV-EE and SV-WE have anticancer activity against WiDr cells at 13.01 ± 2.75 µg/mL and 69.37 ± 24.25 µg/mL, respectively. The IC_{50} value of apoptotic ability of cell inductions from SV-EE was 64.9 ± 1.63%. SV-WE and SV-EE showed higher anticancer activity against WiDr cells and T47D cells. Gamma sea cucumber flour from Indonesia could be utilized as a potential ingredient in functional foods.

Keywords: Anticancer, antioxidants, enzyme hydrolysis (SV-EE), stichopus variegatus, water extract (SV-WE)

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E-mail addresses:
sherlyridhowati@unsri.ac.id (Ridhowati, S.)
fransiskarungkat@gmail.com (Zakaria F. R.)
dahrulsyah74@gmail.com (Syah, D)
ekowatichasanah64@gmail.com (Chasanah, E.)
* Corresponding author

INTRODUCTION

Functional food is a natural food or minimally processed foods that are safe, and have benefits for human health. Functional food can be made from the animal source, such as sea cucumbers that are usually consumed to prevent degenerative diseases. Sea cucumbers are marine invertebrates

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of the phylum *Echinodermata* abundantly found in the world. In Asian folk medicine, sea cucumbers are mainly used for an antibacterial and antifungal, anticoagulant, antihypertensive, and immune system booster (Bordbar, Anwar, & Nzamid, 2011). The potency of them were as a therapeutic properties and medicinal benefits because of their bioactive compounds, such as peptide, phenolic, glycoprotein, glycosaminoglycans, and the others (Bordbar et al., 2011).

Wijesinghea, Perumal, Effendy and Charles (2013) noted that natural products had been used in health management, such as in the prevention and treatment of a cancer. One of the functional properties of sea cucumbers was in alternative treatment for cancer (Bandgar & Gawande, 2010). For the cancer evidence, the solution for eliminating the cancer tissues was the pharmacy, surgery, and chemotherapy (Dong, Dong, Tian, Wang, & Zhang, 2006). In contrast, Sondhi et al. (2010) stated that the pharmaceutical drug for the cancer sometime had a short clinical life, especially serious side effects. Therefore, we do need an anticancer that is perfectly safe and effective.

Ogushi, Yoshie-stark and Suzuki (2006) reported that *Stichopus japonicus* extract could inhibited and reduced the growth of a cancer cells through an apoptosis induction process. Previous research showed that *Stichopus japonicas* hydrolysate obtained from the hydrolysis process using enzymes from its digestive tract had a hydroxyl radical scavenging activity with the highest activity on protein molecular weight fractions between 1 and 5 kDa (Pan, Yao, Zhou, & Wu, 2012).

This research examined the potential of anticancer and antioxidant activities of water-soluble and enzyme extracts from sea cucumber (*Stichopus variegatus*) flour. In this study, sea cucumber was dried into flour using a vacuum oven. The information of enzyme hydrolysis and water extracts from sea cucumber flour have not been widely reported as an anticancer potential against WiDr colon cell line and T47D breast cell line, plus the ability of apoptosis induction.

MATERIALS AND METHODS

Flour Material Preparation

The processing of sea cucumber flour dried vacuum oven was done according to Ridhowati, Chasanah, Syah and Zakaria's (2018) method. The fresh flesh of sea cucumber was washed with seawater after removing the part of internal organs. The fresh flesh of sea cucumber was cut, dried with vacuum oven (50^{o}C, 65 cmHg, 4 h), and milled to obtain 60-mesh flour. Then, all samples were homogenized and kept in frozen at −20^{o}C until used for analysis.

Stichopus Variegatus' Flour Water Extraction (SV-WE)

The extraction was established based on the method published by Farshadpour et al. (2014) with slight modification. A water extract was prepared from *Stichopus variegatus* flour (5 g) then mixed in distilled water (50 mL), then sonicated for 30 min at

4°C with sonicator (Branson 8510, Danbury, USA). After that, it was separated at 5,031 g for 30 min at 4°C. The supernatant was collected. The collected water extracts were freeze-dried and stored at –20°C until analysis.

Stichopus Variegatus' Flour Enzyme Hydrolyzed (SV-EE)

The enzyme extraction was accorded by the method of Perez-Vega, Leticia, Jose and Blanca (2013) with some modification. The digestion of sea cucumber flour was conducted with gastrointestinal enzymes simultaneously. Sea cucumber flour (8 g) was homogenized with 160-mL distilled water in waterbath shaker at 98°C for 20 min. An aqueous solution of flour was initially hydrolyzed with pepsin (10 U/g; P7000-25G, Sigma-Aldrich St Louis, MO, USA) for 120 min at 37°C and pH 2.0. After pepsin hydrolysis, pH was raised to 7.5 with 1 M NaOH, trypsin (0.4 Unit/g, T4799-5G, Sigma- Aldrich), and chymotrypsin (100 Unit/g, C4129-250mgr, Sigma-Aldrich) were added, then continuously incubated at 37°C for 120 min. After hydrolysis, enzymes were inactivated by heating at 85°C for 20 min, followed by cooling to room temperature. Then, sea cucumber hydrolysate was centrifuged at 9820 g at 4°C for 45 min to separate insoluble and soluble fractions. Finally, the soluble phase recovery was freeze–dried and preserved at –20°C until used for further analysis.

Chemical Composition

Approximate chemical composition of SV-EE and SV-WE were determined according to the AOAC (2005) method, such as moisture, ash, crude lipid, and crude protein. The content of amino acids was also conducted according to AOAC (2005) using high-performance liquid chromatography, and expressed as a percentage of amino acids content.

DPPH Scavenging Assay

Determination of antioxidant activity using DPPH Scavenging Assay was based on Blois method (1958) with slight modification. The antioxidant activity test was performed by DPPH method based on the sample's ability to reduce the stable-free radical of DPPH (1,1,1-diphenyl-2-picrylhydrazyl). All measurements were conducted in triplicate.

Total Phenols

Determination of total phenol was assessed by using Folin-Ciocalteau reagent, as described by Anesini, Graciela and Rosana (2008). All determinations were performed in triplicate. The total phenol count of the sample was based on the results of plotting the absorbance values on the standard curve.

Cell Culture

Human colon cancer cell line (WiDr) and breast cancer cell line (T47D) were grown in RPMI 1640 (Gibco, Invitrogen Corporation) media that contains 10% (v/v) FBS (Qualified, Gibco, Invitrogen), and 1000 mg mL streptomycin penicillin

antibiotics 100 µg/mL (Gibco, Invitrogen Corporation), while Vero cells (ATCC CCL 81) were grown in Dulbecco's Modified Eagle's Medium (D-MEM), Phosphate Buffer Saline (PBS) and incubated at 37°C with a 5% CO_2 flow. Anticancer activity was tested using MTT assay. The WiDr, T47D, and Vero cells were distributed into 96 well plates (Nunc) with a total of 5000 cells per well and incubated with both single application and combination samples using DMSO solvent for 24 h on CO_2 (Heraceus). At the end of incubation, the sample was added 100 MTT (Sigma) in RPMI media (Gibco) for WiDr cells, T47D cells, and also in D-MEM media for Vero cells. The plates were incubated for 4 h at 37°C until formazan crystals (see under inverted microscope (Zeiss)). The living cells reacted with the MTT until purple color was formed. After 4 h, the MTT reaction was discontinued by adding 100 µL 10% SDS reagent at each well, then incubated overnight at room temperature by covered with aluminum foil. Absorption was read by ELISA reader (Bio-Rad) at 595 nm wavelength. The data was obtained in the form of absorbance of each well converted into viability cell percentage. It was calculated using searching linear regression equation, and calculated concentration of IC50 that is concentration causing death 50% of cell population so that it can be a known potency of anticancer activity.

Cell Growth Inhibitory Assay

The cytotoxicity of the SV-WE and SV-EE against the tumor cells was assessed via a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide (MTT) assay. WiDr and T47D cells were seeded (1.0×10^5 cells/mL) together. Both WiDr and T47D cells were seeded with various concentrations (2, 10, 50, 250, and 1250 µg/mL) then incubated for 48 h prior to MTT treatment. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide stock solution (50 µL; 2 mg/mL in PBS) was added to each well to achieve a total reaction volume of 200 µL. After 4 h of incubation, MTT reaction was stopped by 10% SDS, 100 µL in each well then incubated overnight. The formazan crystals in each well were dissolved in dimethyl sulfoxide. The amount of purple formazan was assessed by measuring the absorbance at 595 nm using an ELISA reader (Sunrise; Tecan Co.Ltd., Australia).

Detection of Apoptotic using Flow Cytometric Analysis

Differentiation of apoptotic and necrotic cells using FITC Annexin V apoptosis Detection Kit II (BD Biosciences) based on Elmore (2007) methods. WiDr cancer cells with a final density of 7×10^5 in a 6-well microculture were incubated for 12 h in CO_2 (37°C, 5% CO_2 flowing). The extracts were added to cells according to IC50 concentrations for 24 h. Later, 100 µL of extracts were resuspended in 5-µL FTIC Annexin and 5-µL PI, then incubated for

15 min, in dark room at 20 to 25°C. Typical histogram of apoptotic and necrotic cells was performed using FACSCalibur (Becton-Dickinson) flow cytometer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein profile was characterized by SDS-PAGE (Sodium Dodecyl Sulphate Poliacrylamide Gel Electrophoresis) according to Laemmli (1970) method. A 10-µL protein sample plus 10-µL RSB (Reducing Sample Buffer), inserted into the microtube, was then heated to 100°C for 2 min. Electrophoresis is carried out at 60 mA, 220 V for 3.5 h. Protein bands were stained by silver nitrate 0.1% (w/v) (Sigma Aldrich). Analysis of molecular weight (MW) was carried out by using protein Marker (Sigma-Aldrich) as standard. The scanned protein bands were plotted into the linear regression equation curve to obtain the MW protein values in each sample.

FTIR Analysis

Fourier Transform Infrared Spectroscopy (FTIR) () spectrometer (Model Equinox 55, Bruker, Ettlingen, Germany) was used to identify functional groups or side chains of a material, with the range of 400–4,000 cm^{-1} then automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} at 25°C. A 2-mg sample was added with KBr powder up to 200 mg. Previously, KBr powder was heated at 105°C overnight. Samples that have been mixed with KBr are then pounded using agate mortar until homogeneous. The samples were placed onto the crystal cell. The data was obtained using the curve of the relationship between wave number (x axis) and absorbance (y-axis). The frequency of the obtained wave numbers is then determined by the type of bond and functional group compared with the secondary data of FTIR.

Statistical Analysis

The value data are expressed as mean ± standard deviation with three replications. One-Way ANOVA were analyzed using SAS 9.1.3.Portable program.

RESULTS

The Profile of SV-WE and SV-EE

The organisms contained high protein and low fat have been associated with good health (Bordbar et al., 2011). Ridhowati et al. (2018) reported that the fat content of SV-WE and SV-EE were 0.57 ± 0.66% and 0.45 ± 0.02% of total dry weight, respectively. The protein content of SV-EE and SV-WE were 37.09 ± 0.06% and 5.73 ± 0.01% of total dry weight, respectively (Ridhowati et al., 2018). The contents of amino acid profiles were different between SV-EE and SV-WE. Moreover, all amino acid contents from SV-EE and SV-WE have decreased from sea cucumber flour, as shown in Table 1. The differences of protein content and amino acid profile were influenced by the type of sea cucumbers, the extraction process, handling and drying process into a product.

Table 1
Amino acid composition of Stichopus variegatus *(%w/w protein, dry weight)*

Amino acid (% w/w)	SV-Flour*	SV-EE	SV-WE
Acid amino group			
Aspartic acid	2.59 ± 0.01	3.06 ± 0.01	0.43± 0.01
Glutamic acid	4.71 ± 0.01	4.99± 0.01	0.96± 0.01
Polar amino group			
Serine	0.93 ± 0.01	1.76± 0.01	0.17± 0.02
Glycine	4.99 ± 0.01	5.90± 0.01	0.30± 0.01
Threonine	1.31 ± 0.02	2.14± 0.02	0.24± 0.01
Basic amino group			
Arginine	2.33 ± 0.02	2.91± 0.01	0.27± 0.02
Lysine	5.79 ± 0.01	1.28± 0.01	0.21± 0.03
Histidine	0.29 ± 0.02	0.47± 0.02	0.06± 0.02
Hydrophobic amino group			
Alanine	2.61 ± 0.02	2.48± 0.01	0.25± 0.03
Methionine	0.51 ± 0.02	0.96± 0.02	0.13± 0.01
Valine	1.10 ± 0.03	1.28± 0.01	0.15± 0.01
Phenylalanine	0.86 ± 0.02	1.07± 0.02	0.14± 0.01
Isoleucine	0.86 ± 0.03	1.01± 0.01	0.11± 0.01
Leucine	1.16 ± 0.02	1.64± 0.02	0.19± 0.02
Proline	2.61 ± 0.01	2.64± 0.03	0.18± 0.01

Source: Ridhowati et al. (2018)

The Antioxidant Activity and Total Phenol of SV-EW and SV-WE

Table 2 shows that the IC_{50} value of SV-EE and SV-WE have the ability to scavenge DPPH free radicals at 1.67 ± 0.05 mg/mL and 2.30 ± 0.30 mg/mL, respectively. Both of them were failed to capture 50% of DPPH radical even though in high concentrations, compared to ascorbic acid that showed 10.59 ± 0.07 µg/mL on IC_{50} value as the control. In contrast, the highest total phenol contained of 10.90 ± 0.14 mgGAE/g in the SV-WE was significantly different to 10.55 ± 0.07 mgGAE/g in the SV-EE.

Table 2
Total phenolic contents, DPPH scavenging capacities and yields of Stichopus variegatus *flour*

Samples	DPPH scavenging capacity (IC_{50} = mg/mL)*	Total Phenols (mgGAE/g)**	Yields (%)
SV-WE***	2.30 ± 0.30a	10.90 ± 0.14a	3.33 ± 0.18a
SV-EE***	1.67 ± 0.05b	10.55 ± 0.07b	1.12 ± 0.83b
Ascorbic acid	0.01059 ± 0.00007		

Note. *Data expressed as IC_{50} (the concentration of extract that required for scavenging of 50% DPPH) in mg extract/mL DPPH, mean ± SD (n = 3); **Data expressed as gallic acid equivalents (GAE) mg/g extract, mean ± SD (n = 3); *** SV-EE fraction (*Stichopus variegatus*' flour enzyme hydrolyzed) and SV-WE fraction (*Stichopus variegatus*' flour water extraction)

The Anticancer Activity of SV-EE and SV-WE

Evaluations of sea cucumber *Stichopus variegatus* for potential anticancer activity on WiDr and T47D cells were determined the growth inhibitory effects of both SV-EE and SV-WE fractions using the MTT assay. The IC_{50} values against WiDr cells of SV-EE and SV-WE were 13.01 ± 2.75 µg/mL and 69.37 ± 24.25 µg/mL, respectively. The SV-EE was strongly inhibited WiDr proliferation in a dose-dependent manner (Figure 1). While, the IC_{50} value of anticancer activity against T47D cells of SV-WE and SV-EE were 219.10 ± 22.17 µg/mL and 157.92 ± 99.91 µg/mL, respectively (Figure 2). Moreover, SV-EE showed cell proliferation were inhibited more than 82% at IC_{50} value. Otherwise, both of them were more effective against WiDr cell lines than T47D cell lines. Then, the anticancer effect of them on cell viability in normal (Vero) cells was tested as well (Figure 3). Wherein, the IC_{50} value against Vero cells for SV-WE and SV-EE was 1053.49 ± 90.26 µg/mL and 634.22 ± 32.75 µg/mL, respectively.

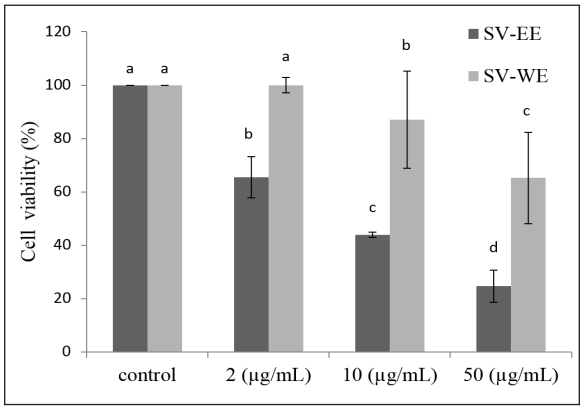

Figure 1. Effect of SV-EE (*Stichopus variegatus*' flour enzyme hydrolyzed) and SV-WE (*Stichopus variegatus*' flour water extraction) on cell viability in WiDr cells. Mean ± SD of three determinations as data

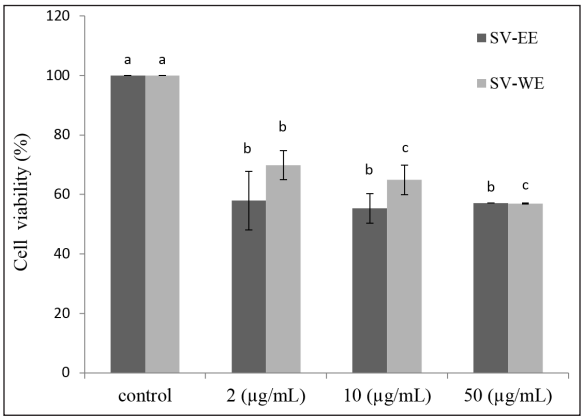

Figure 2. Effect of SV-EE (*Stichopus variegatus*' flour enzyme hydrolyzed) and SV-WE (*Stichopus variegatus*' flour water extraction) on T47D cell viability. Mean ± SD of three determinations as data

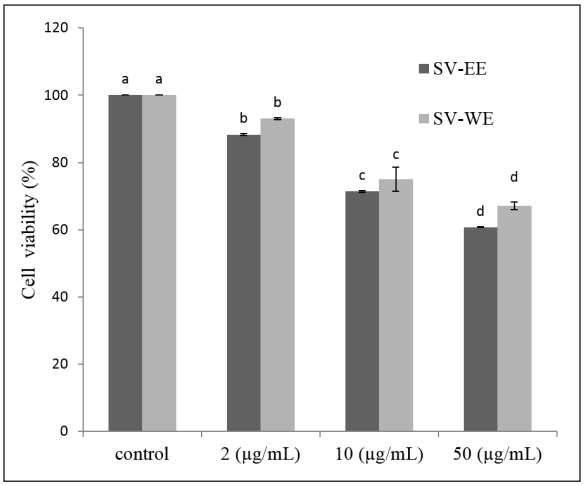

Figure 3. Effect of SV-EE (*Stichopus variegatus*' flour enzyme hydrolyzed) and SV-WE (*Stichopus variegatus*' flour water extraction) on Vero cell viability. Mean ± SD of three determinations as data

DISCUSSION

The content of amino acid was different between SV-EE and SV-WE (Table 1). Nevertheless, both of them have higher hydrophobic amino acid components than polar amino acid group. The quality of marine animals can be affected significantly by free amino acid composition. Lysine, glycine, and hydrophobic amino acids, such as alanine and methionine, may function as immunomodulator and anticancer (Bordbar et al., 2011). Amino acid components of sea cucumbers are involved in the regulation of immune system (Qin et al., 2008). In addition, amino acid content is different because of the variation of organisms, marine geography, species, age and physiological condition (Capillas, Moral, Morales, & Montero, 2002).

Based on Table 2, both of them were failed to capture 50% of DPPH radical even though in high concentrations, compared to ascorbic acid that showed 10.59 ± 0.07 µg/mL on IC_{50} value as the control. The results of this study was almost the same IC_{50} value as with Althunibat's study, the water and organic extract of sea cucumber *Stichopus chloronotus* research that have the ability to capture a DPPH radical at 2.13 ± 0. 05 mg/mL and >10 (Althunibat et al., 2009). In this research, the contribution of total phenol as antioxidant properties was low. According to Zheng and Wang (2003), the capacity of free radical scavenging for phenolic compounds was fluctuated depending on the chemical species and the concentration. In this case, there is no correlation existed between radical scavenging capacity and total phenolic content. The scavenging activity of DPPH radical suggested that from other components, such as salts, sugars, glutathione and peptides (Zhong, Khan, & Shahidi, 2007). However, the phenolic compounds could contribute to

the antioxidant activity of sea cucumber (Zhong et al., 2007). Esmat, Said, Soliman, El-Masry and Badiea (2012) stated that sea cucumbers were rich in phenolic compounds, such as phytoplankton and particles derived from the degradation of marine macroalgae, suspected as a source of phenolic active compounds that accumulated in the sea cucumber body. Polyphenols are the main antioxidants known as free radical terminators. These compounds can neutralize free radicals by acting as a rapid donor from hydrogen to radical atom.

Toxicity evaluation in Vero cells showed less cell deaths at the tested concentration. For the Vero cells, SV-EE was higher in IC_{50} value than SV-WE. The strong inhibitory activity on SV-WE was due to the antiproliferative hydrophilic compounds, triterpene glycosides (Adrian & Collins, 2005). Potential anticancer activity was demonstrated by the ability of the compound material capable of inducing apoptosis. In this study, the cytotoxic effect of SV-EE was investigated in the WiDr cell line. The promising cytotoxic effect showed by SV-EE; it was further continued to evaluate on cell apoptosis induction against WiDr cells. Percentage of apoptotic cells treated with SV-EE fraction was 64.9 ± 1.63% (Figure 4).

It is proposed that the effect of apoptosis induction in this study could be associated with bioactive compound in SV-EE. It was known that hydrophobic and positively charged amino acids existing in peptide C-terminal are contributed to the peptide inhibitory properties. The same pattern almost happened in positively charged amino acids (Arg, Lys), although it is not comparable with those of IC_{50} values (Forghani et al., 2012). The SV-EE has low molecular weight protein fractions. In fact, the position of those amino acids in the peptide sequence is more crucial than

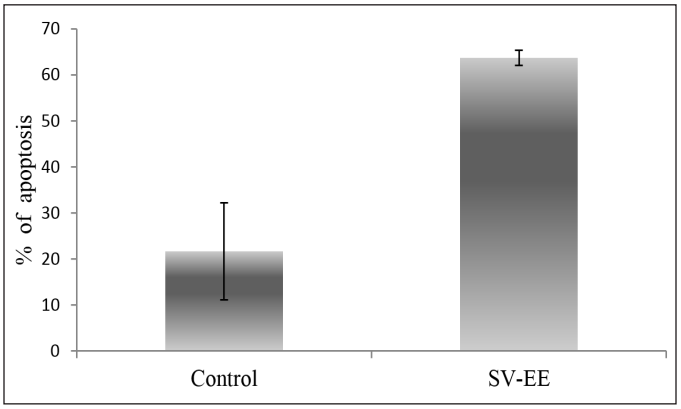

Figure 4. The WiDr cells were subjected to flow cytometric analysis using FITC Annexin V apoptosis detection kit I. The apoptosis induced by SV-EE (*Stichopus variegatus*' flour enzyme hydrolyzed) fraction at the concentration IC_{50} value are shown.

their total amounts (Forghani et al., 2012). It is suspected that a group of hydrophobic amino acids play a role in SV-EE to induce cell apoptosis (Lee, Kim, Lee, Kim, & Lee, 2004), as seen in Table 1.

The higher component of hydrophobic amino acid peptide would penetrate deeper into the hydrophobic core of the cell membrane, thereby causing strong activity forming pores or channels in the cell membrane of cancer. The greater anticancer activity is higher always accompanied with the hydrophobic amino acid content (Huang, Xiao-Fei, & Hong-Ye, 2011). The hydrophobicity of protein or peptide is very important for accessibility to the target hydrophobic, and increases the affinity and reactivity of the peptide with membrane of living cells (Huang et al., 2011).

In this research, SDS-PAGE was conducted to identify the possible molecular weight proteins that may be involved in promoting apoptosis cells in this study. The potential biological activities of SV-EE and SV-WE as a functional food were also supported by the results of electrophoresis SDS-PAGE. The SV-EE has low molecular weight protein fractions of 21, 19, and 9 kDa compared to SV-WE protein that has varying molecular weights between 279 and 9 kDa (Figure 5). The result of this study was similar to the hydrolysis of *Stichopus horrens* with alcalase because the hydrolysis process of sea cucumbers occurred in almost all proteins with large molecular weight to produce polypeptide under 20 kDa (Forghani et al., 2012).

The electrophoretic patterns of protein have shown that SV-EE and SV-WE fraction mostly contained peptides with molecular weight in the range 279 until 9 kDa. Our results were in agreement with those of several previous studies done on different sources, such as Atlantic cod viscera (Aspmo, Horn, & Eijsink, 2005), which revealed high efficiency of alcalase for protein cleavage leading to production of small peptides. The absence of high molecular bands above 100 kDa indicated that gastrointestinal enzyme is able to degrade protein to smaller molecules with molecular weight below 21 kDa. This study

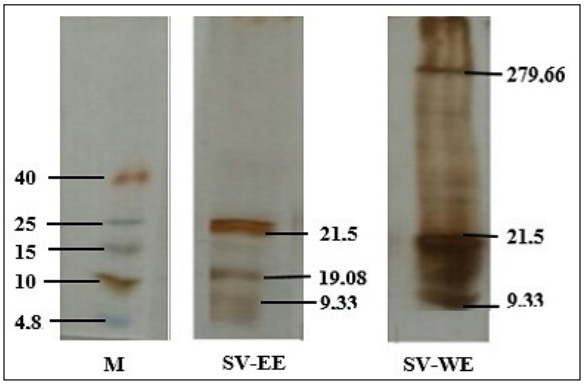

Figure 5. Peptide profile of SV-EE) and SV-WE fractions; M (marker), SV-EE (*Stichopus variegatus*' flour enzyme hydrolyzed) fraction, and SV-WE (*Stichopus variegatus*' flour water extraction) fraction

proposed that the fraction molecules of SV-EE and SV-WE with molecular weight 9 kDa are defined as non-digestible food ingredients.

In this research, the electrophoretic patterns and FTIR spectra were as supporting data. FTIR spectra of both SV-EE and SV-WE from sea cucumber flour are presented in Figure 6. Their FTIR spectra were similar to those of other sea cucumber, *Stichopus japonicus* (Zhu et al., 2012). The SV-EE using gastrointestinal enzyme was found to be the most efficient for degrading protein of *Stichopus variegatus* because of the cleavage of nearly all proteins in the high molecular weight region and generation of polypeptides, SV-EE fraction. The result of SV-EE was supported the FT-IR spectra analysis and MTT assays compared to SV-WE. The major protein compound of sea cucumber was collagen that consisted of 100 kDa α-chain with the molecular weight of and mucopolysaccharides (Saito, Kunisaki, Urano, & Kimura, 2002). The saponin of *Holothuria leucospilota* extract showed several functional groups, namely hydroxyl (OH), alkyl (CH), and ester (-C = O) (Soltani, Parivar, Baharara, & Kerachian, 2014). These functional groups have anticancer activity against A549 cancer cell lines (Soltani et al., 2014).

This study also showed that functional groups were based on spectra analysis. Therefore, SV-EE and SV-WE could inhibit the proliferation of WiDr and T47D cancer cell lines. In this study, sea cucumbers were

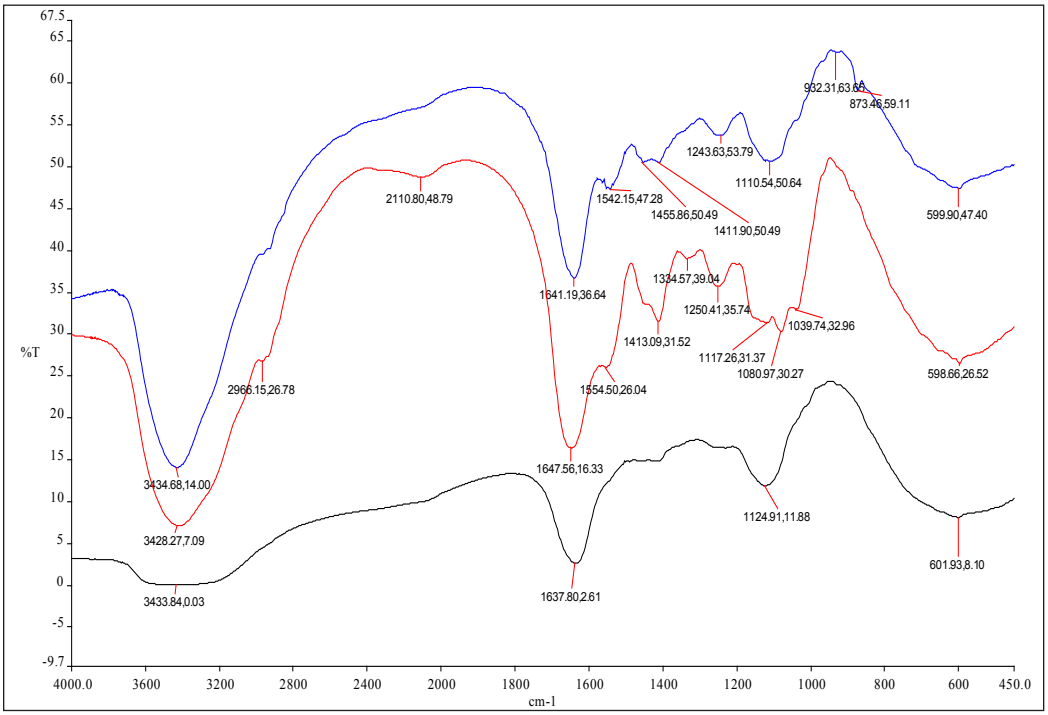

Figure 6. FTIR spectra of black line (sea cucumber flour), red line (SV-EE, *Stichopus variegatus*' flour enzyme hydrolyzed fraction), and blue line (SV-WE, *Stichopus variegatus*' flour water extraction fraction)

caught alive, immediately cleaned, frozen (−20°C) and dried using vacuum oven at 50°C for 4 h, which then caused chemical degradation of the tissues. Sea cucumbers that were being processed after post-mortem then dried in the oven at 60°C for 36 h did not show any tissue degradation (Chang-Lee, Price, & Lampila, 1989).

The results of this study indicated that sea cucumber (*Stichopus variegatus)* flour drying a vacuum oven can be hoped as a functional ingredient. The properties of the sea cucumber flour increased the interaction with components of cancer cells and enhanced anticancer activity (Guadalupe, Armando, & Josafat, 2012). The sea cucumber flour has great potential as an anticancer functional ingredient for cereals, soup, flavor, or biscuits although the flour drying was using a vacuum oven.

CONCLUSION

The findings of this study determined for the new anticancer potential of sea cucumber (*Stichopus variegatus*) flour dried with vacuum oven. The presence of biological activity in the flour of *Stichopus variegatus* extract showed that their consumption could be advantage for healthy. In addition, these findings may facilitate awareness about the potential anticancer properties of *Stichopus variegatus* and help future developments of anticancer therapeutics on industrial scale. In addition, the enzyme-extract fraction of *Stichopus variegatus* flour could be further developed as a complementary cancer remedy, ingredients functional food, and nutraceutical.

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Food and Feeding Habits and Length–Weight Relationship of *Parachanna obscura* from Federal University of Agriculture Reservoir, Abeokuta, Ogun State, Nigeria

Festus Idowu Adeosun

Department of Aquaculture and Fisheries Management, Federal University of Agriculture, Abeokuta, PMB 2240, Ogun State, Nigeria

ABSTRACT

Population increase, indiscriminate fishing, and others, threatening extinction of wild fish species is a major problems in Nigeria fishery. Hence, the food and feeding habits and quality of *Parachanna obscura* from Federal University of Agriculture Abeokuta (FUNAAB) Reservoir were investigated between the months of March and August, 2015. Food materials in fish stomach were analyzed using standard methods, while length and weight were obtained using standard measures. A total of 11 food categories were observed in the stomach of fish sample during the study period 2%, 28%, 9%, 32%, and 29% were empty, one-quarter full, half full, three-quarter full and full, respectively. 8.33% and 5.71% vacuity was experienced in the months of July and August, respectively. Total lengths of *P. obscura* ranged from 12-27 cm with a mean of 17.62 ± 0.27 cm while the weights ranged from 43-198 g with a mean of 102.63 ± 4.32 g. The highest abundance of specimens were observed in August (35%) followed by July (24%) with the lowest observed in March (6%), April (8%), May (12%), and June (15%). Linear regression of the analyzed data resulted in length exponent '*b*' of 2.45 while '*a*' was −1.16 and $r = 0.88$. Approximately 78% of the variance in body weights can be explained by total lengths ($r^2 = 0.78$). Condition factor ranged between 0.70 and 2.49 with a mean of 1.48 ± 0.51. The study confirms the carnivorous nature of *P. obscura* from this water. Also, findings will assist fisheries managers in making fisheries policy regarding appropriate time for fishing of the species.

Keywords: Carnivorous feeder, diet composition, euryphagous, FUNAAB reservoir, linear relationship, stomach contents

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INTRODUCTION

The differences in the mode of feeding showed by fish species could be attributed to evolutionary changes due to structural adaptation for getting food from great diversities of happenings in the wild (Lagler, Bardach, Miller, & Passino, 1977). Taming and culture of any animal species require the determination of feeding habit of such an animal in its natural habitat. Ipinmoroti, Olasunkanmi and Aransiola (2008) expressed their opinion that when studying the type of food and mode of feeding demonstrated by various species of fish for the sake of culture, it should be carried out to enable the identification of the food material that was most preferred by the species as the survival of the fish species in a closed environment depended on how much the natural ecosystem was simulated in the culture system. Fish species found in Inland waters have been observed to have greater access to different food organisms/items in the wild and thus giving room for food selection among these species (Komolafe &Arowomo, 2011). *Parachanna obscura* is a member of the Channidae family and has its root in West Africa (Whenu & Fagade, 2012). The species is commonly abundant in vegetative swamps especially at the offset of the rains when the water level has decreased. Teugels, Reid and King (1992) in their work posited that the species was common in stagnant water bodies in Cross River State of Nigeria - Cameroon. Bonou and Teugels (1985) stated that this species occupied a wide variety of habitats including streams, rivers, lakes, lagoons, and marshes but in flowing water they occupied calm areas. It is one of the two species of the family Channidae found in the coast of West Africa namely, *Channa obscura* and *Channa Africana* (Steindachner, 1879) (syn. *Ophiocephalus obscures* and *Ophiocephalus africanus* respectively). *Parachanna obscura* has been reported by previous studies to be carnivorous in nature (Ajah, Georgewill, & Ajah, 2006). They are observed to feed mainly on earthworms, tadpoles, shrimps, smaller fish, and other aquatic animals (Bonou & Teugels, 1985). Adebisi (1981) and Ajah et al. (2006) reported that adults fed mainly on other fish while the juveniles fed on zooplankton, earthworms, tadpoles, and shrimps. As the mouth part began to form properly, species sizes of 10–16 cm, were observed to feed primarily on detritus and insect larva (Victor & Akpocha 1992). The predatory and piscivorous nature of the species especially in the adult stage was reported by Bolaji, Mfon and Utibe (2011). Although, information regarding the biology of this species has been largely reported, there is however need to shed more light on some aspect of its biology. The present study therefore aims to contribute to the knowledge of the biology of *P. obscura* with a view to further developing its aquaculture.

MATERIALS AND METHODS

Description of Study Site

The study was carried out at the Federal University of Agriculture, Abeokuta (FUNAAB) reservoir (Figure 1) that was constructed in 1994 using excavator, D6-

buldozer and other equipment (inlet and outlet devices). The area and volume of the reservoir are 30,510 m^2 and 63,308,250 L. It lies on latitude 7°13'N and longitude 3°2'E with a prevailing tropical climate and ambient temperature that ranges between 29°C in February and 33°C in May at an average of 31°C. Prior to dam construction, various fish families were observed in the water body; however, no attention or study was carried out to investigate the individual families. The dominant families of fish found in the reservoir include Channidae, Claridae, Cichlidae, Hepsetidae, and Cyprinidae (Adeosun et al., 2012).

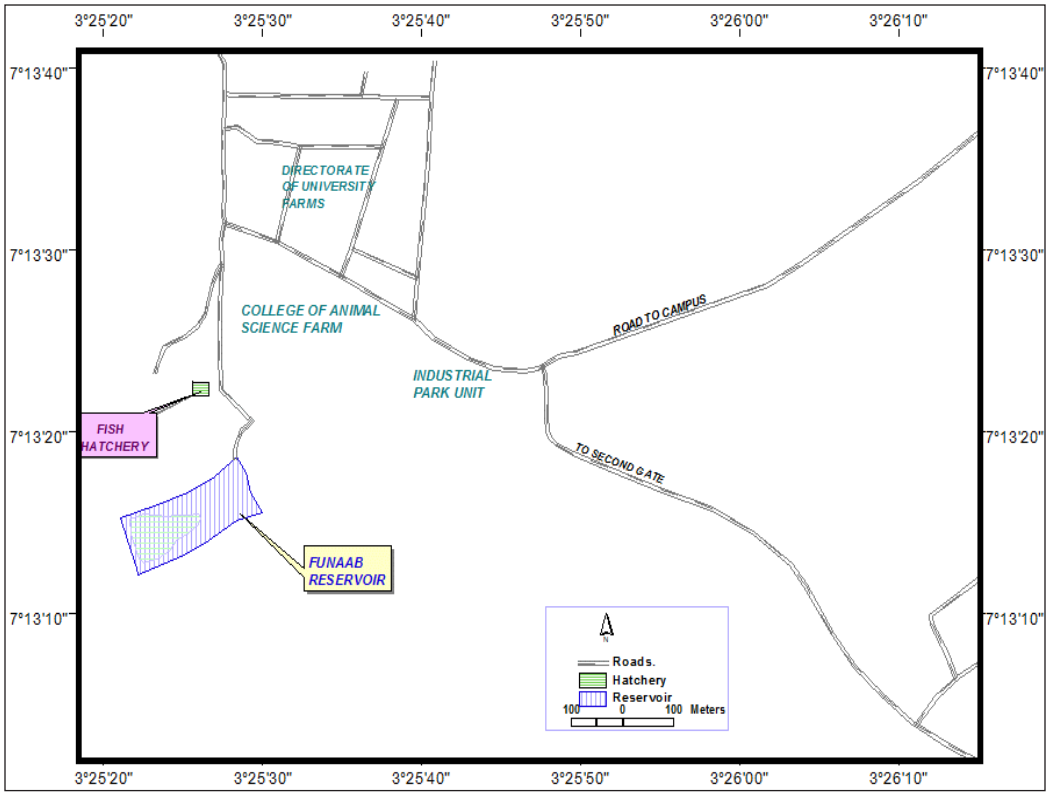

Figure 1. Map of FUNAAB showing the reservoir

Collection of Specimens

A total of 100 fish samples were collected fortnightly between the months of March and August, 2015 with the assistance of Fishermen using gill nets, cast nets, set nets, baskets, and so on. The samples were serially labeled using numbered tag (A_1, A_2,…, B_1, B_2) after which they were transported alive in ice chest to the laboratory for subsequent treatment.

Laboratory Procedure

Fish samples were anesthetized using 4% of chloroform and left for 20 min after which they were arranged and weighed individually to the nearest gram with the aid of Citizon electronic balance. The total and standard lengths of each sample were taken to the nearest 0.01 cm with the aid of measuring meter board. Using a pair of scissors, fish samples were dissected ventrally from the anal opening to the head and stomachs were removed carefully. After observing and recording the degree of fullness as 0/4 for empty stomach, ¼ for one quarter full stomach, ½ for half full stomach, ¾ for three-quarter full stomach and 4/4 as fish with full stomach, weights of each stomach and intestine were measured and recorded (Ugwumba, 1988). Identification of food items (zooplankton and phytoplankton) was done using identification guide by Needham and Needham (1962). About 10% formalin was used to preserve each stomach and its content in a specimen bottle labeled correspondingly with the tag number of each fish sample. Food items were later analyzed using the methods reported by Hynes (1950) and Hyslop (1980).

Analysis of Food Items

Numerical. Stomach contents were poured into a petri dish and placed under different magnifying lens of a binocular microscope. The number of food items present in the preserved stomach of each sample were counted and summed up in other to obtain the grand total number of all food items found in the preserved stomach. The dietary items were identified to taxonomic categories using checklist; the number of each food item was then expressed as a percentage of the grand total number of food items. It is usually express as:

$$\% \text{ number of food items} = \frac{\text{Total number of the particular food items}}{\text{Total number of all food items}} \times \frac{100}{1}$$

Frequency of Occurrence. The total number of stomach with particular food item was counted and expressed as a percentage of the total number of the stomachs with food (empty stomach excluded). This is usually expressed as:

$$\% \text{ Frequency} = \frac{\text{Total number of stomach with particular food item}}{\text{Total number of stomach with food}} \times \frac{100}{1}$$

Index of Fullness

Fullness index was estimated using the formula:

$$\text{Fullness index} = \frac{\text{Dry weight of stomach content}}{\text{Fresh weight of fish}} \times \frac{100}{1}$$

It is usually expressed as parts per 10,000 (% 00, or parts per decimal).

Length–Weight Relationship

Length–weight relationship was expressed by the equation:

$$W = aL^b$$

(Sparre & Venema, 1998, pp. 450)

Where,

W = weight, L= length, 'a' and 'b' are regression constants.

L = total length (cm) and b = regression (growth) coefficient.

Condition Factor

Fulton's condition factor was computed in conformity with Ricker (1975) using the equation:

$$K = 100\ W/L^3$$

(Bagenal & Tesch, 1978)

Where K = condition factor, W = total weight (g), L = total length (cm) and b = regression (growth) coefficient.

RESULTS AND DISCUSSION

Stomach Fullness and Index of Prey Abundance

No empty stomach was observed from March to April while 8.33% and 5.71% vacuity was observed in the months of July and August, respectively (Figure 2). These coincided with the peak of the rains. Of the 100 stomachs of *P. obscura* examined, 2%, 28%, 9%, 32%, and 29% were empty, one-quarter full, half full, three-quarter full and full, respectively (Figure 3). The monthly variation observed in the index of fullness conducted for the species shows that food items were more abundant during the dry season than the wet season. This result is in conformity with the findings of Odo, Onoja and Onyishi (2012) in his survey of 550 samples of *P. obscura* from the lower reaches of Anambra River, Southeastern Nigeria. This accounts for the stomach emptiness observed in some specimen in the months of July and August. Of the fish samples examined, over half had full

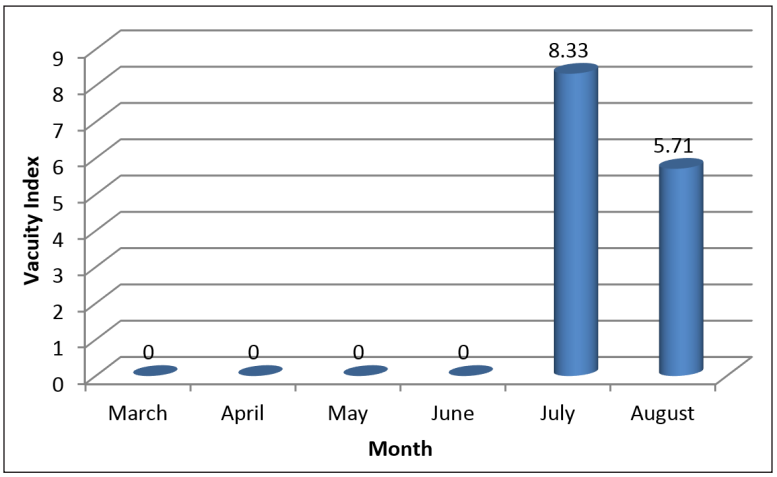

Figure 2. Monthly variation in the number of empty stomachs in *P. obscura* from FUNAAB reservoir

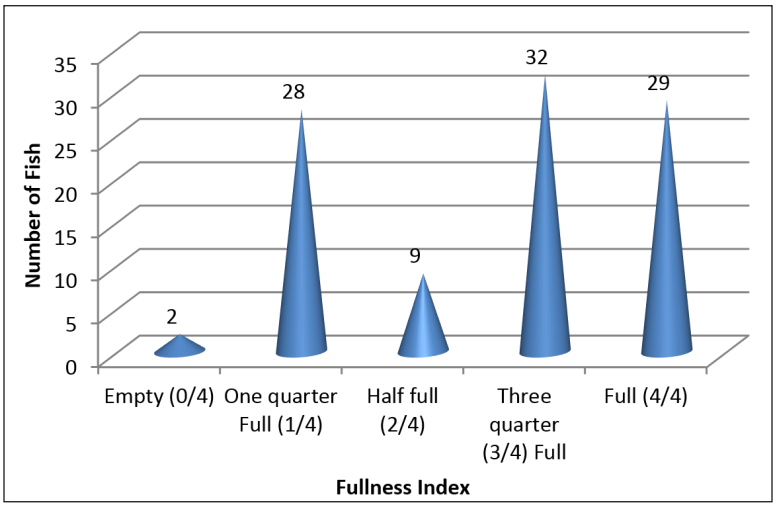

Figure 3. Stomach fullness in *P. obscura* from FUNAAB reservoir

stomachs (Figure 2). However, Komolafe and Arowolo (2011) reported no variation in the fullness of this species in the dry and wet seasons alike. Variation in fullness index observed in this study could be due to the concentration of food items in the dry season than the wet season when the food items are well distributed in the reservoir.

A total of 11 food categories were observed in the stomach of fish samples during the study period (Table 1). These include the green algae, diatoms, protozoa, rotifers, crustaceans, nematodes, fish parts, insect remains, green algae, unidentified food, and desmids. The dietary composition of the stomach of *P. obscura* suggests that the species is a carnivorous feeder as animal materials were observed to be most abundant both in frequency of occurrence and in number. This result corroborates earlier work on the feeding habits of the species (Adebisi, 1981; Ajah et al. 2006). However, the wide range of food items present in the stomach of the species affirms other studies that the species is a euryphagus and non-selective feeder (Uwem, Ekanem, & Eni, 2011). This finding also agrees with the reports of Olaosebikan and Raji (2004) who posited that the species was a carnivorous feeder and prefers food of animal origin.

Table 1
The percentage occurrence and numerical abundance of dietary items in stomachs of P. obscura *samples*

Food Items	Number	% N	Occurrence	% O
Zygnema sp.	457	7.9	33	6.84
Pediastrum sp.	67	1.15	20	4.14
Coelastrum sp.	3	0.05	3	0.62
Closmarium sp.	6	0.1	6	1.24

Table 1 *(continue)*

Food Items	Number	% N		Occurrence	% O
Green Algae	**533**	**9.2**		**62**	**12.84**
Desmids (Gonatozygon*)*	**230**	**3.97**		**20**	**4.14**
Pinnularia sp.	644	11.14		95	19.7
Nitzschia sp.	65	1.1		15	3.11
Amphora sp.	18	0.31		7	1.45
Cyclotela sp.	2	0.03		2	0.41
Diatoms	**729**	**12.58**		**119**	**24.67**
Oscillateria sp.	56	0.96		7	1.45
Coelosphaerium sp.	11	0.19		3	0.62
Microcystis sp.	1	0.01		1	0.2
Blue Green Algae	**68**	**1.16**		**11**	**2.27**
			26.91		**43.92**
Spirotomum sp.	12	0.2		4	0.82
Cladocerans sp.	3	0.05		1	0.2
Paramecium sp.	2	0.03		2	0.41
Amoeba sp.	1	0.01		1	0.2
Protozoans	**18**	**0.29**		**8**	**1.63**
Filinia sp.	2	0.03		2	0.41
Branchious sp.	3	0.05		1	0.2
Rotifers	**5**	**0.08**		**3**	**0.61**
Crayfish parts	1140	19.72		58	12.03
Copepod	110	1.9		35	7.26
Crustaceans	**1250**	**21.62**		**93**	**19.29**
Nematodes	**520**	**8.99**		**41**	**8.5**
Insect remains	**1020**	**17.65**		**57**	**11.82**
Fish parts	**1360**	**23.53**		**60**	**12.44**
			72.16		
Unidentified food	**46**	**0.79**		**8**	**1.65**

% N = percentage of number, % O = percentage of occurrence

Length–Weight Relationship

Linear regression of the transformed data resulted in length exponent '*b*' = 2.45 while '*a*' = −1.16 (Figure 4). Furthermore, a significant positive linear relationship existed between body weight and standard length in the examined samples ($r = 0.88$). Approximately 78% of the variance in body weights can be explained by total lengths ($r^2 = 0.78$). That is, the total length of the juveniles is a fairly good predictor of the body weight. Negative allometry is indicated by the fact that the '*b*' value

obtained for the species was less than 3. This is an indication that samples are lighter than their body lengths (Wootton, 1998, p. 392) that implies poor growths of weight in relation to length. According to Bailey (1994, pp. 937–970), *P. obscura* could grow to the size of 35 cm and Egwui, Okeke and Ezeonyejiaku (2013) recorded a maximum size of 50 cm for the species. The species length as recorded in this study were however less than these values. This could be attributed to either overfishing of the species in this water body or the size of mesh used in capture. Olasunkanmi and Ipirontimi (2014) also recorded negative allometry for this species. This was also corroborated by the findings of Bolaji et al. (2011). The '*b*' value according to Bagenal and Tesch (1978) was however not exceeded as '*b*' values for the species were observed to fall within the growth exponent range of 2 to 4.

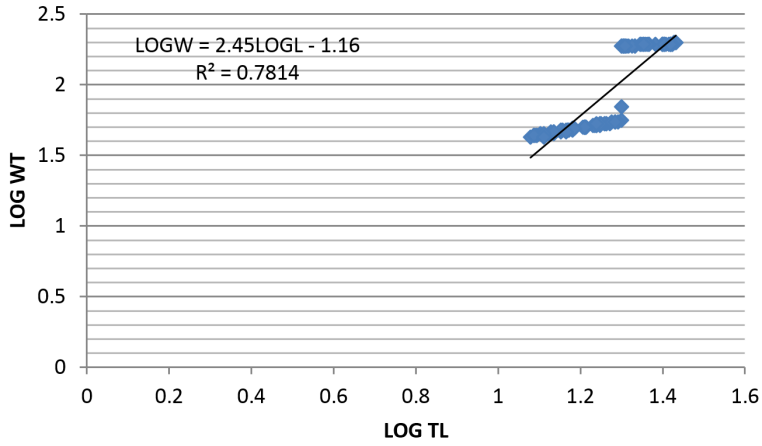

Figure 4. Length–weight relationship of *P. obscura*in FUNAAB reservoir

Length–Frequency Distribution and Abundance

Total lengths of *P. obscura* ranged from 12 to 27 cm with a mean of 17.62 ± 0.27 cm while the weights ranged from 43 to 198 g with a mean of 102 ± 4.32 g. The length histogram as presented in Figure 5 indicates that specimens with total length in the class 16.00–17.99 were the most abundant occupying 17% of the entire sample. The size class 24.00–25.99 cm were the least abundant at 8% frequency. The range of lengths captured in this study was smaller than that reported by Odo et al. (2012) for 550 specimens from Anambra River, Nigeria ranging from 23-28 cm with a mean of 25 ± 0.84. However, specimens examined in this study were weightier that may indicate a better condition. The highest abundance of specimens was observed in August (35%) followed by July (24%) with the lowest observed in March (6%), April (8%), May (12%), and June (15%), respectively (Figure 6).

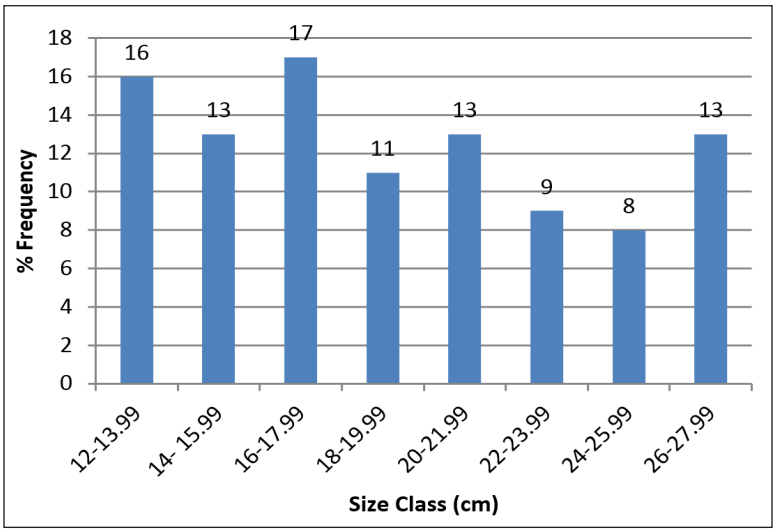

Figure 5. Length-frequency of *P. obscura* from FUNAAB reservoir

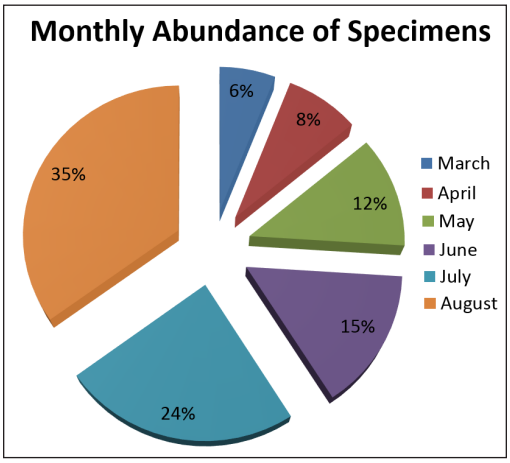

Figure 6. Monthly abundance of *P. obscura* from FUNAAB reservoir

Condition Factor

Condition factor for the 100 specimens of *P. obscura* ranged between 0.70 and 2.49 with a mean of 1.48 ± 0.51 (Figure 7).This is higher than the mean condition factor of the species reported by Odo et al. (2012) for *P. obscura* from Anambra River. It was observed that only 21% of the fish had condition factor greater than 2 while 63% had condition factors that fell between 1 and 1.99. It could therefore be said that the fish species water body in question was in good state of health. Although the fish seem to be well conditioned, since the value was greater than 1, the average value was less

than that of other well-conditioned fishes. This may be an indication that the fish is likely to be easily affected if there is any alteration in the environmental condition as well as unavailability of prime food items.

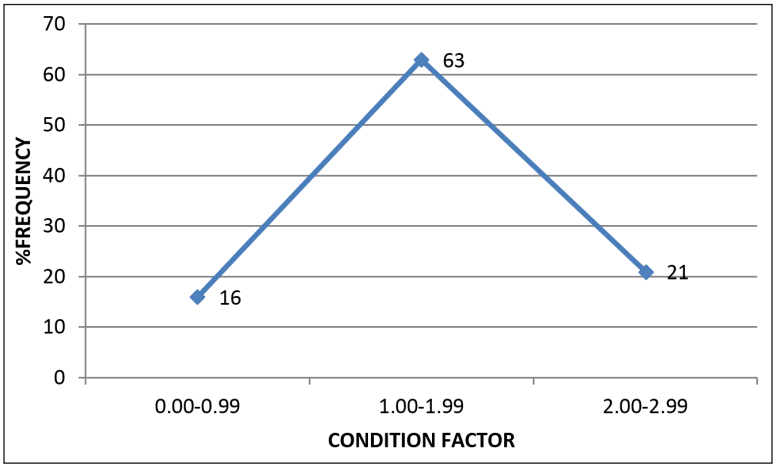

Figure 7. Condition factor of *P. obscura* from FUNAAB reservoir

CONCLUSION

The study concludes that there is abundance of food materials in this water body all year round. Findings will assist fisheries managers in making fisheries policy regarding appropriate time for fishing of the species thereby preventing overfishing and threat of extinction. The various groups of food items in the diet of *P. obscura* from this water body confirmed fish parts to be the prominent and most preferred food consumed by *P. obscura*. Base on this, the species will be good for polyculture as it can be used to control proliferation of cichlids. Also, it has a good quality with condition factor above 1.

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Potential of *Albizia lebbeck*-Cassava Peel Silage as Dry Season Feed for West African Dwarf Sheep

Festus Temitope Ajayi and Sunday Oloruntoba Omotoso*

Institute of Agricultural Research and Training, Moor Plantation, PMB. 5029, Ibadan, Nigeria

ABSTRACT

The study evaluated the growth performance, digestibility, and nitrogen utilisation of West African Dwarf (WAD) sheep fed with *Albizia lebbeck* - cassava peel silage and *Panicum maximum*. Sixteen female WAD sheep (6–7 months old and average live weights of 11 kg) were randomly allotted into four dietary treatments in a complete randomized design. Dietary treatments of *A. lebbeck* - cassava peel silage are: Diet 1 (60% Cassava peel + 25% *A. lebbeck* + 15% *P. maximum*), Diet 2 (45% Cassava peel + 40% *A. lebbeck* +15% *P. maximum*), and Diet 3 (30% Cassava peel + 55 % *A. lebbeck* + 15% *P. maximum*), while Diet 4 (100% *P. maximum*) is the control. The pH values (4.27–5.39), NH_3-N (0.089–0.125%), lactic (0.72–1.08%), and butyric (7.04–10.53%) acids, contents of silages differed ($p < 0.05$). Intakes of dry matter (68.6–71.3 g/kg $W^{0.75}$/d) and crude protein (15.9–18.4 g/kg $W^{0.75}$/d) of sheep were similar ($p > 0.05$) across treatments, while total feed intake (840 g/d) and weight gain (46.07 g/d) of sheep fed diet 1 were superior ($p < 0.05$) to other treatment groups. Although, sheep fed diet 4 had the best ($p < 0.05$) feed conversion ratio, silage diet was efficiently utilized by the sheep fed diet 1. Nutrient digestibility varied ($p < 0.05$) across treatment groups. The values of apparent nitrogen digestibility (69.33%), nitrogen absorbed and retained was highest (2.08 and 1.97 g/d) in sheep fed diet 1, while nitrogen absorbed and retained was lowest (1.37 and 1.12 g/d) in sheep fed diet 3. In conclusion, WAD sheep fed diet 1 performed remarkably in terms of feed intake, weight gain, and N-utilisation. Therefore, *A. lebbeck*-cassava peel silage can be a viable option for sheep feeding in the dry season when grass quality is low.

Keywords: *Albizia lebbeck* foliage, cassava peel, digestibility, nitrogen utilisation, performance, silage

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E-mail addresses:
festus2ajay@gmail.com (Festus Temitope Ajayi)
tobsum2006@gmail.com (Sunday Oloruntoba Omotoso)
* Corresponding author

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INTRODUCTION

In the humid or subhumid regions of Nigeria, small ruminant (sheep and goats) production contribute to the income resource of the rural poor and supply of animal protein (milk and meat) of most households. However, the dry season (usually from December to March) is a critical period when the performance of small ruminant decreases. This is due to the rapid decline in quantity and quality of pastures and the high cost of concentrate feed, which rural smallholder farmers cannot afford to supplement the available low-quality forages. Under these conditions, the animals have to survive on highly lignified roughages (Ademosun, 1992), which are relatively deficient in the essential nutrients required for improved performance.

Cassava peel constitutes the bulk of dry season feed and the most outstanding sources of energy for small ruminant in the intensive and semi-intensive system of production (Adegbola, Smith, & Okeudo, 2010). However, when cassava peel is fed solely to a ruminant, it is not adequate to meet the energy and nitrogen requirements for improved animal performance, even when the animals scavenge household wastes or graze low-quality roughages (standing hay). In the light of the aforementioned, efficient utilisation of low-quality roughages for improved performance would be enhanced through nitrogen supplementation from multipurpose tree legumes.

Utilisation of multipurpose tree legumes as a cheap protein-rich supplement to balance nutrient deficiencies of feed during the dry season in the tropics has been documented (Bansi, Wina, Matitaputy, & Turafelli, 2014). In the same vein, optimal utilisation of low-quality roughages by ruminants through supplementation with foliages of multipurpose tree legumes has also been reported (Attaelmnan, Fadel Elseed, & Salih, 2009; Mousa, 2011) to increase dry matter intake and improve animal performance.

Meanwhile, it is worth mentioning that despite the nutritional potentials of leguminous tree fodder**,** smallholder ruminant farmers in the humid South Western parts of Nigeria rarely utilize foliages of some leguminous tree fodder as ruminant feed. One of the numerous under-utilized leguminous tree fodders is *Albizia lebbeck.* Objections to its utilisation might be due to lack of awareness about the inherent nutritive value and how to integrate them in feeding system, fear of losing their animals on account of likely "poisonous substances" (anti-nutritional factors) and the stress of harvesting particularly cut and carry in the intensive or semi-intensive system of ruminant production.

A. lebbeck, (family: Fabaceae) is an introduced leguminous fodder tree species in Nigeria (Keay, Onochie, & Standfied, 1964), widely adapted to mild and high climates of tropical and sub-tropical countries (Lowry, Prinsen, & Burrows, 1994). Being an evergreen and woody perennial plant, they are grown purposely to provide shade. It is often noticeable with its dry pods held on the tree year round. While the nutritive value and anti-nutritional contents (tannin

and phenolic compounds) of the leaves have been documented (Balgees, Elmnan, Elseed, & Salih, 2011; Chitra & Balasubramanian, 2016), higher dry matter intake (286 g/d and 335.5 g/d) and digestibility (64.22 % and 72.22 %) have also been reported (Adeloye, Daramola, Yousuf, & Ogundun, 2006; Balgees et al., 2011) in West African Dwarf (WAD) and Nubian goats, fed crop residues supplemented with *A. lebbeck* leaves, respectively.

Ensilage is a complex biochemical process in which arrays of compounds are generated as a result of fermentation. However, variations in nutritional value and fermentation quality of silage may have an impact on feed intake (Krizsan & Randby, 2007), and consequently on the performance of animals. Although foliages of *A. lebbeck* mixed with grass makes good quality silage for lactating goats (Solorio-Sanchez, Sol-Jiménez, Sandoval-Castro, & Torres-Acosta, 2007), there is limited information about the optimal level of *A. lebbeck* supplementation with cassava peel and utilisation of *A. lebbeck*-cassava peel silage by WAD sheep.

Since *A. lebbek* foliage can be fed as hay or silage, alone or mixed with grasses to ruminants, there is a need to ensile *A. lebbeck* leaves, cassava peel, and *Panicum maximum* mixtures for dry season feed. The aim of this experiment was to evaluate the fermentation quality of *A. lebbeck* - cassava peel silage, performance, digestibility, and nitrogen utilisation of WAD sheep fed *A. lebbeck* - cassava peel silage.

MATERIALS AND METHODS

Study Location, Animal Management, and Experimental Design

The experiment was conducted at the sheep and goat unit of the Institute of Agricultural Research and Training, Moor Plantation, Ibadan, South West Nigeria, latitude (7°15'–7°30'N) and longitude (3°45'–4°0'E). The area has a tropical humid climate with a mean annual rainfall of 1415 mm and an average daily temperature between 28°C and 35°C. Sixteen female WAD sheep (6–7 months old, average live weight of 11 kg) were weighed and randomly allocated to individual pens, and to one of four treatments diets in a complete randomized design with four animals per replicate in a 105-day feeding trial. Experimental house and individual pens were washed and disinfected, and floors of the pens were covered with wood shaving.

Before the commencement of the experiment, animals were given prophylactic treatments once (administration of injectable oxytetracycline and a multivitamin preparation at the rate of 1 mL per 10 kg body weight via intramuscular route), treated against ecto and endoparasites (administration of ivermectin, subcutaneously at the rate of 0.2 mL per 10 kg body weight), and vaccinated against *Peste de Petit Ruminant* (PPR). The animals were adjusted to their treatments over a 2-week preliminary period, which was followed by a 105-day feeding trial.

Preparation of the Silages

Fresh foliages of *A. lebbeck* was harvested from the National Cereals Research Institute (NCRI) Moor Plantation, Ibadan. Fresh cassava peel was collected from a gari processing mill and spread on a concrete floor to wilt to about 50% DM. *P. maximum* (Guinea grass) was harvested from an established pasture of the Institute of Agricultural Research and Training, Moor Plantation, Ibadan. Foliages of *A. lebbeck* and guinea grass were harvested at 6-week re-growth interval, chopped into 3-5 cm size in length and allowed to wilt. All the materials to be ensiled were weighed in varying proportions, as indicated below and mixed to form a homogenous mixture. Each mixture was ensiled separately for 28 days as previously described (Ajayi, Omotoso, & Dauda, 2016).

Feed, Feeding, and Growth Trials

The diets consisted of concentrate feed as depicted (Table 2), guinea grass (*P. maximum)* and *A. lebbeck*-cassava peel silage mixtures in varying proportions as highlighted below:

Diet 1 = 60% Cassava peel + 25% *A. lebbeck* + 15% *P. maximum*

Diet 2 = 45% Cassava peel + 40% *A. lebbeck* + 15% *P. maximum*

Diet 3 = 30% Cassava peel + 55% *A. lebbeck* + 15% *P. maximum*

Diet 4 = 100% *P. maximum*

Sheep were randomly allotted to the four dietary treatments as indicated above in complete randomized design, with four animals per replicate in a 105-day feeding trial. *A. lebbeck* - cassava peel silage (basal diet) and guinea grass (control diet) were offered separately at 3% body weight. The control diet (*P. maximum*) was harvested at 6-week re-growth intervals, wilted and chopped to 5 cm before feeding. Concentrate feed was offered to all the sheep at 2% body weight. Experimental diets and concentrate mixtures (10 kg maize, 30 kg wheat offal, 10 kg corn bran, 25 kg palm kernel cake, 13.7 kg limestone, 10 kg Groundnut cake, 0.5 kg Fish meal, 0.5 kg Growers premix, 0.3 kg salt) were offered separately at 09:00 and 16:00 h, respectively.

There was provision for a daily feed allowance of 10% above the previous week's consumption. Fresh and clean water was served *ad libitum* to all the animals. Daily feed intake was determined by subtracting feed refused from feed offered over a 24-h period. Animals were weighed at the commencement of the experiment and subsequently live weight change was measured once each week before feeding in the morning throughout the feeding trial.

Digestibility and Nitrogen Balance Trials

After the feeding trial, sheep were transferred to metabolic cages and adapted for 7 days. The study was carried out over a period of 7 days for each treatment for measurements of intake, faeces, and urine of each sheep. Feed offered, feed refused, faeces and urine samples were sampled daily for dry matter determination and chemical analysis. Loss

of nitrogen by volatilization was prevented by adding 10 mL of 10% concentrated sulfuric acid (H_2SO_4) into plastic containers for the collection of urine (Chen & Gomes, 1992). Daily collections of faeces and urine were bulked and 10% sub-sample of each was taken for analysis. Urine samples were frozen for nitrogen determination. Faecal samples were oven dried to a constant weight in a forced air oven at 60ºC, ground and analyzed for chemical analysis.

Chemical Analysis

Samples of un-ensiled feedstuffs (*A. lebbeck* foliage, guinea grass, and cassava peel), silage diets and feces were dried in an oven to a constant weight, milled to pass through a 2-mm sieve, and then analyzed for Crude protein (Micro-Kjeldahl method, N × 6.25), dry matter, ash, ether extract, calcium and phosphorus according to the standard proximate procedures (AOAC, 1995). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined according to the procedure of Van Soest, Robertson and Lewis (1991).

Silage samples were extracted with distilled water and used for measuring the pH, volatile fatty acids (VFAs), ammonia nitrogen (NH_3-N), water-soluble carbohydrates (WSCs), and alcohol contents.

The pH of silages was read on a digital pH meter (model 3510; Jenway). Concentrations of the lactic, acetic, propionic, and butyric acids were measured by the titration method according to the procedure by Gilchrist Shirlaw (1967). Ammonia nitrogen (NH_3-N) content was determined by a colorimetric method using a Spectronic21-D as described by Bolsen et al. (1992). WSC was determined by the colorimetric method as described by Dubois, Giles, Hamilton, Rebes and Smith (1956), and the alcohol content was determined according to AOAC (2005).

Statistical Analysis

All data obtained were subjected to analysis of variance for a completely randomized design using the general linear model (PROC GLM) of SAS (1998), with the following statistical model: $Y_{ij} = \mu + D_i + e_{ij}$. Where Y_{ij} = observed variation, μ= population mean, D_i = fixed effect of diets (1–4), and e_{ij} = residual error. Duncan's Multiple Range Test (Duncan, 1955) was used to detect significant differences among means at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical Composition of Experimental Diets and Raw materials

Chemical and nutrient compositions (g/100 g DM) of feedstuffs and experimental diets are presented in Tables 1 and 2. The dry matter contents of silage diets were relatively similar with a mean value of 85.13% (Table 2). Dry matter (DM) values were highest in diet 3 (86.83%) and lowest in diet 4 (43.62%). The crude protein (CP) contents of diet 1 to diet 3 (11.29-13.32%) were above the 10-12% recommendation for growth or maintenance of small ruminants (NRC, 1985) while control diet (6.81% CP)

was below the minimum threshold level (7% CP) required for optimal rumen microbial activities (Norton, 2003). Ether extract also followed a similar trend, with highest values in diet 3 (5.17%) and lowest in diet 4 (1.84%).

The increased CP (11.29–13.32%) content of silage diets (Table 2) observed in this study corroborated the findings of Balgees et al. (2011) who supplemented baggase with varying proportions (100 g and 150 g) of *A. lebbeck* leaves. The

Table 1
Nutrient composition and fiber fractions (g/100 100 g DM) of feedstuffs for the experimental diets

Composition	*A. lebbeck* foliage	Cassava peel	*P. maximum*
Dry matter	89.43	89.9	92.93
Ash	10.72	9.15	10.11
Crude protein	15.96	6.04	8.40
Ether extract	3.25	1.92	1.13
Neutral detergent fibre	41.35	43.59	61.26
Acid detergent fibre	35.17	26.96	33.89
Acid detergent lignin	8.36	7.71	4.47
Hemicellulose	6.18	16.63	27.37
Cellulose	26.81	19.25	29.42

Table 2
Chemical compositions (g/100 g DM) of A. lebbeck - *cassava peel silage and* P. maximum *fed to West African Dwarf sheep*

Composition	Silage Treatments				*P. maximum* Concentrate
	Diet 1	Diet 2	Diet 3	Diet4	
Dry matter	86.07	82.49	86.83	43.62	90.61
Crude protein	12.80	11.29	13.32	6.81	16.43
Ether extract	2.53	3.54	5.17	1.84	7.32
Ash	3.62	3.31	4.25	4.27	9.40
NDF	31.17	25.55	35.26	55.30	63.85
ADF	29.40	24.61	28.77	34.18	48.31
ADL	26.13	22.32	18.73	8.12	5.67
Hemicellulose	1.77	0.93	6.49	21.12	15.54
Cellulose	3.27	2.29	10.04	26.06	42.64
Calcium	0.66	0.54	0.75	0.01	0.94
Phosphorus	0.85	0.88	0.67	0.03	1.02

CPL: Cassava peel; AL: *A. lebbeck*; PM: *P. maximum*;
Diet 1: 60 % CPL + 25% AL + 15 % PM; Diet 2: 45% CPL + 40 % AL +15 % PM;
Diet 3: 30 % CPL + 55 % AL + 15 % PM; Diet 4: 100% PM; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin

higher percentage of CP obtained in silage diets compared to the control diet was probably due to the release of CP from *A. lebbeck* leaves into the ingredient mixtures. Meanwhile, the values of NDF, ADF, and ADL obtained among silage diets (diet 1 to diet 3) were not above the range (55–60 g/100 g DM) that could limit feed intake and degradation of tropical forages by ruminant (Meissner & Paulsmeier, 1995). The NDF (55.30%) and ADF (34.18%) values were highest in the control (diet 4) compared to silage diets. Among silage diets, ADL content varied from 18.73-26.13%, while NDF (35.26%) and ADF (29.40%) values were highest in diet 3 and diet 1, respectively. The values of hemicellulose (0.93–6.49%) and cellulose (2.29–10.04%) contents of silage diets in this study fell below the range of hemicellulose (8.00–22.00%) and cellulose (13.00–22.00%) contents reported (Inyang, Babayemi, Adeniji, & Ifut, 2013) for cassava wastes - *A. saman* pod silage.

The low levels of NDF and ADF in silage diets could be attributed to the breakdown of hemicellulose in the ingredient mixtures through hydrolysis by organic acids produced during the ensiling process (McDonald, Henderson, & Heron, 1991). Increased ADL content could also be a consequence of the synthesis of Maillard polymers during the fermentation process, which have properties similar to lignin (Van Soest, 1994). The wide variations observed in the hemicellulose and cellulose contents between diet 1 (1.77% and 3.27%) and diet 3 (6.49% and 10.04%) could have arisen from the ensiling conditions (silage pH, and temperature, bacterial species and the population of bacteria) within each diet during the ensiling process and the proportion of cell wall fractions exposed to hydrolysis by organic acid (McDonald et al., 1991).

However, the levels of fiber fractions in this study could be categorized as moderate and might favor the proliferation of fiber digesting microorganisms in the rumen. The DM, CP, and fibre contents of silage diets (diets 1-3) are also suggestive of inherent nutritive value that could impact positively on animal performance. The observed differences in values of chemical compositions could be due to the proportions of young and matured leaves of *A. lebbeck* and varying proportions of ingredient mixtures. Others include cassava cultivar, the age of harvest of cassava tuber and the extent of removal of tuber from the skin during processing.

Fermentation Characteristics of Silage Diets

The fermentation characteristics of silage diets fed to WAD sheep differed ($p < 0.05$) significantly across the treatments (Table 3). Evaluation of silage quality is a subjective process in which, silage pH reflects the rate of fermentation and to a limited extent, the quality of ensiled forages. The pH range (4.27–5.39) obtained in this study, being highest in diet 3 and lowest in diet 1 is contrary to the pH range (3.38–4.61) reported (Inyang et al., 2013) for cassava wastes – *A. saman* pod silage. However, it falls within the pH range (4.4–5.5) for

good silage (Menenses et al., 2007). The differences in values of silage pH from this study compared to that reported by Inyang et al. (2013) might be due to the buffering capacity and amount of WSCs of the leguminous fodder (*A. lebbeck* foliage). In addition, the quantity of fermentable starchy mesocarp or endocarp layer of cassava tuber attached to the peel and the extent of utilisation of the limited WSC of ingredients mixtures by lactic acid producing bacteria to drop the pH.

The acid profile of silage is the best indicator of silage quality (Bethard, 2006), particularly, lactic acid. In a good quality silage, lactic acid is the primary acid and it is stronger than other acids (acetic, propionic, and butyric). The concentration of acetic acid increased ($p < 0.05$) marginally with increasing levels of *A. lebbeck*, but similar ($p > 0.05$) in diets 1 and 2. The lactic, propionic, and butyric acids differed ($p < 0.05$) significantly with values ranging from 0.72-1.08%, 5.92-8.88%, and 7.04-10.53%, respectively. Hence, lactic (0.72–1.08 %) and acetic (0.74–1.07%) acids concentrations in this study falls within the category of acceptable silage that may increase intake as recommended (Ward & Ondara, 2008). Inadequate fermentable carbohydrates from the ingredient mixtures (especially from the leguminous fodder) for usage by lactic acid bacteria and other epiphytic aerobic microorganisms (Pahlow, Muck, Driehuis, Oude Elferink, & Spoelestra, 2003), could be a reason for the lower lactic acid concentration compared to other volatile fatty acids (propionic and butyric acids).

On the contrary, the amount of propionic and butyric acids in this study were not comparable with the contents recommended (Allen et al., 1995) for well-preserved silage. Less than 0.5% (propionic acid) and 0.1-0.2% (butyric acid) were recommended as a critical concentration for an adequate

Table 3
Fermentation characteristics of A. lebbeck - *cassava peel silage fed to West African Dwarf sheep*

Composition	Diet 1	Diet 2	Diet 3	SEM	P-value
P^H	4.27^c	5.25^b	5.39^a	0.39	<.000
Lactic acid (%)	1.08^a	0.81^b	0.72^c	0.12	<.000
Acetic acid (%)	0.74^b	0.75^b	1.07^a	0.05	<.000
Propionic acid (%)	6.65^b	5.92^c	8.88^a	0.44	<.000
Butyric acid (%)	7.04^c	7.80^b	10.53^a	0.53	<.000
NH_3N (%)	0.089^c	0.095^b	0.125^a	0.01	<.000
Water-soluble carbohydrates (%)	65.67^a	65.42^a	57.88^b	1.49	0.020
Alcohol content (%)	3.68^a	3.25^a	2.35^b	0.23	0.023

^{abc} =Means with the same superscripts along the same row are significantly different ($p < 0.05$).NH_3N: Ammonia nitrogen; SEM: Standard error of mean; P-value: Probability value; CPL: Cassava peel; AL: *A. lebbeck*; PM: *P. maximum*; Diet 1: 60 % CPL + 25% AL + 15 % PM; Diet 2: 45% CPL + 40 % AL +15 % PM; Diet 3: 30 % CPL + 55 % AL + 15 % PM

fermentation process in well-preserved silage (Ward & Ondara, 2008). The higher values of butyric and propionic acids in this study compared to the recommended concentration could have been caused by the low lactic acid content obtained in this study. It is an indication of degradation of protein content and large amount of dry matter loss as reported (Seglar, 2003).

Although propionic acid has a sharp sweet smell and taste, it is an acceptable silage acid to inhibit the development of undesirable microorganisms in silages exposed to aerobic conditions (Selwet et al., 2008). Ammonia nitrogen (NH_3-N) concentrations were highest ($p < 0.05$) in diet 3 (0.125%) and lowest in diet 1 (0.089%). The NH_3-N per total nitrogen values in all the silage mixtures were below 7-10% maximum value that could depict extensive proteolysis during ensiling (McDonald et al., 1991). Therefore, the silage in this study could be considered as excellent because the NH_3-N/N content was below 7 g/100 g of the total N as reported (Lima, Lourenço, Díaz, Castro, & Fievez, 2010).

Expectedly, WSCs and alcohol concentrations from this study increased with increasing amount of cassava peel in the silage. Hence, WSC and alcohol contents were similar ($p > 0.05$) in diets 1 and 2, but differed ($p < 0.05$) from diet 3. The percentages of WSC of *A. lebbeck*-cassava peel silage in this study suggest that it could be "residual WSC" that was not efficiently utilized by lactic acid bacteria during ensiling. This might be a reason for the high values (2.35–3.68%) of alcohol contents observed in this study contrary to <0.5% alcohol recommended (Seglar, 2003) during the fermentation process. Even though, higher residual WSC in silage supplies energy to ruminants (Weinberg, Ashbell, Hen, & Azreli, 1993), alcohol, a fermentation end-product, when it is above acceptable levels may reduce silage intake. Generally, the variations in fermentation characteristics in this study could be attributed to the varying proportion of *A. lebbeck* foliage and cassava peel in the feed mixtures, individual crop characteristics and other factors influencing silage quality.

Performance Indices of Sheep

The feed intake and growth performance of the sheep fed experimental diets are shown in Table 4. The DM and CP intakes (g/kg $W^{0.75}$) of the sheep fed the control (diet 4) and the silage diets (diets 1–3) were not significantly ($p > 0.05$) different. Meanwhile, sheep fed diet 4 had the highest (71.3 g/kg $W^{0.75}$) values of DM intake, but lowest (15.9 g/kg $W^{0.75}$) for CP intake. Among the sheep fed silage diets, DM and CP intakes (g/kg $W^{0.75}$) were highest in sheep fed diet 1 (68.6 and 18.4 g/kg $W^{0.75}$) but lowest (64.5 and 17.5 g/kg $W^{0.75}$) in sheep fed diet 3, respectively. ADL intake of the sheep on diets 1 to 3 differed ($p < 0.05$) significantly from the sheep fed control diet. However, NDF and ADL intakes were similar ($p > 0.05$) for sheep on silage diets. Meanwhile, NDF intake was significantly higher ($p < 0.05$) for the sheep fed diet 2 (60.6 g/kg $W^{0.75}$) compared to the sheep on diet 4 (55.6 g/kg $W^{0.75}$). Furthermore, the

ADF intake of sheep fed diet 3 differed ($p < 0.05$) from the animals fed diets 1 and 4, respectively, but similar ($p > 0.05$) with the sheep fed diet 2.

Nutrient intake is a function of the concentration of the DM intake. However, highest DM intake of the sheep fed control diet in this study did not translate into increased total feed intake and weight gain. This could be a reflection of the <7.0% CP content of the diet that is required for optimal rumen microbial activities (Minson, 1990). The highest DM intake of sheep fed diet 4, amongst other factors, could be due to the palatability of guinea grass. Guinea grass is highly relished by small ruminants (Babayemi, 2009).

The total feed intake (g/d) and the weight gain (g/d) differed ($p < 0.05$) significantly across the treatments. Total feed intake and weight gain of sheep fed silage diets were highest in sheep fed diet 1 (840 g/d and 46.07 g/d) and lowest in sheep fed diet 3 (768 g/d and 34.40 g/d). The significantly ($p < 0.05$) highest total feed intake and weight gain of sheep fed diet 1 (*A. lebbeck,* cassava peel and *P. maximum* silage mixtures) compared to the control in this study is

Table 4
Nutrient intake (g/KgW$^{0.75}$/d) and performance indices of West African Dwarf sheep fed A. lebbeck - *cassava peel silage and* P. maximum

Parameters	Diet 1	Diet 2	Diet 3	Diet 4	SEM	P-value
Nutrient intake						
Dry matter	68.6	68.2	64.5	71.3	1.30	0.361
Crude protein	18.4	17.8	17.5	15.9	0.49	0.337
NDF	58.2^{ab}	60.6^{a}	59.4^{ab}	55.6^{b}	0.84	0.180
ADF	28.3^{c}	31.4^{ab}	31.5^{a}	29.4^{bc}	0.49	0.015
ADL	8.6^{a}	8.4^{a}	8.0^{a}	6.7^{b}	0.26	0.009
Feed intake (%)						
Silage intake	61.20	63.16	55.37	____		
Concentrate	38.80	36.84	44.63	39.34		
P. maximum	____	____	____	60.66		
Total feed intake (g/day)	840^{a}	810^{b}	768^{c}	566^{d}		<.000
Initial live weight (kg)	10.21	10.44	11.37	10.82		
Final live weight (kg)	14.08	13.71	14.26	13.84		
Weight gain (g/day)	46.07^{a}	38.93^{b}	34.40^{c}	35.95^{c}	1.67	<.000
FCR	18.23^{c}	20.80^{b}	22.33^{a}	15.74^{d}	1.31	<.000

^{abcd}= Means with the same superscripts along the same row are significantly different ($p < 0.05$). NDF: beutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; SEM: Standard error of mean; *p*-value: Probability value; FCR: Feed conversion ratio; CPL: Cassava peel; AL: *A. lebbeck*; PM: *P. maximum*; Diet 1: 60 % CPL + 25% AL + 15 % PM; Diet 2: 45% CPL + 40 % AL +15 % PM; Diet 3: 30 % CPL + 55 % AL + 15 % PM; Diet 4: 100% PM

an indication of effective degradability of the silage, which resulted to the higher nutrient intakes and good performance. The relatively good performance of sheep fed diet 1 is also indicative of optimal levels of ingredient mixtures to enhance synchronization of energy and nitrogen to the rumen for improved growth performance of animals (Cole et al., 2008; Seo et al., 2010). Findings from this study further corroborated the reports of several authors (Attaelmnan et al., 2009; Solorio-Sanchez et al., 2007), who observed increased feed intake and weight gain of small ruminants by supplementation of low-quality forages with foliages of protein-rich fodder.

Feed conversion ratio was significantly ($p < 0.05$) lower for sheep fed diet 4 compared to the other treatment groups. The lower feed conversion ratio obtained for sheep fed diet 4 compared to other animals on silage treatments could be linked to the intake of concentrate diet to overcome the nitrogen deficiency and enhanced proliferation of rumen microbes to promote degradability of nutrients. Incorporation of concentrate diet rich in protein in animal diets enhanced more intakes of energy and protein (Suarez, Van Reenen, Stockhofe, Dijkstra, & Gerrits, 2007), the supply of microbial nitrogen to stimulate rumen microbial activities and optimal efficiency of feed utilisation (McDonald et al., 2010) for growth, gestation, or lactation.

However, silage diet was efficiently utilized by sheep fed diet 1 (60% cassava peel, 25% *A. lebbeck*, and 15% *P. maximum* mixtures) compared to other silage treatments. Variations in efficiency of feed utilisation could be a reflection of the proportion of *A. lebbeck* foliage in silage mixtures and intake of concentrates, individual animal differences caused by development of a series of biochemical reactions in the rumen, and ability of protein-binding capacity of tannins on the ingredient mixtures to bind the fermentable carbohydrates and proteins to by-pass the rumen into the abomasum and intestines (Diaz-Hernandez, Nixon, Ball, Leng, & Rowe, 1997).

Apparent Nutrient Digestibility of Experimental Diets

The digestibility of DM, CP, ADF, and ADL differed significantly ($p < 0.05$) across the sheep fed silage diets (Table 5). Except for DM digestibility, CP, and ADF, digestibility values decreased with increasing levels of *A. lebbeck* foliage, while ADL digestibility values increased marginally with decreasing levels of *A. lebbeck* foliage The values of CP digestibility were significantly highest (88.1%) in the sheep on diet 1 and lowest (74.5%) in sheep fed diet 4, respectively. Digestibility of ether extract did not differ ($p > 0.05$) across the treatments. Digestibility of DM and NDF was similar ($p > 0.05$) in sheep on diets 1 and 2, likewise for sheep fed diets 3 and 4, respectively. However, DM digestibility values was highest ($p < 0.05$) in sheep fed diet 2 (69.5%) and lowest in sheep fed diet 3 (59.3%), but digestibility of ADF values was highest (89.1%) in sheep fed diet 1 and least (81.4%) in sheep fed diet 4. Digestibility of ADL was similar (p

> 0.05) across silage treatment groups but significantly different ($p < 0.05$) from the sheep on the control diet.

Increased nutrient digestibility of roughage-based diet has been reported with increasing levels of legume supplementation (Tolera & Sundstol, 2000). However, in this study, a numerical decrease of CP, EE, and ADF digestibility values (silage diets) was observed across the treatments with increasing levels of *A. lebbeck* foliage in silage mixtures. Irrespective of the proportions of ingredient mixtures, all the nutrients were adequately digested and digestibility values of silage diets were impressive, being >50% compared with the control.

This implied that dry matter digestibility increased when highly degradable roughages are synchronized with a readily fermentable protein required for optimal activities of rumen microbial flora (Baah, Tait, & Tuah, 1999). The observed marginal decrease in values of apparent nutrient digestibility particularly in sheep fed diet 3 despite increasing levels of *A. lebbeck* foliage across the treatments (sheep fed silage diets) could be due to the accumulation of lignin fraction that resulted from the varying proportions of cassava peel and *A. lebbeck* foliage in the feed mixtures. Lignin has been implicated to interfere with microbial degradation of fiber polysaccharides by acting as a physical barrier (Buxton & Redfearn, 1997).

Table 5
Apparent nutrient digestibility (%) of West Africa Dwarf sheep fed A. lebbeck - *cassava peel silage and* P. maximum

Parameters (%)	Diet 1	Diet 2	Diet 3	Diet 4	SEM	P-value
Dry Matter	67.4[a]	69.5[a]	59.3[b]	60.9[b]	1.38	0.001
Crude Protein	88.1[a]	83.2[b]	77.5[c]	74.5[c]	1.68	0.001
Ether extract	76.5	75.3	75.4	79.2	1.87	0.404
Neutral detergent fiber	85.7[a]	86.4[a]	81.8[b]	82.4[b]	0.73	0.025
Acid detergent fiber	89.1[a]	88.8[a]	84.5[b]	81.4[c]	1.04	0.001
Acid detergent lignin	78.3[a]	80.1[a]	80.6[a]	71.4[b]	1.28	0.009

[abc] =Means along the same row with different superscripts are significantly different (P<0.05). SEM: Standard error of mean; *p*-value: Probability value; CPL: Cassava peel; AL: *A. lebbeck*; PM: *P. maximum*; Diet 1: 60 % CPL + 25% AL + 15 % PM; Diet 2: 45% CPL + 40 % AL +15 % PM; Diet 3: 30 % CPL + 55 % AL + 15 % PM; Diet 4: 100% PM

Nitrogen Utilisation of Experimental Diets by Sheep

The nitrogen utilisation of WAD sheep fed experimental diets is shown in Table 6. Nitrogen intake was significant ($p < 0.05$) across the treatments, but N-intake values were similar ($p > 0.05$) in sheep fed diets 1, 2, and 4, respectively. While nitrogen intake of the sheep fed diet 3 differed ($p < 0.05$) from sheep on diets 1 and 2 respectively, fecal nitrogen were similar ($p > 0.05$) across the treatment groups. Conversely,

urinary nitrogen differed ($p < 0.05$) across the treatments, but values were relatively similar ($p > 0.05$) in sheep fed diets 3 and 4, respectively. Since nitrogen absorbed and retained is dependent on N-digestibility, apparent N-digestibility and N-absorbed were similar ($p > 0.05$) in sheep fed diets 1 and 2, likewise in sheep fed diets 3 and diet 4, respectively. Apparent N-digestibility values were highest in sheep fed diet 1 (69.33%) and lowest in the sheep fed control diet (57.06%). However, values of nitrogen absorbed and retained were highest ($p < 0.05$) in sheep fed diet 1 (2.08 and 1.97 g/d) and lowest in sheep fed diet 3 (1.37 and 1.12 g/d).

The positive nitrogen balance observed across the treatment groups in this study implied that the experimental diets were adequate in nutrients, well digested and utilized by the animals. The remarkable N-absorption and N-retention by the sheep fed diets 1 and 2, respectively, compared to animals on diet 3 could be attributed to higher N-intake and lower N-excretion, which suggests a better protein metabolism and efficient nitrogen utilisation. However, the lower values of N-absorption and N-retention in sheep fed diet 3 compared to other animals on silage diets could likely have arisen from reduced rate and extent of ruminal nitrogen degradability (Souza et al., 2009), orchestrated by tannin–nitrogen complexes of leguminous tree fodder.

Similarly, the variations in N-absorption and N-retention in sheep fed silage diets compared to animals fed control (diet 4) also suggest that the protein in the diet resists microbial degradation due to tannin–protein complexes on leaves of leguminous tree fodder. Tannin-rich fodders have pronounced effect on nitrogen utilisation of goats (Olafadehan, Adewumi, & Okunade, 2014). Interestingly, the remarkable nitrogen utilisation of the sheep fed diet 1 and nutrient utilisation of all the sheep fed silage diets in this study resulted in significant growth performance (weight gain) compared to the sheep fed control diet.

Table 6
Nitrogen (N) utilisation (%) of West Africa Dwarf sheep fed A. lebbeck - *cassava peel silage and* P. maximum

Parameters	Diet 1	Diet 2	Diet 3	Diet 4	SEM	P-value
N-intake (g/d)	3.0^{a}	3.0^{a}	2.38^{b}	2.41^{ab}	0.12	0.073
Fecal-N (g/d)	0.92	0.96	1.01	1.03	0.02	0.386
Urinary-N (g/d)	0.11^{c}	0.44^{b}	1.01^{a}	1.03^{a}	0.14	0.000
N-retained (g/d)	1.97^{a}	1.60^{b}	1.12^{c}	1.20^{c}	0.13	0.003
N-absorbed (g/d)	2.08^{a}	2.04^{a}	1.37^{b}	1.38^{b}	0.13	0.000
Apparent N digestibility (%)	69.33^{a}	68.00^{a}	57.56^{b}	57.06^{b}	2.19	0.003

^{abc}=Means along the same row with different superscripts are significantly different (P<0.05).
SEM: Standard error of mean; *p*-value: Probability value; CPL: Cassava peel; AL: *A. lebbeck*; PM: *P. maximum*; Diet 1: 60 % CPL + 25% AL + 15 % PM; Diet 2: 45% CPL + 40 % AL +15 % PM; Diet 3: 30 % CPL + 55 % AL + 15 % PM; Diet 4: 100% PM

CONCLUSION

From this study, silage quality and nutritive value of cassava peel improved when ensiled with *A.-lebbeck* foliage. Hence, *A. lebbeck*-cassava peel silage was acceptable and efficiently utilized by the sheep. The higher feed and nutrient intake of sheep fed diet 1 (60% cassava peel, 25% *A. lebbeck* and 15% *P. maximum* mixtures) resulted in higher weight gain compared to sheep on the control diet. Therefore, *A. lebbeck*-cassava peel silage can be a viable option for sheep feeding in the dry season when grass quality is low.

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Stress Analysis of *Amaranthus hybridus* L. and *Lycopersicon esculentum* Mill. Exposed to Sulphur and Nitrogen Dioxide

Dennis Emuejevoke Vwioko[1], Innocent Okoekhian[1] and Matthew Chidozie Ogwu[1,2]*

[1]Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, PMB 1154, Ugbowo, Benin City, Edo State, Nigeria
[2]School of Biological Sciences, Seoul National University, 1 Gwanak, Gwanak ro, Gwanak gu, Seoul 08826, Republic of Korea

ABSTRACT

The effects of SO_2 and NO_2 on stress-related enzymes in *Amaranthus hybridus* [AH] and *Lycopersicon esculentum* [LE] were studied. The plants were exposed to SO_2 and NO_2 generated by chemical reaction in a chamber for one hour every day for three weeks. After exposure, chlorophyll content, ascorbic acid, catalase, proline content, superoxide dismutase and peroxidase from leaf samples were assessed. Catalase contents decreased (AH: 84.31 ± 2.56 and LE: 50.52 ± 1.74) in the plants relative to control samples, which showed appreciable retention (AH: 98.59 ± 4.70 and LE: 91.84 ± 7.12). Plants showed more reduction in peroxidase due to exposure to NO_2 compared to values obtained in plants treated with SO_2. Meanwhile, lower values of ascorbic acid and chlorophyll contents were obtained in plants exposed to SO_2 and NO_2 gases. On the other hand, proline content increased after exposure to the gases. Morphological disorders, such as leaf browning, chlorosis and shrinkage of leaves were also noticeable. So, it is apparent that plants exposed to elevated SO_2 and NO_2 gases did not exhibit sufficient capacity to counteract the stress imposed by these gases.

Keywords: Air pollutants, *Amaranthus hybridus*, catalase content, *Lycopersicon esculentus*, nitrogen dioxide, plant stress, sulphur dioxide

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E-mail addresses:
matthew.ogwu@snu.ac.kr/matthew.ogwu@uniben.edu (Matthew Chidozie Ogwu)
vwioko@yahoo.com (Dennis Emuejevoke Vwioko)
cent@hotmail.com (Innocent Okoekhian)
* Corresponding author

INTRODUCTION

Air pollution is a major challenge and a key contributor to climate change. It damages the ecosystem through enhanced fluctuations in atmospheric constituents,

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which can directly affect plants through their leaves or indirectly through soil acidification (Tripathi & Gautam, 2007). When exposed to airborne pollutants, most plants experience physiological and biochemical changes before exhibiting visible damage to leaves (Javed *et al*., 2009; Liu & Ding, 2008). Reduction in the cost of acquiring technology have led to the proliferation of industries and vehicles, thereby, increasing toxic gases and particulate matters in our environment (Agbaire & Esiefarienrhe, 2009; Joshi, Chauhan, & Joshi, 2009). Tiwari, Agrawal and Marshall (2006) suggested that sulphur dioxide (SO_2) and nitric oxides (NO_x) are major pollutants, whose concentrations are higher in urban than in suburban and rural areas. These have been worsened by rapid industrialisation through non-ecofriendly approaches (Uka, Hogarh, & Belford, 2017).

Plants show species-specific effects when exposed to harmful levels of air pollutant. The stress induced by gaseous pollutants have not been given sufficient attention compared with other abiotic factors, such as water, salinity, and herbicides. This paper contributes towards addressing that void in the light of increased concentrations of air pollutants due to industrialisation and climate change. Injury from gaseous pollutants ranges from visible markings on the foliage, reduced growth and yield, premature death of the plant, leaf injury, stomatal damage, premature senescence, decreased photosynthetic activity, disturbance in membrane permeability, and reduction of growth and yield in sensitive plant species (Tiwari *et al*., 2006; Black, Stewart, Roberts, & Black, 2007). Oxides of sulphur and nitrogen dissolve in rain water and ultimately enters the soil, change soil chemistry resulting in nutrient deficiency and reduces uptake by roots of plants (Agrawal, Singh, Agrawal, Bell, & Marshall, 2006; Agrawal & Deepak, 2003; Tankha & Gupta, 1992).

Air pollution can directly affect plants through leaves or indirectly via soil acidification with the potential of causing physiological and biochemical changes before exhibiting visible damage to leaves (Liu & Ding, 2008). These biochemical and physiological changes may include chlorophyll content, proline absorption, ascorbic acid, peroxidase and catalase contents. Burning of fossil fuels containing sulphur compounds will result in release of fine particulate sulphates and sulphuric acid mist (Ogunstein & Smith, 2007). In humans, SO_2 can aggravate existing respiratory diseases, such as asthma, bronchitis and emphysema as it constricts air passages making it difficult to breathe (Grahame & Schlesinger, 2009). NO_x, which is needed for the formation of nitrogen dioxide (NO_2), is produced during high temperature combustion of fossil fuels in electric power generating facilities, industrial operations, automobiles and chemical processing plants may affect a human's health by causing acute bronchitis or pneumonia and may reduce resistance to respiratory infections, such as chronic lung impairment (Ifeanyichukwu, 2002).

Green leafy vegetables Green leafy vegetables are crucial part of human diet (Oguntona & Oguntona, 1986). Generally, they are consumed as side dishes to major staples, such as cassava, cocoyam, guinea corn, maize, millet and plantains. Okoli, Nmorka and Unaegbu (1998) estimated that over 60 species of green leafy plants were consumed in Nigeria alone with an estimated *per capita* daily consumption of 65 g (Gruda, 2005). This includes plant species within families, such as *Amaranthaceae*, *Compositae*, *Portulacaceae* and *Solanaceae*.

Amaranth *Amarathus hybridus* [L.], Amaranthaceae is a traditional food plant in Africa. It has a potential to improve nutrition, boost food security, and support sustainable land care (NRC, 2006). Amaranth leaf is known in Swahili as *mchicha* (Olufemi, Assiak, Ayoade, & Onigemo, 2003). In Nigeria, it is a common vegetable known in Yoruba as *efo tete* or *arowo jeja*, *aleifo* (Hausa), and *Inine* (Igbo). Amaranth has been used widely by the Chinese for its healing chemicals to treat illnesses, such as infections, rashes, migraines and also used as fodder and dyes (Schippers, 2000). The leaves are also consumed as a nutritious leafy vegetable, being used both in cooking and in salads. These are excellent sources of vitamins, minerals, sugars and folic acid, which lower the risk of cancer and heart diseases (Steinmetz & Potter, 1996). They contain a variety of bioactive non-nutritive health promoting factors as antioxidants, photo-chemicals, essential fatty acids and dietary fibres (Mephba, Eboh, & Banigo, 2007; Negi and Roy, 2000).

Tomato (*Lycopersicun esculentum* [Mill.], Solanaceae) has many varieties that are now widely cultivated, sometimes in greenhouses and cool climates (Agbabiaje & Bodunde, 2002). Tomato is consumed in diverse ways, including raw, as an ingredient in many dishes, sauces, salads, and drinks. It is a perennial in its habitat, although often grown outdoors in temperate climates (Sheeja, Mondal, & Rathore, 2004). It is sensitive to water logging and flooding and prefers well-drained soils.

The objective of this research is to assess the effects of common urban air pollutants (SO_2 and NO_2) on *A. hybridus* [AH] and *L. esculentum* [LE]. This study highlights the non-visible effects of pollutants by measuring the levels of stress-related enzymes on these plants after exposure to SO_2 and NO_2 in a gas chamber. The changes in their biochemical components will produce visible morphological changes noticeable only as an after effect. By understanding the effects of these gaseous pollutants on chlorophyll, proline, ascorbic acid, superoxide dismutase and catalase contents, the effects of these enzymes can be predicted with greater accuracy. The economic viability of these vegetables crops makes them a suitable subject to understand the economic and ecological impacts of these gaseous pollutants. Therefore, our results will contribute towards understanding the effects of these air pollutants on the production of these vegetables vis-a-vis food security and sustainable development.

MATERIALS AND METHODS

Study Area

The experiment was conducted in the Experimental laboratory of the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State.

Sources of Soil Samples

Top soil (0 – 10 cm depth) was collected from the Botanical Garden of Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State. The physicochemical and microbial properties of the soil have been reported by Ogwu and Osawaru (2014). About 2 kg of the soil samples were transferred into forty experimental bowls perforate at the base and used for planting. Twenty bowls were used for each AH and LE.

Plant Material

Seeds of AH and LE were obtained from the previous harvest collection of a local farmer specialising in vegetable production in a community near Benin City, Edo State. The seeds were tested for viability and only viable seeds were selected and used for the study. Four seeds were sown per bowl in the experimental plots of the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State and later reduced to one.

Producing Nitrogen Dioxide (NO_2) and Sulphur Dioxide (SO_2) Gases

Nitrogen dioxide was produced according to Lin, Xiao, Yuan, Choi and Chan (2005) herein summarised. About 50 g of lead nitrate was transferred into a Buchner flask with an air-tight cork. The flask was put on top of a tripod stand with wire gauze, the other end of the Buchner flask was connected with a glass tubing to the experimental chamber. After successfully setting up the apparatus, the Bunsen burner was ignited and the Buchner flask was heated gently. As lead nitrate decompose, NO_2 gas was steadily released into the experimental chamber through the glass tubing. After complete decomposition, the Bunsen burner was turned off and the stopper connecting the glass tubing and the chamber was locked to avoid reverse flow of the NO_2 gas.

Sulphur dioxide was produced according to Klemm and Talbot (1991), herein summarised. About 50 g of sodium sulphite was transferred into a Buchner flask and a hollow cork used to cover the flask. A burette filled with concentrated H_2SO_4 was directly connected to the cork with a stopper and clamped to a retort stand. The Buchner flask was connected to the experimental chamber using a thick rubber tube connected end-to-end. After setting up, the burette was gradually opened to flow into the Buchner flask and the reaction began. Subsequently, SO_2 gas released was channelled into

the experimental chamber through the connected rubber tub. At the end of the experiment, 100 ml of the concentrated H_2SO_4 was used.

$$H_2SO_{4\,(aq)} + Na_2SO_{3\,(s)} \longrightarrow Na_2SO_3 + H_2O_{\,(l)} + SO_{2(g)}$$

The NO_2 and SO_2 gases-exposure were carried out in an air tight wooden chamber of about 1.5 ft square. The gas generation set up was directly linked to the experimental chamber using sealed glass tubing with a stopper in-between the chamber and the glass tubing. The potted plants were kept in the experimental chamber few minutes prior to the initiation of gas generation. Once set up was complete, the generation of the gas was initiated and terminated after 30 minutes. The stopper was closed and locked to prevent reverse diffusion of the gas in the chamber. The plants were taken out of the gas chambers after exposure and later watered.

Measurement of SO_2 and NO_2 Concentrations and Exposure of Plants

The concentrations of generated gases (SO_2 and NO_2) were measured using aeroqual air monitoring kit (AeroQual model series 500, made in U.S.). Before gas generation, the background NO_2 and SO_2 concentrations in the Laboratory and chamber were recorded. The measurements were repeated immediately after generation of the gases (Table 1). The instrument used to measure the generated gas was Aeroqual air monitoring kit, with model number: Series 500, made in U.S.

The temperature of the experimental chambers was also measured before, during and after generation of the gaseous pollutants and exposure of the plants (Table 2).

Three bowls each of the pre-cultivated plants were placed in the chambers for 1 hour every day for three weeks, after which the plants were removed for biochemical analysis. The leaves of AH and LE used for this study were 3 and 4 weeks-old respectively.

Table 1
Concentration of SO_2 and NO_2 gases in laboratory and chambers before and after generation

	Gaseous pollutant	Laboratory room	Gas chambers
Before Generation	SO_2	0.00 ppm	0.037ppm
	NO_2	0.64 ppm	0.190 ppm
After Generation	SO_2	31.41 ppm	133.30 ppm
	NO_2	0.063 ppm	1.266 ppm

Table 2
Temperature (°C) of experimental chamber before and after one hour of gas generation without the plant

	Day	Pollutants	Temperature (°C)
Before exposure	Day 1	SO_2	18.8
	Day 1	NO_2	17.4
After exposure	Day 1	SO_2	26.1
	Day 1	NO_2	23.9
Before exposure	Day 2	SO_2	22.8
	Day 2	NO_2	22.6
After exposure	Day 2	SO_2	29.7
	Day 2	NO_2	28.6

Preparation of Leaf Samples for Biochemical Analysis

After the plants were removed from the gas chamber, leaves were detached from the stem to prevent translocation and avoid enzyme denaturing because higher temperatures denature enzyme and retard growth of plants. The leaves were transferred into polythene bags, placed in a cooler containing ice until analysis. The plant leaves were immediately subjected to extraction with chloroform and distilled water respectively. The acetone extract was analysed for its chlorophyll content while the distilled water extract was stored in the refrigerator at -4°C for antioxidant assay.

The study adopted method of Roe and Kuether (1943) to obtain the level of vitamin C content. Ascorbic acid was oxidized to dihydroascorbic acid by the action of 2,6-dichlorophenol indophenol. The dihydroascorbic acid was then hydrolysed to diketogluconic acid in a strong acidic medium. This forms an osazone and re-arranges to a stable reddish-brown product, which is measured colorimetrically at 500 nm.

The method of Misra and Fridovich (1972) was adopted to estimate Superoxide dismutase. Catalase (CAT) activity was determined based on the method of Cohen, Dembiec and Marcus (1970).

Estimation of Glutathione peroxidase was done by preparing a reaction mixture of 3.5 ml distilled water, 0.5 ml of 5 % pyrogallol solution, 0.25 ml of 0.147 M H_2O_2 solution and 0.5 ml phosphate buffer (pH 6.0). The mixture was equilibrated at 20°C for about 5 minutes and 0.25 ml of 2 N H_2SO_4 was added to stop the reaction after exactly 20 seconds. The optical density was measured at 420 nm against the blank solution (water).

$$= \frac{OD_{430nm}/30_{sec} \text{ x } Vt \text{ x } Df}{M \text{ x } V \text{ x } L \text{ x } y}$$

Where OD = Absorbance of test sample at 430 nM

Vt = Total volume of the reaction mixture

Df = Dilution factor

M = Molar extinction coefficient of purpurogallin 12.0 $M^{-1}cm^{-1}$

L = Light path, 1 cm

V = Volume of sample homogenate used

Y = mg of protein in tissue used

Proline determination was carried out based on the methods of Bates, Waldran and Teare (1973).

Total Chlorophyll content (TCh), Chlorophyll *a* and *b* was measured based on to Yadawa (1986).

Statistical Analysis

The experiment had used a completely randomised design in the field during cultivation and exposure in the laboratory. All analyses in this study were the means of three replicates and standard error of deviation. Statistical analysis was conducted to determine the level of significance of the stress induced on the AH and LE by NO_2 and SO_2 measured using one-way analysis of variance and Tukey-Kramer multiple comparison posthoc test on SPSS version 19. The analysis considered AH and LE as the independent variable and the gaseous pollutants (NO_2 and SO_2) as the dependent variables with statistical significance cut-off of P less than 0.05.

RESULTS

Chlorophyll Content

Chlorophyll *a*, *b*, and total chlorophyll contents of AH and LE leaves exposed to SO_2 and NO_2 are presented in Figures 1 and 2.

Chlorophyll content in leaves of both AH and LE was greater in control plant than SO_2 and NO_2 exposed plant. However, chlorophyll was higher at early growth stage.

Chlorophyll content of AH exposed to NO_2 showed a decrease (Figure 1). The decrease in the chlorophyll contents (chlorophyll *a, b* and total chlorophyll) of AH plants exposed to NO_2 was significantly higher ($P < 0.0001$) in the 4 weeks-old plants compared with the 3 week-old plants. Significant differences ($P < 0.0001$) were thus recorded in the chlorophyll contents of AH plants for control as against exposed. The AH exposed to SO_2 showed an increase in the chlorophyll contents in the 4 week-old plant compared with the 3 week-old plants. A slight increase was observed in the control for the 4 week-old plants compared with the 3 week-old plants.

Chlorophyll contents in LE plants exposed to NO_2 showed a comparative increase for the control in the 4 week-old plants compared with the 3 week-old plants (Figure 2). An appreciable increase was observed in the chlorophyll contents of LE for the 4 week-old plants exposed to NO_2. The LE exposed to SO_2 showed a significant increase for both the exposed and control of the 4 weeks-old plants compared with the 3 week-old plants.

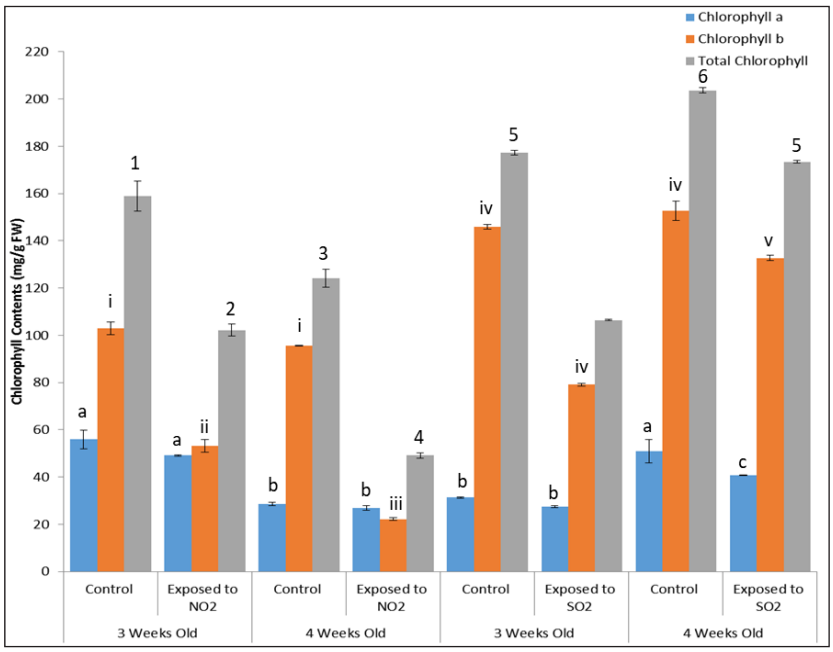

Figure 1. Chlorophyll content of *Amaranthus hybridus* plants exposed to NO_2 and SO_2 after 3 and 4 weeks. *Superscripts represents result of post-hoc test, where means sharing the same superscripts are not significantly different from each other.

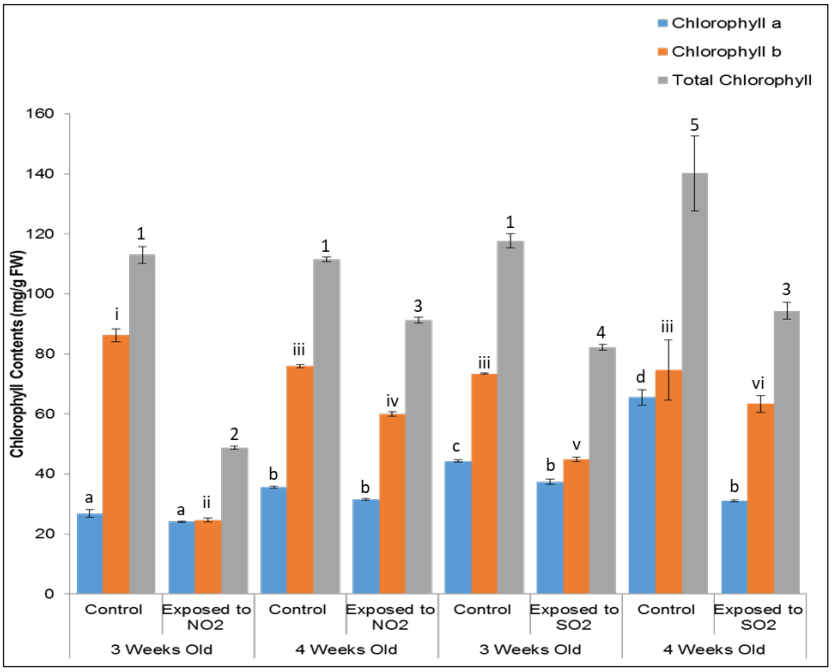

Figure 2. Chlorophyll content of *Lycopersicon esculentum* plants exposed to NO_2 and SO_2 after 3 and 4 weeks. *Superscripts represents result of post-hoc test, where means sharing the same superscripts are not significantly different from each other.

Catalase Content

The results obtained for the analysis of catalase content using leaf tissues of AH and LE is shown in Figure 3, where P value was significant for both three and four weeks-old plant exposed to NO_2 and SO_2 (at $p < 0.0001$). Catalase content of AH was shown to reduce more than 10 % due to exposure to NO_2 from 98.59 ± 4.70 to 84.31 ± 2.56 after three weeks and from 54.71 ± 10.80 to 36.57 ± 4.91 after four weeks. The catalase content of LE also dropped from 91.84 ± 7.12 to 50.52 ± 1.74 after three weeks and from 31.26 ± 1.28 to 22.61 ± 2.22 after four weeks. The effects of SO_2 on catalase content of AH suggested more than 50 % reduction after three and four weeks respectively. The effects of SO_2 on catalase content of LE suggested a more than 70 % reduction.

These results suggested catalase content was higher in the control than gas exposed plants. Also, it seemed that under stress, AH, tended to produce catalase as the major antioxidant enzyme. Catalase activities were very high in the leaf tissues of LE. Exposure to SO_2 and NO_2 probably caused reduction in catalase activities in AH.

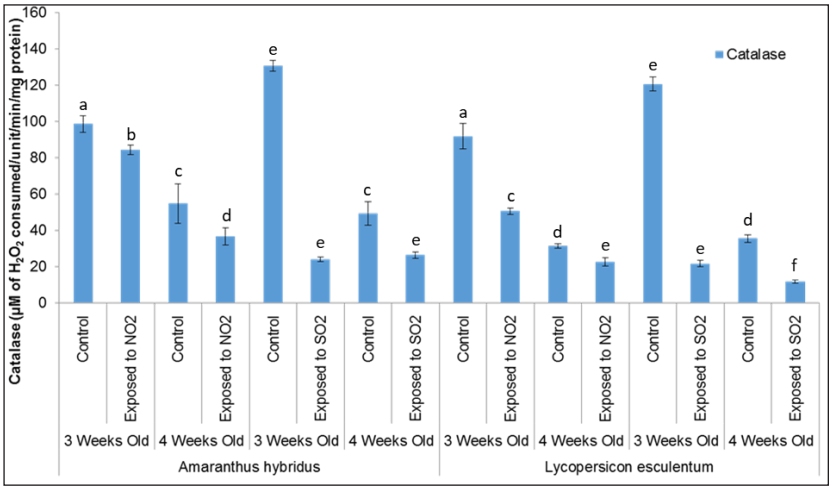

Figure 3. Catalase synthesis of *Amaranthus hybridus* and *Lycopersicon esculentum* plants exposed to SO_2 and NO_2 gases. *Superscripts represents result of posthoc test, where means sharing the same superscripts are not significantly different from each other.

Superoxide Dismutase Content

The results obtained for the analysis of superoxide dismutase (SOD) contents using leaf tissues of AH and LE are shown in Figure 4. Superoxide dismutase content showed slight reduction after three and four weeks of exposure to SO_2 and NO_2 although these were significantly different with P value < 0.0001. The results indicate that the exposure to SO_2 and NO_2 caused reduction in SOD activities in the leaf tissues. A gradual decrease in SOD content was noticeable in the leaf tissues of LE exposed to SO_2 and NO_2 (after 3 and 4 weeks) compared with AH.

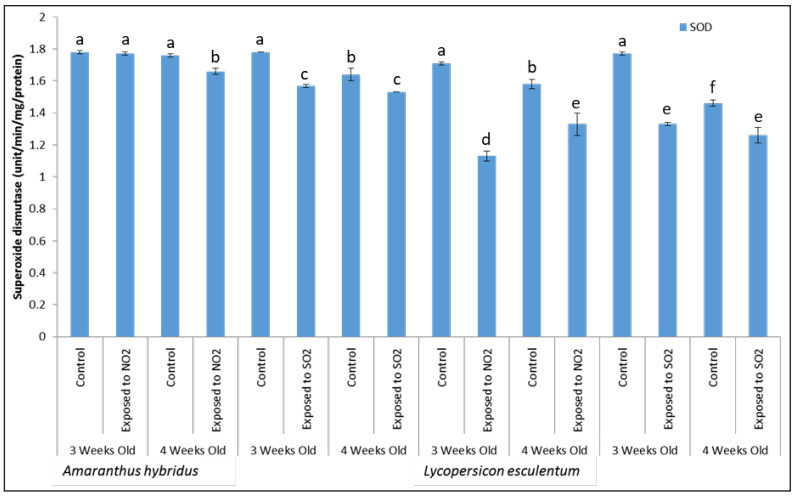

Figure 4. Superoxide dismutase synthesis of *Amaranthus hybridus* and *Lycopersicon esculentum* plants exposed to SO_2 and NO_2 gases. *Superscripts represents result of post-hoc test, where means sharing the same superscripts are not significantly different from each other.

Glutathione Peroxidase Content

The results obtained for the analysis of glutathione peroxidase content using leaf tissues of AH and LE are shown in Figure 5. Glutathione peroxidase activities were higher in LE plants in control conditions. In addition, exposure of plants to NO_2 did not affect glutathione peroxidase activities as such. It seems that under stress, glutathione peroxidase has a sharp reduction in the antioxidant enzyme activities of AH and LE plants exposed to SO_2 compared with NO_2 with respect to period of exposure to the gases.

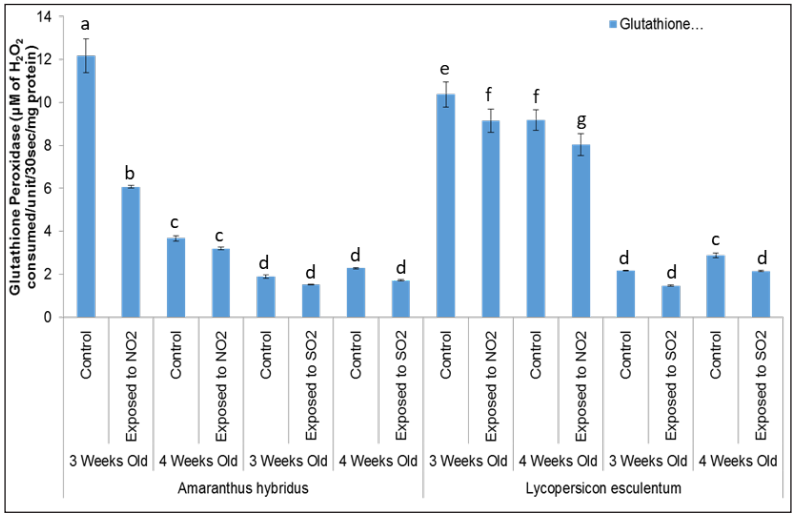

Figure 5. Glutathione Peroxidase synthesis of *Amaranthus hybridus* and *Lycopersicon esculentum* plants exposed to SO_2 and NO_2 gases. *Superscripts represents result of post-hoc test, where means sharing the same superscripts are not significantly different from each other.

Proline Synthesis

The exposure AH and LE to NO_2 and SO_2 stimulated the plants to increase the production of proline (Figure 6), which is an antioxidant compound. The increase in proline content is an indication of stress. It was observed that less amounts of proline were recorded for the older plants (4 weeks) exposed to the gases.

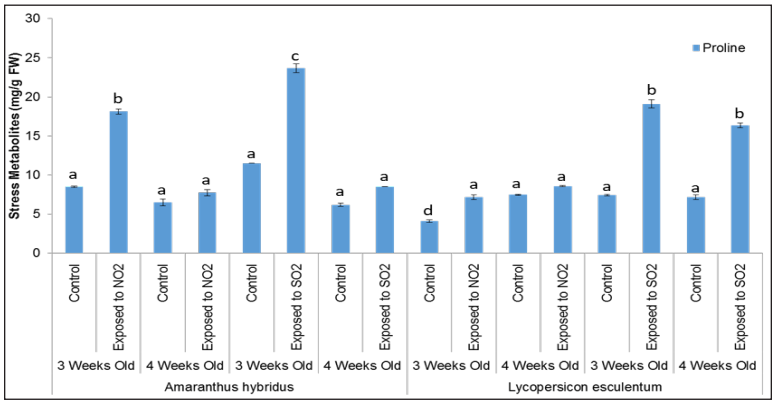

Figure 6. Proline synthesis of *Amaranthus hybridus* and *Lycopersicon esculentum* plants exposed to SO_2 and NO_2 gases. *Superscripts represents result of posthoc test, where means sharing the same superscripts are not significantly different from each other.

Ascorbic Acid Content

Tables 3 and 4 shows the results for the ascorbic acid assay of AH and LE exposed to NO_2 gas after 3 and 4 weeks respectively. The controls of the ascorbic acid contents of the 3 weeks-old AH and LE plants were observed to have higher ascorbic acid contents than 4 weeks-old plants exposed to NO_2.

The results for the ascorbic acid assay of AH and LE *plants* exposed to SO_2 after 3 and 4 weeks) are shown in Tables 5 and 6. The study showed that the ascorbic acid contents were very high in the leaf tissues of AH controls compared to the exposed plants. More so, exposure to SO_2 caused a reduction in ascorbic acid activities. The consequences of this significant reduction in ascorbic acid activities were observed in the leaf tissues of LE plants exposed to SO_2 compared with the control plants.

Table 3
Ascorbic acid content of Amaranthus hybridus plants exposed to NO_2

Samples	Condition	*Age of plants	Vitamin C (mg/g FW)
Amaranthus hybridus	Control	3 weeks-old	28.13 ± 0.21^{a}
Amaranthus hybridus	Exposed	3 weeks-old	17.75 ± 0.79^{b}
Amaranthus hybridus	Control	4 weeks-old	11.73 ± 0.18^{c}
Amaranthus hybridus	Exposed	4 weeks-old	7.92 ± 0.71^{d}

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement; P value was < 0.0001; *Superscripts represents result of posthoc test, where means sharing the same superscripts are not significantly different from each other

Table 4
Ascorbic acid content of Lycopersicon esculentum plants exposed to NO_2

Samples	Condition	*Age of plants	Vitamin C (mg/g FW)
Lycopersicon esculentum	Control	3 weeks-old	16.13 ± 0.05^{a}
Lycopersicon esculentum	Exposed	3 weeks-old	4.00 ± 0.20^{b}
Lycopersicon esculentum	Control	4 weeks-old	6.15 ± 0.39^{b}
Lycopersicon esculentum	Exposed	4 weeks-old	4.52 ± 0.07^{b}

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement; P value was < 0.0001; *Superscripts represents result of posthoc test, where means sharing the same superscripts are not significantly different from each other

Table 5
Ascorbic acid content of Amaranthus hybridus plants exposed to SO_2

Samples	Condition	*Age of plants	Vitamin C (mg/g FW)
Amaranthus hybridus	Control	3 weeks-old	11.78 ± 0.05^{a}
Amaranthus hybridus	Exposed	3 weeks-old	8.30 ± 0.14^{b}
Amaranthus hybridus	Control	4 weeks-old	18.73 ± 0.15^{c}
Amaranthus hybridus	Exposed	4 weeks-old	9.61 ± 0.34^{b}

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement; P value was < 0.0001; *Superscripts represents result of posthoc test, where means sharing the same superscripts are not significantly different from each other

Table 6
Ascorbic acid content of Lycopersicon esculentum plants exposed to SO_2

Samples	Condition	*Age of plants	Vitamin C (mg/g FW)
Lycopersicon esculentum	Control	3 weeks-old	6.10 ± 0.14^{a}
Lycopersicon esculentum	Exposed	3 weeks-old	4.13 ± 0.05^{b}
Lycopersicon esculentum	Control	4 weeks-old	13.18 ± 0.09^{c}
Lycopersicon esculentum	Exposed	4 weeks-old	3.48 ± 0.07^{b}

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement; P value was < 0.0001; *Superscripts represents result of posthoc test, where means sharing the same superscripts are not significantly different from each other

Morphological Changes

The morphological changes observed in the plants because of their exposure to the gaseous pollutants are presented in Table 7. The plants became weak and showed yellowing of leaves possibly as a result of chlorophyll degradation of chlorophyll. The leaves also shrunk.

The condition of the plants prior to exposure to the gaseous pollutants is shown in Figure 7. They appear healthy.

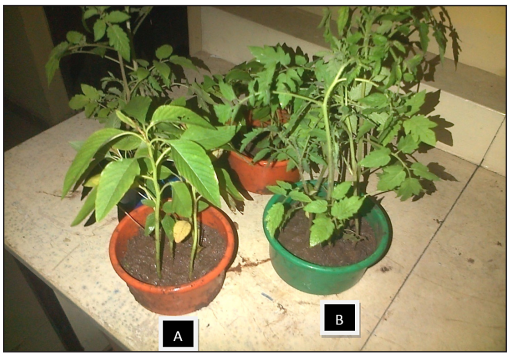

Figure 7. Amaranthus hybridus (A) and *Lycopersicum esculentum* (B) plants prior to exposure to NO_2 and SO_2.

Table 7
Morphological observations of Lycopersicun esculentum and Amaranthus hybridus plants after exposure

Plant species		SO_2		NO_2
Lycopersicun esculentum	1.	Plants appeared very weak as if it was suffering from drought	1.	Plants appeared very weak as if it was suffering from drought
Amaranthus hybridus	2.	Leaves appeared yellow probably due to rapid chlorophyll degeneration.	2.	Leaves appeared yellow probably due to rapid chlorophyll degeneration.
Lycopersicun esculentum	3.	Leaf shrinkage and stem weakening.	3.	Leaf shrinkage and stem weakening.
Amaranthus hybridus	4.	Severe leaf browning.	4.	Severe leaf browning.

After four weeks of exposure to NO_2, the plants appear shrunken and the leaves are no longer green (Figure 8).

Figure 9 shows the *Amaranthus hybridus* and *Lycopersicon esculentum* after four weeks of exposure to SO_2 gas.

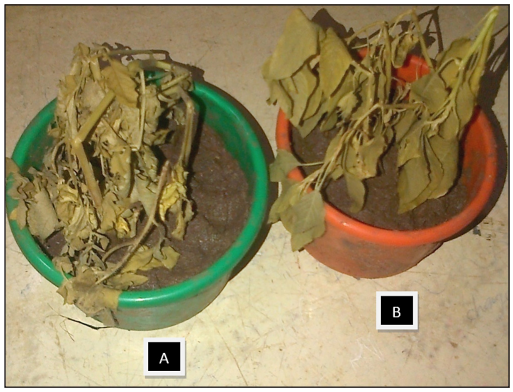

Figure 8. Lycopersicon esculentum (A) and *Amaranthus hybridus* (B) and plants after four weeks of exposure to NO_2

Figure 9. Lycopersicon esculentum (A) and *Amaranthus hybridus* (B) and plants after four weeks of exposure to SO_2

DISCUSSION

The results of this study clearly showed that NO_2 and SO_2 triggered the rapid induction of antioxidant stress enzymes in the leaves of AH and LE. Superoxide dismutase, catalase, glutathione peroxidase, ascorbic acid, proline and chlorophyll contents showed a reduction in the antioxidant enzyme levels when exposed to SO_2 and NO_2 gases. Therefore, inducing stress reactions capable of affecting metabolic pathway in chloroplasts, mitochondria, peroxisomes, plasma membrane, apoplast, endoplasmic reticulum, and cell-wall is vital (Sen, 2012). For instance, according to Woo, Lee and Lee (2007) NO_2 and SO_2 induced the excessive excitation energy within the chloroplasts, which ultimately resulted in increased

generation of reactive oxygen species (ROS) and **oxidative stress**. This may be responsible for the cell death observed in the present study due to oxidative damage to cellular macromolecules. Excess production of ROS due to environmental stress leads to progressive oxidative damage, which may cause cell death as the antioxidative defence mechanisms cannot scavenge the overproduced ROS (Sharma, Jha, Dubey, & Pessarakli, 2012). More so, the sessile nature of plants compounds this challenge from gaseous pollutants.

Increased activities of these enzymes and non-enzymatic antioxidants are considered indicators of oxidative stress in plants (Ruuhola, Rantala, Neuvonen, Yang, & Rantala, 2009). Peroxidase is an antioxidant enzyme whose activity in plants may be used as an indicator of gaseous pollutant to evaluate urban air pollution (Li, 2003). The report of Wu and Tiedemann (2002) showed that the increase of SOD, POD, CAT and APX was induced by the accumulation of singlet oxygen (O_2^{-1}) and hydrogen peroxide (H_2O_2). The results of this study showed that in response to air pollution, the activities of CAT, SOD, and GPx in AH and LE in leaves were decreased. However, an increase in proline content was recorded. This is similar to the study Seyyednejad, Niknejad and Yusefi (2009) where significant increase in proline content was recorded in *Albizia lebbeck*. The results of the present study are consistent with those of Tripathi and Gautam (2007) and Tiwari *et al*. (2006). They observed a decreased in chlorophyll and catalase level in leaves subjected to ambient air pollution, except for proline contents which increased during plant stress. More so, the changes in chlorophyll content, ascorbic acid content, SOD, GPx and CAT activities were significant ($p < 0.0001$) compared with the control.

The influence of different pollutants on plant is associated with oxidative damage at cellular level and the plants response to this oxidative stress by changes in the activities of ROS scavenging enzymes (Pukacka & Pukacki, 2000). Thus, increased anti-oxidative enzymes activity in the leaves is strongly associated with their resistance to air pollution (Woo & Je, 2006). However, the sensitivity to different pollutants differed between plants and within plant species. In addition, it has been reported that ageing leaves contain lower antioxidant levels (Wu & Tiedemann, 2002). Peroxidase activity varies with plant species and with the concentration of pollutants. Much experimental work has been conducted on the effects of air pollutant on crops and vegetation at various levels ranging from biochemical to ecosystem levels. When exposed to airborne pollutants, most plants experience physiological changes before showing visible damage to leaves (Liu & Ding, 2008). SO_2 and NO_2 are the most phytotoxic pollutants; these polluting gases enter leaves through stomata, following the same diffusion pathway as CO_2 (Streets & Waldhoff, 2000). The current study observed a reduction in catalase contents of the control as well as study plants. This reduction in catalase may be due to degradative processes

in the plant owing to photo oxidation. Brisson, Zelitch and Havir (1998) studied the effects of antisense on photorespiration, where the CO_2 compensation point at a leaf temperature of 38°C showed a significant linear increase with a reduction in catalase content. Plant catalase are known to be associated with protection against accumulation and toxicity of hydrogen peroxide [H_2O_2]. Since the peroxide contents of the environment was not measured prior to the study, reduction in catalase may be associated with accumulation of H_2O_2 by the study plants. MacRae and Ferguson (1985) posited that reduction in catalase maybe due to the inability of damaged peroxisomal membranes to transport catalase precursors into the peroxisome. Catalase also has several complementary biochemical roles within the plant, and these interactive actions may also account for the reduction observed in the study. Catalase isozyme is associated with mitochondria and in the cytosol (Scandalios, Guan, & Polidoros, 1997). In general, changes in catalase is likely due to the plant response to changes in environmental condition or biotic challenges (Mhanmdi *et al.,* 2010).

Ascorbic acid content is another parameter that may be used to decide the tolerance of plant to air pollution. It plays a significant role in light reaction of photosynthesis, activates defence mechanism under stress condition and functions as a powerful antioxidant (Arora, Sairam, & Sirvastava, 2002; Caviglia & Modenesi, 1999; Conkin, 2001).

Environmental, physiological and morphological tolerance mechanism to gaseous pollutants may be due to avoidance and stress tolerance through strain avoidance or tolerance (Taylor, 1978). The changes in these biochemical compounds as well as in their morphology suggest that the tolerance threshold of these two plant species exceeded the concentrations of toxic gaseous pollutants. To elucidate the tolerance levels of these plants to these gases, subsequent study may involve varied concentrations of NO_2 and SO_2 with considerations given to strain/species as well as biochemical and physiological limits of the tolerance. Plant tolerance may be also be affiliated with their physical structures, such as stomata, which serve as a potential entry point and the length of exposure to these pollutants. Anderson and Mansfield (1970) found that critical tolerance level to pollutants correlated with the soil concentration although this may vary.

Numerous studies indicate that oxidative stress due to atmospheric pollutants enhances the quantity of low molecular antioxidants such as ascorbic acid in cells. Ascorbic acid is a very important primary antioxidant which reacts with hydroxyl radical, superoxide and singlet oxygen, as well as secondary antioxidant. In this study, ascorbic acid level in polluted or exposed leaves of AH and LE decreased in response to air pollution stress. Species differed in the magnitude of response to pollutant exposure, although differences were not

consistent within taxonomic or functional groups (Honour, Bell, Ashenden, Cape, & Power, 2009). The current knowledge of effects of air pollution on plants is mostly based on experiments where plants have been exposed to high concentrations of air pollutants for short periods under experimental conditions. However, less is known about responses of plants to air pollutants at environmentally relevant concentrations and for long durations in field conditions (Li, 2003). It is well known that oxidative stress decreases the activities of antioxidant enzymes and produces many changes in plant morphological activities and growth as observed in the present study. Therefore, plant stress caused by these air pollutants is a costly process that induces plants to redirect useful resources to produce a cascade of biochemical, cellular, molecular and morphological responses to survive.

Jahan and Igbal (1992) emphasised that significant reduction in different leaf variables in a polluted environment was traceable to the constituent chemicals in the environment. In a study on SO_2 effect on *Platanus acerifolia*, changes in leaf blade and petiole size was observed (Dineva, 2004). In urban environments, trees play an important role in improving air quality by taking up gases and particles (Woo & Je, 2006). A change in vegetation may be indicative of the impacts of air pollution.

CONCLUSION

Exposure of AH and LE to SO_2 and NO_2 initiated a stress response that showed visible morphological changes. The energy required for these stress responses is costly to the plants. The decrease in chlorophyll, superoxide dismutase, catalase, glutathione peroxidase and ascorbic acid contents of the leaves may potentially affect normal growth and development of these vegetables. The marketability of these crops depends on the buoyancy of their leaves, which are affected by exposure to these gases. There is a need to regularly check the concentration of these gases in urban centres where these crops are cultivated in home gardens to meet the subsistence needs of the family.

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APPENDIX

Supplementary Results

Supplementary Table 1
Chlorophyll contents of Amaranthus hybridus plants exposed to NO_2

Samples	Condition	*Age of plants	Chlorophyll *a* (mg/g of Sample)	Chlorophyll *b* (mg/g of Sample)	Total chlorophyll (mg/g of Sample)
Amaranthus hybridus	Control	3 weeks-old	55.90 ± 3.86	103.00 ± 2.64	158.90 ± 6.30
Amaranthus hybridus	Exposed	3 weeks-old	49.16 ± 0.30	53.07 ± 2.70	102.23 ± 2.53
Amaranthus hybridus	Control	4 weeks-old	28.53 ± 0.76	95.57 ± 0.23	124.1 ± 3.84
Amaranthus hybridus	Exposed	4 weeks-old	26.87 ± 0.92	22.23 ± 0.49	49.10 ± 1.21

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement
P value is < 0.0001

Supplementary Table 2
Chlorophyll contents of Lycopersicon esculentum plants exposed to NO_2

Samples	Condition	*Age of plants	Chlorophyll *a* (mg/g of Sample)	Chlorophyll *b* (mg/g of Sample)	Total chlorophyll (mg/g of Sample)
Lycopersicon esculentum	Control	3 weeks-old	26.77 ± 1.32	86.23 ± 2.12	113.00 ± 2.80
Lycopersicon esculentum	Exposed	3 weeks-old	24.03 ± 0.15	24.63 ± 0.64	48.67 ± 0.49
Lycopersicon esculentum	Control	4 weeks-old	35.57 ± 0.25	75.92 ± 0.59	111.49 ± 0.81
Lycopersicon esculentum	Exposed	4 weeks-old	31.37 ± 0.32	59.87 ± 0.76	91.23 ± 1.07

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement
P value is < 0.0001

Supplementary Table 3
Stress enzyme contents of Amaranthus hybridus plants exposed to NO_2

Samples	Condi-tion	*Age of plants	Superoxide dismutase (unit/min/ mg protein)	Catalase (µM of H_2O_2 consumed/ unit/min/mg protein)	Glutathione peroxidase (µM of H_2O_2 consumed/ unit/30sec/ mg protein)	Proline (mg/g of Sample)
Amaranthus hybridus	Control	3 weeks	1.78 ± 0.01	98.59 ± 4.70	12.19 ± 0.79	8.50 ± 0.10
Amaranthus hybridus	Exposed	3 weeks	1.77 ± 0.01	84.31 ± 2.56	6.07 ± 0.07	18.13 ± 0.32
Amaranthus hybridus	Control	4 weeks	1.76 ± 0.01	54.71 ± 10.80	3.67 ± 0.1153	6.50 ± 0.44
Amaranthus hybridus	Exposed	4 weeks	1.66 ± 0.02	36.57 ± 4.91	3.19 ± 0.06	7.74 ± 0.42

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement
P value is < 0.0001

Supplementary Table 4
Stress enzyme contents of Lycopersicon esculentum plants exposed to NO_2

Samples	Condition	*Age of plants	Superoxide dismutase (unit/min/mg protein)	Catalase (µM of H_2O_2 consumed/unit/min/mg protein)	Glutathione peroxidase (µM of H_2O_2 consumed/unit/30sec/mg protein)	Proline (mg/g of Sample)
Lycopersicon esculentum	Control	3 weeks	1.71 ± 0.01	91.84 ± 7.12	10.37 ± 0.59	4.10 ± 0.17
Lycopersicon esculentum	Exposed	3 weeks	1.13 ± 0.03	50.52 ± 1.74	9.15 ± 0.53	7.17 ± 0.29
Lycopersicon esculentum	Control	4 weeks	1.58 ± 0.03	31.26 ± 1.28	9.18 ± 0.49	7.47 ± 0.06
Lycopersicon esculentum	Exposed	4 weeks	1.33 ± 0.07	22.61 ± 2.22	8.03 ± 0.51	8.57 ± 0.06

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement
P value is < 0.0001

Supplementary Table 5
Chlorophyll contents of Amaranthus hybridus plants exposed to SO_2

Samples	Condition	*Age of plants	Chlorophyll *a* (mg/g of Sample)	Chlorophyll *b* (mg/g of Sample)	Total chlorophyll (mg/g of Sample)
Amaranthus hybridus	Control	3 weeks	31.30 ± 0.20	146.00 ± 1.00	177.30 ± 0.92
Amaranthus hybridus	Exposed	3 weeks	27.40 ± 0.36	79.06 ± 0.50	106.47 ± 0.15
Amaranthus hybridus	Control	4 weeks	51.00 ± 4.94	152.67 ± 4.16	203.67 ± 1.09
Amaranthus hybridus	Exposed	4 weeks	40.70 ± 0.17	132.67 ± 1.16	173.37 ± 0.55

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement
P value is < 0.0001

Supplementary Table 6
Chlorophyll contents of Lycopersicon esculentum plants exposed to SO_2

Samples	Condition	*Age of plants	Chlorophyll *a* (mg/g of Sample)	Chlorophyll *b* (mg/g of Sample)	Total chlorophyll (mg/g of Sample)
Lycopersicon esculentum	Control	3 weeks	44.27 ± 0.40	73.40 ± 0.27	117.67 ± 2.42
Lycopersicon esculentum	Exposed	3 weeks	37.33 ± 0.85	44.80 ± 0.79	82.13 ± 1.00
Lycopersicon esculentum	Control	4 weeks	65.47 ± 2.57	74.67 ± 10.03	140.14 ± 12.48
Lycopersicon esculentum	Exposed	4 weeks	31.07 ± 0.35	63.30 ± 2.72	94.37 ± 2.87

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement
P value is < 0.0001

Supplementary Table 7
Stress enzyme contents of Amaranthus hybridus plants exposed to SO_2

Sample	Condition	*Age of plants	Superoxide dismutase (unit/min/mg protein)	Catalase (μM of H_2O_2 consumed/unit/min/mg protein)	Glutathione peroxidase (μM of H_2O_2 consumed/unit/30sec/mg protein)	Proline (mg/g of Sample)
Amaranthus hybridus	Control	3 weeks	1.78 ± 0.00	130.67 ± 3.06	1.89 ± 0.07	11.50 ± 0.00
Amaranthus hybridus	Exposed	3 weeks	1.57 ± 0.01	24.00 ± 1.28	1.52 ± 0.02	23.67 ± 0.58
Amaranthus hybridus	Control	4 weeks	1.64 ± 0.04	49.13 ± 6.50	2.28 ± 0.02	6.20 ± 0.20
Amaranthus hybridus	Exposed	4 weeks	1.53 ± 0.00	26.24 ± 1.75	1.72 ± 0.029	8.50 ± 0.00

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement
P value is < 0.0001

Supplementary Table 8
Stress enzyme contents of Lycopersicon esculentum plants exposed to SO_2

Sample	Condition	*Age of plants	Superoxide dismutase (unit/min/mg protein)	Catalase (μM of H_2O_2 consumed/unit/min/mg protein)	Glutathione peroxidase (μM of H_2O_2 consumed/unit/30sec/mg protein)	Proline (mg/g of Sample)
Lycopersicon esculentum	Control	3 weeks	1.77 ± 0.01	120.67 ± 3.79	2.16 ± 0.02	7.40 ± 0.10
Lycopersicon esculentum	Exposed	3 weeks	1.33 ± 0.01	21.49 ± 1.75	1.47 ± 0.02	19.10 ± 0.52
Lycopersicon esculentum	Control	4 weeks	1.46 ± 0.02	35.45 ± 2.10	2.88 ± 0.11	7.17 ± 0.29
Lycopersicon esculentum	Exposed	4 weeks	1.26 ± 0.05	11.72 ± 0.84	2.15 ± 0.02	16.33 ± 0.29

Key = mean ± *S.D* (n = 3), * = Period of exposure before measurement
P value is < 0.0001

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Effect of Plant Extracts on Growth and Yield of Maize (*Zea mays* L.)

Nailul Rahmi Aulya[1], Zozy Aneloi Noli[2], Amri Bakhtiar[3] and Mansyurdin[2*]

[1]Faculty of Mathematics and Natural Sciences, Andalas University, Padang, 25163 West Sumatra, Indonesia
[2]Biology Department, Faculty of Mathematics and Natural Sciences, Andalas University, Padang, 25163 West Sumatra, Indonesia
[3]Faculty of Pharmacy, Andalas University Padang, 25163 West Sumatra, Indonesia

ABSTRACT

An experiment was conducted at Andalas University area to evaluate the efficacy of plant extracts on the growth and yield of maize. The first trial was testing the crude extracts of several plants on vegetative growth of maize factorial completely randomized design (CRD). The first factor crude extract sources from five species of plants, and the second factor was levels of extract concentration. The second trial was application of the purified extracts on growth and yield of maize using factorial CRD. First factor was the purified extract concentrations, and second factor was application frequencies. Results showed that application of 100 mg/L crude extract of *Gleicheni linearis* leaves was the most effective in increasing plant height and leaf area, compared to control. Treatment with 100 mg/L crude extract was better than the purified one. However, application of purified extract resulted in lower growth and yield of maize compared to control. The highest growth and yield attributes were recorded in 100 mg/L crude extract of *G. linearis* when applied at 15 days after planting (DAP). Further experimentation is needed for confirmation of the results.

Keywords: Crude extract, growth, maize, purified extract, yield

INTRODUCTION

Maize (*Zea mays* L.) is an important food commodity occupying the second rank of plant after rice in Indonesia. National maize demand in 2015 was 12.4 million tons, whereas national production was only 5.178 ton/ha (CBS, 2016). Therefore, maize production failed to fulfill the national demand.

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E-mail addresses:
rahmiaulya96@gmail.com (Nailul Rahmi Aulya)
zozya@yahoo.com (Zozy Aneloi Noli)
amribakhtiar@gmail.com (Amri Bakhtiar)
mansyurdin@fmipa.unand.ac.id (Mansyurdin)
* Corresponding author

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Indonesian government has set up the target to increase maize production 2.3% by 2019 with the productivity more than 5.178 ton/ha every year (Directorate General of Crop Plants, 2014). One of the strategies to reach the target is through maize extensification using suboptimal land. According to Utomo (2002), there was still huge suboptimal lands in Indonesia with an estimate about 60.7 million hectares. However, there is an obstacle in using suboptimal land because in general it consists of Ultisol soil having low fertility.

The fertility of Ultisol soil has been increased by using organic and inorganic fertilizers but it does not give good results. Therefore, other alternative ways are needed. One of them is using biostimulants that can promote plant growth (Abbas, 2013), increase plant response on stress (Du Jardin, 2012), and enhance plant physiological process (Abbas, 2013). Biostimulants can be obtained from microbial inoculant, humic acid, fulvic acid, amino acid, sea grass extracts (Calvo, Nelson, & Kloepper, 2014), and plant secondary metabolite compounds (Du Jardin, 2015).

Culver, Fanuel and Chiteka (2012) reported that application of crude extract of *Moringa oleifera* leaves on tomato leaves 2 weeks after germinating could increase growth and yield, dry roots weight, and height of tomatoes. Abdalla (2013) also reported that application of 2% leaf extract and 3% branch extract of *M. oleifera* with the frequency two times (7 and 14 days after planting) in a planting season, significantly increased height, fresh and dry weight of *Eruca vesicaria* subsp. s*ativa*. Ertani et al. (2015) found that extract of grape fruit skin with a dose 50 mg/L sprayed at 2 and 4 weeks after planting could increase biomass and dry weight of chili pepper.

Groups of secondary metabolite compounds that have been isolated from plants and used as biostimulants among other thing are triterpenoid saponin (Andresen & Cedergreen, 2010), flavonoid (Prabhu, Kumar, & Rajamani, 2010), and alkaloid (Aniszewski, 2007). In Indonesia, some plants dominantly contain secondary metabolites are leaves of cassava (*Manihot esculenta*) containing rutin glicoside and leaves of fern (*G. linearis*) containing kaempferol glicoside, which is the highest component of flavonoid found in plants (Bakhtiar, Jubahar, Ahmad Mahyudin, & Rivai, 1994). Alkaloid was found in stem bark of *Alstonia scholaris* (Marliana & Ismail, 2011), terpenoid in *Centella asiatica* (Singh, Singh, Gupta, Solanki, Sharma, & Nema, 2012) and xanthon in fruit pericarp of mangosteen (*Garcinia mangostana*) (Orozco and Failla, 2013). Effect of plant extract on maize has not been reported in Indonesia. Therefore, the present study has been initiated to evaluate the efficacy of plant extracts on the growth and yield of maize.

MATERIALS AND METHODS

A research was conducted at Andalas University area surrounded with screen cloth wall and a netting roof for insect protection. This study consisted of two trials, test of

crude extracts of several plants on vegetative growth and test of purified extracts (results from trial 1) on growth and yield of maize. The first trial was conducted from April to August 2016, and the second one was from October 2016 to February 2017.

Design

This trial was done using experimental method arranged in two factors of completely randomized design (CRD). The factor in the first stage was crude extract sources from five species of plants: (A1) leaves of cassava, (A2) leaves of *G. linearis*, (A3) *C. asiatica*, (A4) fruit pericarp of mangosteen, and (A5) stem bark of *A. scholaris*. The nested factor in the second stage was levels of extract concentration: (B1) control, (B2) 25 mg/L, (B3) 50 mg/L, and (B4) 100 mg/L. The experiment was replicated three times.

Source of Crude Extracts

Crude extracts of cassava and *G. linearis* leaves, *C. asiatica*, fruit pericarp of mangosteen, and stem bark of *A. scholaris* were obtained on Laboratory of Sumatran Biota, Andalas University. Extracts were prepared by standard method by Zakiah, Suliansyah, Bakhtiar and Mansyurdin. (2017). et al. (2017) and Ummah, Noli, Bakhtiar and Mansyurdin (2017). Crude extracts of cassava and fern leaves were prepared by boiling method (Bakhtiar et al., 1994) and those of *C. asiatica*, fruit pericarp of mangosteen and stem bark of*A. scholaris* by macerated with methanol (Orozco & Failla, 2013; Singh et al., 2012).

Test Crop and Planting

Planting media were Ultisol soil mixed with compost (5:1 in volume) that were placed in polybags (60 × 40 cm). Maize cv. *Bima-19 URI* was used as test crop and it was collected from the Research Station of Cerealia, Makasar, Indonesia. Two seeds were planted per polybag with a depth of 3 to 5 cm, and the distance among polybags was 25 × 75 cm^2. Removing worse seedling, the healthy seeding was allowed to grow. Seedlings were fertilized with urea (1.05 g/polybag) and MoP (0.53 g/polybag), based on the recommended dose of chemical fertilizer for maize.

Application of Crude Extracts

Crude extracts were diluted in organic solvent, dimethyl sufoxide (DMSO), and then diluted with 1-L water. Crude extracts, ±25 mL per plant, were sprayed evenly on maize leaves 2 weeks after planting. Application of crude extracts was carried out in morning when relative humidity was close to saturation (Kalaivanan, Chandrasekaran, & Venkatesalu, 2012).

Measurement of Growth Parameters

Parameters measured were vegetative growth of maize up to 49 DAP (days after planting) that covered the growth parameters such as plant height, number, and leaf area were measured at every week up to 49 DAP. Then plants were removed from the soil, fresh, and dry weight of shoot and roots were also measured.

Statistical Analysis

The analysis of variance (ANOVA) for various growth characters was performed following *F* test. When *F* was significant at the $p < 0.05$ level, treatments means were separated using Duncan's New Multiple Range Test (DNMRT). Data were analyzed following standard procedure using SPSS software.

Trial II

Design. In trial II, the extract used was the purified one that showed the best result (the most effective) in trial I. The experiment was arranged in two factors CRD replicated three times. First factor was concentrations of extract: F1 (control), F2 (100 mg/L crude extract), F3 (0.4 mg/L purified extract), F4 (0.8 mg/L purified extract), and F5 (1.6 mg/L purified extract). Second factor was application frequency: (T1) once (at 15 DAP) and (T2) twice (15 and 30 DAP).

Preparation of Purified Extract. Purified extracts were prepared from the most effective extract in increasing vegetative growth of maize in the first stage. Preparation of purified extract was meant to obtain the major compounds from crude extract by discarding some other compounds. Purified extract from cassava leaves crude extract was prepared by adding ethanol and screened, then filtrate was steamed in vacuo and purified extract was formed (Bakhtiar et al., 1994). Purified extract from crude fern leaves extract was prepared by adding ethanol and then screened. The filtrate formed was steamed in vacuo, then the purified extract was formed (Bakhtiar et al., 1994; Jubahar et al., 2006). The one from *C. asiatica* crude extract was prepared by adding active carbon and screened, then filtrate was steamed in vacuo, and purified extract was formed (Singh et al., 2012). Purified extract from mangosteen fruit shell crude extract was prepared by adding ethyl acetate and screened, and filtrate was steamed and then was added with hexane, precipitation formed was screened and dried until purified extract was formed (Orozco & Failla, 2013). Purified extract from stem bark of *A. scholaris* was prepared by adding HCl 2M and ethyl acetate and then screened. Water and ethyl acetate fractions were added with NH_4OH then steamed, and purified extract was formed (Marliana & Ismail, 2011).

Phytochemical Analysis. Phytochemical analysis on crude extract was done qualitatively using thin layer chromatography (TLC) plate. Phytochemical screening on secondary metabolites such as flavonoid, terpenoid, steroid, alkaloid, phenolic and saponin was done using standard laboratory method (Harborne, 1973; Trease & Evans, 1983).

Confirmation on the content of purified extracts using TLC for flavonoid content was done using stationary phase with Silica Gel 60 $F_{254}$and mobile phase with *n* butanol:ethyl acetate:water (3:1:1), and for terpenoid it was done using stationary phase with Silica Gel 60 F_{254} and chloroform:metanol (4:1) at mobile phase.

Application of Extracts. Extracts were diluted in organic solvent, *dimethyl sufoxide* (DMSO), and then diluted with 1-L water. Crude and purified extracts, ±25 mL per plant, were sprayed evenly on maize leaves 2 weeks after planting. Application of crude extracts was carried out in morning when relative humidity was close to saturation (Kalaivanan et al., 2012).

Measurement of Growth and Yield Parameters. Previously mentioned growth parameters were measured from early vegetative phase to harvest. Fresh and dry weight of shoot and roots, length and diameter of cob as well as 100 grains weight were recorded at harvest.

Statistical Analysis. The analysis of variance (ANOVA) for various growth characters was performed following *F* test. When *F* was significant at the $p < 0.05$ level, treatments means were separated using DNMRT. Data were analyzed following standard procedure using SPSS software.

RESULTS AND DISCUSSION

Trial I

The growth parameter of maize such as plant height and leaf area were significantly influenced by the interaction effect of different crude extract and concentration. The plant height was found in A2B4 where crude extract of *G. linearis* leaves 100 mg/L were used, which was statistically similar to A2B3 (*G. linearis* leaves 50 mg/L). The lowest plant height was recorded in A4B3 (crude extract of pericarp of mangosteen 50 mg/L). The highest leaf area was found in A2B4, which was significantly higher than the other treatments. The lowest leaf area was recorded in A4B2 (crude extract of stem bark *A. scholaris* 25 mg/L) (Table 2). The interaction effect of crude extract and concentration were not significant. The number of leaves, fresh and dry weight of shoot were significantly influenced by the different concentration of crude extracts.

The highest number of leaves per plant (10.3) was found in cassava leaves at 25 mg/L. The highest fresh weight of shoot was found at concentration 25 mg/L (540.50 g) that was statistically similar to concentration 50 mg/L (526.73 g), and the highest dry weight of shoot was found in concentration 100 mg/L (188.28 g). However, the highest fresh weight of root was found in crude extract of *G. linearis* leaves extract (104.83 g).

Application of 100 mg/L crude extract of *G. linearis* was the most effective in increasing plant height and leaf area, 243.4 cm and 655.43 cm^2, while controls were 174.1 cm and 447.96 cm^2. Crude extract of *C. asiatica* was effective in increasing plant height, number of leaves, and fresh weight of maize plant shoot. Application of crude extract of *C. asiatica* at concentration 25 mg/L was able to increase plant height (216.3 cm) and number of leaves (11 per plant) and showed the highest fresh weight of shoot (618.33 g). Crude extract of mangosteen fruit pericarp was effective in increasing plant height, number of leaves, fresh and dry weight of maize canopies.

Application of crude extracts of mangosteen fruit pericarp at concentration 100 mg/L was able to increase plant height (213.3 cm), number of leaves (10.0 per plant) and showed the highest fresh weight (616.00 g) and dry weight (224.51 g) of shoots.

The result of this research in contrast with Zakiah et al. (2017), vegetative growth of soybean (*Glicine max*) was inhibited by a crude extract of *G. linearis* leaves, mangosteen fruit pericarp and *A. scholaris* stem bark, but crude extract of *C. asiatica* promoted height and leaf area. Ummah et al. (2017) reported that the application of crude extracts of mangosteen fruit pericarp significantly increased the plant height and fresh weight of root of upland rice (*Oryza sativa*). On the other hand, crude extract of *G. linearis* leaves and stem bark of *A. sholaris* decreased vegetative growth. Other studies also showed that the response of plant species to crude extracts was different from one other . Phiri (2010) reported that extract of moringa leaf increased radical length of maize but reduced radical of rice. Shikur (2015) showed that the water extracts of alfalfa (*Medicago sativa*) influenced the root length and yield of beetroot (*Beta vulgaris*).

The effects of crude extracts five plant species on the growth of maize might be due to the content of secondary metabolites in the crude extracts. Secondary metabolites contained in crude extract of *G. linearis* leaves were flavonoid, terpenoid, phenolic, and saponin compounds. Organic compounds of *C. asiatica* were steroid, terpenoid, and polar phenolic. Those of mangosteen fruit pericarp were terpenoid, phenolic, and saponin compounds (Ummah et al., 2017). Terpenoid and terpenoid saponin from the plant extracts might have a role to promote plant growth. Terpenoid is a compound that engages in plant growth and development (Kabera, Semana, Mussa, & He, 2014). Some hormones are terpenoid such as giberelin and abscisic acid. Saha, Walia, Kumar and Parmar (2010) reported that saponin triterpenoid isolated from seed and pericarp of *Sapindus mucorossi* and *Diploknema butyracea* fruits showed the activity to promote growth of maize and rice.

Stem bark extract of *A. scholaris* containing steroid, terpenoid, and saponin that can promote growth also contains alkaloid, phenolic compounds that are toxic to plants. Acording to Ummah et al. (2017), the cassava leaf extract contained terpenoid, flavonoid, and phenolic compound. Flavonoid can inhibit auxin transport that results in decreasing plant height and stem diameter (Brown et al., 2001). Some phenolic compounds inhibit seed germination (Colvas, Ono, Rodriques, & de Sauza Passos, 2003). Polar phenolic compound is more toxic and inhibits cell devision (Zhao-Hui, Qiang, Xiao, Cun-De, & De-An, 2010). Generally, alkaloid group has allelopathic effect that can inhibit growth of monocotyle and dicotyle plants (Shao, Huang, Zhang, & Zhang, 2013).

Based on the result of trial I, application of 100 mg/L crude extract of *G. linearis* leaves showed the most effective effect in increasing plant height and leaf area, that is 1.39 times than the control plant (243.4 cm:174.1 cm) and 1.46 times of control leaf area (655.43 cm^2:447.96 cm^2). Therefore, crude extract of *G. linearis* leaves was continued to test its effects on growth and yield of maize at trial II.

Trial II

The yield contributing characters of maize were significantly influenced by the interaction effect of different concentration of extract and application time. The leaf area was found in F2T1 where crude extract of *G. linearis* leaves 100 mg/L was applied once at 15 DAP, which was statistically similar to F5T1 (purified extract of *G. linearis* leaves 1.6 mg/L was applied once at 15 DAP). The lowest leaf area was recorded in F4T1 (0.8 mg/L PE + 15 DAP) (Table 2). The highest fresh dry weight of shoot was also found in F2T1. The lowest fresh weight of shoot was recorded in F5T1 while the lowest dry weight of shoot was found in F5T2 (purified extract of *G. linearis* leaves 1.6 mg/L was applied twice at 15 and 30 DAP). The highest fresh weight of root, dry weight of root and weight of 100 grains were found in F2T2 (crude extract of *G. linearis* leaves 100 mg/L was applied twice at 15 and 30 DAP). The lowest fresh weight of root was recorded in F5T1, and the lowest dry weight of root and weight of 100 grains were found in F5T2.

Crude and purified extracts of *G. linearis* leaves significantly influenced the leaf area, fresh and dry weight of shoot, fresh and dry weight of roots, length of cob, and weight of 100 grains weight. The highest leaf area (250.95 cm^2), fresh weight of shoot (495.6 g), and dry weight of shoot (114.78 g) were found in 100 mg/L crude extract applied once at 15 DAP. The highest fresh weight (77.7 g) and dry weight (36.3 g) of roots, and weight of 100 grains (177.00 g) were observed in 100 mg/L crude extract applied twice at 15 and 30 DAP.

Mvumi, Tgwira and Chiteka (2013) reported that the application of moringa leaf extract in field treatment significantly increased the grain yield of maize, but in greenhouse condition it was not significant on all parameters. Spraying moringa extract once at 2 weeks after germination increased the grain yield by 59%, while spraying every 2 weeks until physiological mature increased grain yield by 128%.

The lower effect of purified extracts on growth and yield of maize compared to crude ones, and even lower than the one in control, presumably due to the content of purified extract contains kaempferol (R_f^2 = 0.41) and other flavonoid (R_f^1 = 0.15) groups (Figure 1). According to Samanta, Das and Das (2011), flavonoids are the low molecular weight polyphenolic secondary metabolic compounds, and function as allelopathic compounds. Allelopathy flavonoid can inhibit cell growth by inhibiting the production of ATP and the function of auxin (Mierzeak, Kostyn, & Kulma, 2014).

Based on crude extract potential of *G. linearis* leaves, it was able to increase fresh and dry fresh of roots, and weight of 100 grain, while two groups of flavonoid of purified extract was decreased leaf area, fresh weight of shoot. Further studies are needed to evaluate the bioactivity of terpenoid and saponin of purified extract to promote growth and yield of maize. According to Saha et al. (2010), activity of saponin and terpenoid was indicated to promote growth of maize and rice.

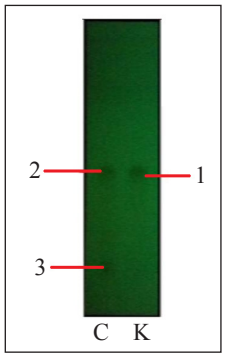

Figure 1. Elucidation of purified extract of *G. linearis* by TLC: Mobile phase: *n*-hexane:ethyl acetate (1:1); stationary phase: silica gel 60 F_{254}; detection: UV_{254}; C = purified extract of *G. lineris* leaf; K = kaempferol as standard; 1 = spot of standard kaempferol (R_f = 0,41); 2 = spot of kaempferol of purified extract (R_f =0.41 =); 3 = spot of other flavonoid group of purified extract (R_f =0.15)

Table 1
Effect of crude extract of several plants with different level of concentration on plant height, number of leaves, leaf area, fresh and dry weight of shoot and root of maize

Crude extracts	Concentrations				Average
	Control	25 mg/L	50 mg/L	100 mg/L	
			Plant height (cm)		
Cassava leaves	174.80efg	176.20efg	176.00efg	166.30efg	173.33
G. linearis leaves	174.10efg	231.70ab	240.50a	243.40a	222.43
C. asiatica	178.00defg	216.30abc	157.00fg	198.50bcde	187.45
Fruit pericarp of mangosteen	176.60defg	193.10cdef	148.80h	213.30abcd	182.95
Stem bark *A. scholaris*	171.20efg	178.80defg	184.80cdefg	159.00fg	173.45
Average	174.94	199.22	181.42	196.10	
			Leaf area (cm^2)		
Cassava leaves	486.70bc	392.26cd	391.55cd	429.90bcd	425.10
G. linearis leaves	447.96bcd	498.18bc	427.18bcd	655.43a	507.19
C. asiatica	453.97bcd	513.59bc	394.47cd	451.43bcd	453.37
Fruit pericarp of mangosteen	423.02bcd	422.47bcd	493.20bc	525.48b	466.04
Stem bark *A. scholaris*	487.01bc	351.81d	486.23bc	426.87bcd	437.98
Average	459.73	435.66	438.53	497.82	

Table 1 *(continue)*

Crude extracts	Concentrations				Average
	Control	25 mg/L	50 mg/L	100 mg/L	
	Number of leaves/plant				
Cassava leaves	9.30ns	10.70ns	10.30ns	10.30ns	10.15A
G. linearis leaves	9.00	10.30	10.00	10.70	10.00A
C. asiatica	9.00	11.00	8.70	9.30b	9.50AB
Fruit pericarp of mangosteen	8.7	9.70	8.00	10.00	9.10B
Stem bark *A. scholaris*	9.30	9.70	8.70	9.00	9.18B
Average	9.06 B	10.28 A	9.14 B	9.86 A	
	Fresh weight of shoot (g/plant)				
Cassava leaves	456.67ns	566.67ns	405.00ns	440.00ns	467.09ns
G. linearis leaves	420.00	615.00	637.50	635.00	576.88
C. asiatica	456.67	618.33	290.00	489.33	463.58
Fruit pericarp of mangosteen	363.33	542.50	276.67	616.00	449.63
Stem bark *A. scholaris*	463.33	360.00	496.67	453.33	443.33
Average	432.00 B	540.50 A	421.17 B	526.73A	
	Dry weight of shoot (g/plant)				
Cassava leaves	171.88ns	173.74ns	167.48ns	157.05ns	167.54ns
G. linearis leaves	186.29	233.20	141.59	195.19	189.07
C. asiatica	141.25	208.11	145.55	192.99	171.98
Fruit pericarp of mangosteen	139.83	155.33	117.07	224.51	159.19
Stem bark *A. scholaris*	145.58	139.37	161.59	171.68	154.56
Average	156.97 AB	181.95 A	146.66 B	188.28 A	
	Fresh weight of root (g/plant)				
Cassava leaves	71.67ns	75.00ns	71.67ns	82.33ns	75.17B
G. linearis leaves	84.33	103.33	111.67	120.00	104.83A
C. asiatica	65.00	70.00	53.33	70.00	64.58A
Fruit pericarp of mangosteen	62.50	87.50	46.67	97.50	73.54A
Stem bark *A. scholaris*	76.67	55.67	111.67	80.00	81.00AB
Average	72.03ns	78.30	79.00	89.97	
	Dry weight of root (g/plant)				
Cassava leaves	26.37ns	15.25ns	14.98ns	25.30ns	20.48ns
G. linearis leaves	22.34	34.99	28.69	22.10	27.03
C. asiatica	15.52	24.26	13.63	20.09	18.38
Fruit pericarp of mangosteen	19.64	29.64	16.26	33.23	24.69
Stem bark *A. scholaris*	27.41	17.50	27.29	19.07	22.82
Average	22.26ns	24.33	20.17	23.96	

Note: In a column and row, within treatment, same letter(s) indicate do not differ significantly according to DNMRT ($p < 0.05$). ns :nonsignificant.

Table 2

Effect of crude and purified extract of G. linearis *leaves and application frequency on growth and yield of maize*

Treatments		Plant height (cm)	No. of leaves/ plant	Leaf area (cm^2/plant)	Fresh weight of shoot (g/plant)	Dry weight of shoot (g/plant)	Fresh weight of root (g/plant)	Dry weight of root (g/ plant)	Length of cob (cm)	Diameter of cob (cm)	100 grains weight (g)	No. of cob/ plant
Concentration (mg/L)	Application frequency											
Control	1×	156.83ns	8.3ns	225.87bc	420.7d	82.40d	60.6e	32.4b	17.0ns	4.6ns	138.33f	1ns
	2×	156.83	8.3	171.57d	456.7b	102.18b	64.3d	26.9efg	15.2	4.3	136.67g	1
100 mg/L CE	1×	159.83	8.7	250.95a	495.6a	114.78a	64.7d	25.9fg	17.3	4.9	167.00b	1
	2×	161.00	9.0	216.90c	457.6b	97.40b	77.7a	36.3a	17.7	4.5	177.00a	1
0.4 mg/L PE	1×	153.67	9.0	185.00d	381.0e	83.72d	63.6d	27.8def	15.8	4.5	142.33e	1
	2×	156.67	8.3	134.34e	431.0c	81.11d	62.3de	29.5cd	16.8	4.6	150.50d	1
0.8 mg/L PE	1×	145.17	8.0	131.98e	389.7e	93.45c	67.3c	28.8cde	17.0	4.5	120.83i	1
	2×	161.67	8.3	188.28d	345.3f	93.60c	62.7de	30.7bc	16.8	4.2	157.00c	1
1.6 mg/L PE	1×	167.00	8.7	238.48a	443.0bc	76.31e	57.3f	25.3gh	15.3	4.3	104.53j	1
	2×	164.00	8.7	142.31e	323.7g	103.8b	70.7b	23.5h	16.0	4.9	132.23h	1

Note: CE: crude extract; PE: purified extract

Different letters within each same column indicate significant differences according to DNMRT ($p < 0.05$)

ns: nonsignificant

CONCLUSION

Crude extract of *G. linearis* leaves at the rate of 100 mg/L was the most effective in increasing plant height (243.4 cm) and leaf area (655.43 cm^2), followed by 50 mg/L of crude extract to plant height (240.5 cm) and 25 mg/L to leaf area (498.18 cm^2) , while in controls they were 174.1 cm and 447.96 cm^2. In the second trial, crude extract was more effective than the purified one. Growth and yield of maize treated with purified extract were lower than the ones of control. The highest fresh weight (77.7 g) and dry weight (36.3 g) of roots, and weight of 100 grains (177.00 g) were found in 100 mg/L crude extract applied twice at 15 and 30 DAP. The application of crude extract of *G. linearis* leaves could be used as biostimulants to increase growth and yield of maize.

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Effects of Crude Glycerin from Palm Oil Biodiesel Production as a Feedstuff for Broiler Diet on Growth Performance and Carcass Quality

Nusawan Boonwong, Chaiyawan Wattanachant* and Sutha Wattanasit

Department of Animal Science, Faculty of Natural Resources, Prince of Songkla University, 90112 Songkhla, Thailand

ABSTRACT

The effects of crude glycerin, a by-product from palm oil biodiesel production, supplemented in broiler diet on growth performance and carcass quality were studied. Four hundred 1-day-old-male Cobb 500 were randomly allotted into a completely randomized design of five groups consisting of four replications per group. Control diet and diets containing with 2.5%, 5%, 7.5%, and 10% of glycerin in pellet form were fed *ad libitum* to the broilers from 1 day of age until the age of 42 days. At the end of the experiment, eight broilers per treatment were slaughtered for carcass quality study. From the results, no significant difference was found in live weight change and feed intake with those receiving diets with and without glycerin supplementation. However, low growth performance and carcass yield were indicated when the levels of glycerin increased, particularly at 10%. No effect of dietary treatments on physical properties of the breast meat ($P > 0.05$). Therefore, this study recommended for mixing crude glycerin not >5% in the broiler diets. Furthermore, the inclusion of glycerin in the diets did not show any influence on the pH, tCIE color, shear force, drip, and cooking loss percentages of the breast meat ($P > 0.05$).

Keywords: Broiler, carcass quality, crude glycerin, growth performance

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E-mail addresses:
nusawan_nr@hotmail.com (Nusawan Boonwong)
chai_tum@yahoo.com (Chaiyawan Wattanachant)
sutha.v@psu.ac.th (Sutha Wattanasit)
* Corresponding author

INTRODUCTION

Biodiesel production has grown over the past several years due to the rising of world fossil fuel prices. Crude glycerin is the main by-product of biodiesel production, which is usually used for various purposes such as fuel, lubrication, animal diet, and so on. However, the quality of crude glycerin in terms of physical property and chemical

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composition is quite variable depending on sources of oil used for the biodiesel production (Dasari, 2007; Kerr, Dozier, & Bregendahl 2007; Thompson & He, 2006).

In terms of animal feed production, crude glycerin is mostly supplemented in animal diet, which is a by-product from biodiesel plants, that can substitute corn or sorghum as a source of energy (Thompson & He, 2006; Dasari, 2007; Kerr et al., 2007). In broiler feed, it could be substituted corn for about 5%–10% (Silva et al., 2012), although low weight gain in the second stage of broiler raising was indicated when fed diet with 10% of crude glycerin (Cerrate et al., 2006). In Thailand, preliminary work of Settapong et al. (2015) summarized that 10% of crude glycerin from the medium-scale biodiesel plant, which used waste cooked oil and a source of production (consisting of 80.25% purity, 4,387.45 GE/kg, and 0.44% crude fat), did not affect the growth performance of broiler. Nevertheless, not much information on the utilization of crude glycerin by-product from a palm oil biodiesel plant in animal feeds has been reported, in particular for broiler. Therefore, the objective of this study was to evaluate the influence of crude glycerin from palm oil biodiesel production added as an energy source in broiler diets on growth performance and carcass quality.

MATERIALS AND METHODS

Broilers and Management

This study was conducted at the Chicken Unit, Department of Animal Science, Faculty of Natural Resources, Prince of Songkla University (PSU), Hat Yai, Thailand. A total number of four hundred of 1-day-old-male Cobb 500 chicks with 44.45 ± 0.19 g of initial live weight from Charoen Pokphan Food (Thailand) Co. Ltd. (CPF) were allotted into five treatments, four replicates of 20 birds each. All birds were raised to receive experimental diets in the evaporative closed house system for 42 days (6 weeks). All procedures involved with this experimental broilers were in accordance with the animal ethical standard of the responsible committee on animal experimentation, PSU, while broiler farm management followed the recommended standard code number TAS 6901-2009 of the National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives, Thailand (ACFS, 2009).

Feeds and Feeding

Five diets consisting of 0%, 2.5%, 5.0%, 7.5%, and 10% of crude glycerin were formulated to supply the broiler's nutrient requirements according to the National Research Council (NRC, 1994). Diets were then mixed and pelleted at Animal Feed Mill Unit, Department of Animal Science, PSU. Crude glycerin used in this study was acquired from New Biodiesel Co., Ltd., Suratthani province, Thailand (a commercial palm oil biodiesel production plant). And its composition was determined by Settapong (2013) as 87.65% purity, 4,650 kcal/kg, 0.22% EE, 0.48% CP, and 1.44% ash. Ingredients and calculated nutritional values of the five mixed diets are presented in Table

1. The feeding program during this study was divided into two stages according to the management system: starter (1st stage) from 1 to 21 days (or 1–3 weeks) and finisher (2nd stage) from 22 to 42 days (4–6 weeks) of age. During the study, chickens were offered feed and water *ad libitum*.

Table 1
Ingredient composition and calculated nutrient content of the five experimental diets (1–21 and 22–42 days of age)

Items	Glycerin (%)*									
	1st stage (1–21 days of age)					2nd stage (22–42 days of age)				
	0	2.5	5	7.5	10	0	2.5	5	7.5	10
Ingredients										
Corn	55.25	52.21	49.17	46.14	43.10	62.61	59.75	56.89	53.52	50.92
Soy bean meal	29.59	30.13	30.66	31.20	31.74	21.78	22.31	22.84	23.38	23.91
Fish meal	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Vegetable oil	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Dicalcium phosphate	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-met	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
Lys	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Crude glycerin[1]	0.00	2.50	5.00	7.50	10.00	0.00	2.50	5.00	7.50	10.00
Rice hulls	0.00	0.00	0.00	0.00	0.00	0.44	0.27	0.10	0.00	0.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Calculated values										
Crude protein, %	23	23	23	23	23	20	20	20	20	20
ME, kcal/kg	3,200	3,200	3,200	3,200	3,200	3,200	3,200	3,200	3,200	3,200
Ca, %	1.00	1.00	1.00	1.00	1.00	0.90	0.90	0.90	0.90	0.90
Met, %	0.50	0.50	0.50	0.50	0.50	0.38	0.38	0.38	0.38	0.38
Lys, %	1.10	1.10	1.10	1.10	1.10	1.00	1.00	1.00	1.00	1.00
Thr, %	0.80	0.80	0.80	0.80	0.80	0.74	0.74	0.74	0.74	0.74
Sodium, %	0.20	0.20	0.20	0.20	0.20	0.15	0.20	0.15	0.15	0.15
Calcium, %	1.00	1.00	1.00	1.00	1.00	0.90	0.90	0.90	0.90	0.90
Non-phytate phosphorus, %	0.45	0.45	0.45	0.45	0.45	0.35	0.35	0.35	0.35	0.35
Lysine, %	1.10	1.10	1.10	1.10	1.10	1.00	1.00	1.00	1.00	1.00
Methionine, %	0.50	0.50	0.50	0.50	0.50	0.38	0.38	0.38	0.38	0.38
Threonine, %	0.80	0.80	0.80	0.80	0.80	0.74	0.74	0.74	0.74	0.74
Sodium, %	0.20	0.20	0.20	0.20	0.20	0.15	0.15	0.15	0.15	0.15

*Containing 87.65% purity, 4,650 kcal GE/kg, 0.22% EE, 0.48% CP, 1.44% ash, and 6.40 of pH (Settapong, 2013)

Managements and Data Collection

Performance parameters such as live weight, average daily gain (ADG), feed intake, and feed conversion ratio (FCR) were obtained for each raising stage. At the end of this study, eight chickens per treatment were sampled to fasting for about 12 h before being slaughtered at the Department of Animal Science, PSU slaughterhouse. Fasted live weight was determined before slaughter, while the slaughtering procedure used in this study was done according to the Islamic procedure of Thai Agricultural Standard 8400-2007 (ACFS, 2007). After slaughter, weight of carcasses and organs were determined, whereas pH at 45 min *post mortem* (pH_0) of breast muscle (m. *Pectoralis major*) at the right side of each carcass was obtained. Then the carcasses were individually packed in plastic bags and stored at 4°C for about 24 h. After this period, each carcass was weighed and the ultimate pH (pH_μ) of the breast muscle determined. After that, commercial parts; breast, fillet, thigh, drumstick, and wing were cut and weighted. The procedure of the chicken meat cutting part was done according to the guidelines of Thai Agricultural Standard 6700-2005 (ACFS, 2005). Color, shear force, and cooking loss values of breast muscle of each carcass were determined according to Wattanachant (2004). In this study, the pH of muscle was measured using a MettlerTeledo portable pH meter model SG2 with Mettler pH probe (Lot 406-M63DXK-57/25). Breast muscle color was determined with a CR-10 Chromometer (Minolta Color Meter, Osaka, Japan) and reported in the CIE color system. The shear force value was measured by a Texture Analyzer (TA-XT2i, Texture expert Vision 1.17, Stable Micro System, Godalming, Surrey, UK) by using a Warner-Bratzler shear blade.

Statistical Analysis

The data were analyzed as a completely randomized design by using the GLM procedure. Mean comparisons were performed by the Duncan's multiple range test according to Kaps and Lamberson (2004).

RESULTS AND DISCUSSION

Growth Performance

The effects of the dietary treatments on growth performance are presented in Table 2. During the first stage of rearing, broilers that received control diet with no crude glycerin inclusion had higher live weight, live weight gain, ADG, and feed intake ($P < 0.01$) than other treatment diets. However, those fed 2.5% and 5.0% glycerin had significantly better live weight gain compared to those fed diet with 10% glycerin inclusion. No effect of dietary treatments on FCR ($P > 0.05$) was found at this stage.

In the second stage, broilers fed control diet with no glycerin and the inclusion of 2.5%–10% of crude glycerin had no significant effects on final live weight, live weight gain, and ADG ($P > 0.05$), although the final weight of the control diet tended to be higher than others. In this stage, broilers

fed control diet with no glycerin inclusion had similar feed intake as those fed diet with 2.5%–7.5% of glycerin, but the feed intake of all these three groups were significantly higher than those fed 10% glycerin ($P < 0.05$). After calculation of FCR, this study indicated that dietary treatments either with or without glycerin inclusion did not show any significant effect on FCR of broilers ($P > 0.05$).

During the period of 1–42 days of age, no significant effects of dietary treatments were found in live weight change, ADG, and FCR ($P > 0.05$). Feed intake in broilers fed control diet and those fed with diets supplemented with 2.5% and 5.0% of crude glycerin ($P > 0.05$) was similar, but statistically higher than those fed 7.5% and 10% of crude glycerin ($P < 0.05$). From this study, it was observed that feed intake of broilers decreased when the percentage of crude glycerin inclusion in the diets increased. Thus, FCR of broilers tended to decrease due to the lesser feed intake when the level of glycerin increased.

The reduction in growth performance was related to the amount of crude glycerin inclusion in the dietary treatment,

Table 2
Live weight change, feed intake, FCR of broilers receiving diets containing 0, 2.5, 5, 7.5, and 10% of crude glycerin (n = 400)

Items	Crude glycerin (%)					SEM	*P*-value
	0	2.5	5	7.5	10		
Initial weight (g)	44.50	44.20	44.45	44.10	44.65	0.28	0.7518
1–21 days (1st stage)							
Final weight (g)	887.50[a]	797.50[b]	812.50[b]	746.25[bc]	717.50[c]	22.42	0.0008
Weight gain (g)	843.00[a]	753.30[b]	768.05[b]	702.15[bc]	672.85[c]	22.33	0.0008
ADG (g)	40.14[a]	35.87[b]	36.57[b]	33.44[c]	32.04[d]	1.06	<0.0001
Feed intake (g/bird)	1,131.30[a]	989.63[b]	1,003.98[b]	934.13[bc]	877.05[c]	23.48	<0.0001
FCR	1.34	1.31	1.31	1.33	1.31	0.13	0.1339
21–42 days (2nd stage)							
Final weight (g)	,710.00	2659.00	2690.00	2570.00	2560.00	35.67	0.0589
Weight gain (g)	1822.50	1861.50	1877.50	1833.75	1842.50	112.43	0.9799
ADG (g)	86.79	88.64	89.40	86.39	87.74	5.35	0.9799
Feed intake (g/bird)	3920.51[a]	3949.31[a]	3940.83[a]	3782.08[ab]	3545.48[b]	48.12	0.0480
FCR	2.15	2.12	2.10	2.06	1.92	0.11	0.5042
1–42 days							
Final weight (g)	2710.00	2659.00	2690.00	2570.00	2560.00	35.67	0.0689
Weight gain (g)	2665.50	2614.80	2645.55	2535.90	2515.35	28.97	0.0429
ADG (g)	63.46	62.26	62.99	60.38	59.89	1.04	0.0305
Feed intake (g/bird)	5091.81[a]	4938.90[a]	4964.80[a]	4736.21[b]	4322.53[c]	147.59	0.0335
FCR	1.88	1.86	1.85	1.86	1.72	0.09	0.0794

[a,b,c]Means on the same rows with different superscripts differ significantly ($P < 0.05$)

particularly at 10% inclusion, during the first stage of rearing (1–21 days). This finding confirms the work of Cerrate et al. (2006) who reported that the addition of 10% glycerol to the diet caused a similar live weight to fed diets with 0% and 5% glycerol at 14 days of age. Nevertheless, the results of this study are different from the trial executed by Silva et al. (2012) who found no statistical difference of the dietary inclusion of 10% of glycerin during the rearing period on growth performance. In addition, other reports with adding glycerin at 8% in broiler diet (Abd-Elsamee et al., 2010) and 10% in broiler diet (Settapong, 2013) did not observe any impairment of growth performance. However, this study can conclude that broilers fed diets with 2.5% and 5% of glycerin inclusion might perform better than those received diet with 7.5% and 10% of crude glycerin. During the second stage (22–42 days), although growth performance of broilers fed diets with crude glycerin inclusion from 2.5% to 10% did not show any statistically differences, growth performance tended to be lower than those broilers fed control diet. This finding was similar to the report of Silva et al. (2012) although the result of this study for live weight was about 16.7% lower. Feed intake tended to decrease when the level of glycerin inclusion increased, similar to the findings of Jung and Batal (2011) and Silva et al. (2012). This reduction of weight gain might not be related to the effect of methanol in glycerin ingredient due to the low percentage of methanol (1.02%). Thus, the low weight gain is probably related to the hardness of the pellet feed that altered the flowability of feed in the gut described by Cerrate et al. (2006).

Carcass Characteristics and Physical Properties of Meat

Table 3 illustrates the carcass trait results of broilers at 42 days of age. No significant effect of treatment diets on the slaughter weight, warm carcass weight, chilled carcass weight, dressing percentage, and visceral percentage were obtained ($P > 0.05$), although warm and chilled carcass weights tended to decrease when the percentage of glycerin inclusion increased. Considering the retail parts (Table 4), broilers receiving control diet with no crude glycerin had similar breast percentage with those fed 2.5%–7.5% of glycerin ($P > 0.05$), whereas the lowest breast percentage was indicated in broilers fed 10% crude glycerin ($P < 0.05$). No significant differences were detected in increasing glycerin levels on fillet, drumstick, wing, and thigh percentages ($P > 0.05$). It was noticed that the thigh percentage tended to increase, while the breast percentage tended to decrease when the level of glycerin inclusion increased. This work was different from the report of Cerrate et al. (2006) who found a significant increase of breast percentage in broilers fed with 5% as compared to those fed diets with 0% and 2.5% of glycerin with no significantly difference in breast percentage of broilers fed diets containing 5% and 10% of glycerin ($P > 0.05$). The low meat yield in this study might be related to the low protein depot in the carcass. When comparing

results of this study to other literatures, there was a positive effect found on carcass traits. This could be described that with suitable amount of glycerin, it might increase the protein deposition in broiler due to the reduction of gluconeogenic amino acid via the inhibition of phosphoenolpyruvate carboxykinase as described by Young, Shrago and Lardy (1964), Cryer and Bartly (1973), and Silva et al. (2012). Glycerin was also observed to decrease the activity of glutamate dehydrogenase (Steele, Winkler, & Altszuler, 1971).

The inclusion of glycerin in the diets did not show any influence on the pH and the color of the breast meat neither at 45 min post-mortem time nor 24 h post-mortem time ($P > 0.05$) (Table 5). In addition, shear

Table 3
Carcass components of broilers received diets containing 0, 2.5, 5, 7.5, and 10% of crude glycerin

Items	Crude glycerin (%)					SEM	*P*-value
	0	2.5	5	7.5	10		
Number of broiler, birds	8	8	8	8	8	–	–
Slaughter weight (g)	2600.00	2598.50	2600.00	2541.88	2536.00	94.84	0.2447
Warm carcass weight (g)	1860.50	1838.50	1832.50	1796.50	1790.20	78.56	0.1901
Chilled carcass weight (g)	1839.00	1804.50	1804.00	1772.50	1760.93	65.97	0.0769
Dressing percentage							
Warm carcass, (%)	71.56	70.75	70.48	70.68	70.59	0.49	0.6977
Chilled carcass (%)	70.73	69.44	69.38	69.73	69.44	0.55	0.6880
Viscera (%)	10.68	10.55	10.86	10.40	10.37	0.22	0.5577

[a,b,c]Means on the same rows with different superscripts differ significantly ($P < 0.05$)

Table 4
Carcass composition of broilers receiving diet containing 0, 2.5, 5, 7.5, and 10% of crude glycerin

Items (%)	Crude glycerin (%)					SEM	*P*-value
	0	2.5	5	7.5	10		
Breast	25.8[a]	24.55[a]	24.75[a]	24.13[ab]	23.21[b]	0.64	0.0301
Fillets	5.40	5.46	5.67	5.61	5.45	0.14	0.0798
Drumsticks	13.5	13.43	14.09	13.85	13.95	0.35	0.9960
Wings	11.4	11.12	10.97	11.18	11.3	0.41	0.5813
Thighs	17.68	18.47	18.21	18.30	18.39	0.42	0.0590
Meat	58.42	57.14	57.20	56.30	56.85	1.25	0.0653
Bone	27.86[b]	28..89[a]	29.12[a]	30.55[a]	30.99[a]	0.63	0.0456
Total fat	8.32	8.26	8.20	7.90	7.91	0.22	0.5127

[a,b,c]Means on the same rows with different superscripts differ significantly ($P < 0.05$)

force, drip, and cooking loss percentages of the breast meat from broilers fed all treatment diets did not show any significant differences ($P > 0.05$). This was probably related to the amount of crude glycerin inclusion in this study that was in the typical range recommended by other works of Cerrate et al. (2006) and Silva et al. (2012). No negative effects on meat traits were indicated, although weight change and feed intake were decreased when the level of glycerin increased. The pH of meat in this study was in the normal range (6.10–6.19) mentioned by Ristic and Damme (2010), although the pH at 45 min in this study was slightly lower (6.25) compared to the report of Karaoğlu, Aksu, Esenbuga, Macit and Durdag (2006). This was probably related to the amount of glycogen stored in the muscle during slaughter period described by Haslinger, Leitgeb, Bauer, Ettle and Windisch (2007). The lighter L* value of breast meat at 24 h post-mortem was in agreement with the work of Qiao, Fletcher, Smith and Northcutt (2001), and Petracci and Fletchert (2002).

Table 5
pH at 45 min and 24 h post-mortem and color of breast muscle from broilers received diet containing 0, 2.5, 5, 7.5, and 10 % of crude glycerin

Item	Crude glycerin (%)					SEM	*P*-value
	T1	T2	T3	T4	T5		
45 min (pH_0)							
pH	6.16	6.14	6.11	6.10	6.19	0.04	0.4693
L	43.39	43.23	43.27	44.57	44.23	0.48	0.1731
a*	1.65	1.68	1.65	1.69	1.71	0.06	0.9566
b*	2.60	2.69	2.59	2.68	2.64	0.07	0.8095
24 h (pH_u)							
pH	5.79	5.75	5.77	5.83	5.84	0.02	0.0675
L	46.44	45.98	44.99	47.14	46.44	0.56	0.1109
a*	1.66	1.59	1.60	1.69	1.66	0.04	0.2587
b*	1.79	1.74	1.75	1.72	1.69	0.05	0.6244
Drip loss (%)	1.45	1.48	1.57	1.67	1.71	0.10	0.2642
Cooking loss (%)	18.83	17.93	18.76	18.42	17.62	0.66	0.5898
Shear force (g*)	2,790.88	2,762.74	2,801.24	2,980.81	2,998.68	170.38	0.5615

*Muscle samples were cut approximately to a dimension of 1 × 2 × 0.5 cm. Crosshead speed was set to 2 mm/s and 25 kg load cell

CONCLUSION

The results of this study indicated that crude glycerin from palm oil biodiesel production could be used to substitute corn in broiler diet. However, low growth performance and carcass yield were indicated when the levels of glycerin exceeded 5% of the diet. From the results of this study, the optimum amount of crude glycerin recommended in the broiler diets is no >5%. However, the

replacement of corn by crude glycerin will not be suitable if the price of corn is lower than the price of glycerin.

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Soil CO_2 Efflux of Oil Palm and Rubber Plantation in 6-Year-Old and 22-Year-Old Chronosequence

Cindy Usun Sigau* and Hazandy Abdul Hamid

Institute of Tropical Forestry and Forest Products, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia

ABSTRACT

Soil CO_2 efflux, in relation with chronosequence at oil palm and rubber plantations, was measured monthly, each with both 6- and 22-year-old stands. Other environmental factors such as soil temperature and relative humidity (RH), as well as soil properties, were also measured at 0–30 cm depth. Soil CO_2 efflux was found to be highly affected by forest types and chronosequence factor. The 22-year-old age stand ($M = 0.91$; $SD = 0.17$ g CO_2 m^{-2} h^{-1}) had significantly higher soil CO_2 efflux than the 6-year-old stand ($M = 0.54$; $SD = 0.18$ g CO_2 m^{-2} h^{-1}). Soil RH plays a significant role controlling soil CO_2 efflux compared with soil temperature, especially at younger stands of tropical oil palm and rubber plantations spatially. Lower Q_{10} values were found to be caused by higher temperature that had reduced enzymatic and substrates activities for soil respiration. Non-discernible trends of temporal soil CO_2 efflux, soil temperature, and RH indicated that other significant factors could be the catalyst, and thus further research is required to explain the relations between soil CO_2 efflux and environmental factors. Research findings indicated that older stand age of oil palm and rubber plantations in Malaysia released higher soil CO_2 efflux, but with no degrading effects towards the environment.

Keywords: Chronosequence, environment, land use, oil palm, plantation, rubber, soil respiration, tropical

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E-mail addresses:
cindyusunsigau@gmail.com (Cindy Usun Sigau)
hazandy@gmail.com (Hazandy Abdul Hamid)
* Corresponding author

INTRODUCTION

Globally, net carbon (C) emission from land use is approximately 1.0 ± 0.8 pg C yr^{-1} (Le Quéré et al., 2015). Therefore, the slightest alteration of the terrestrial ecosystems may lead to a considerable change of the atmospheric CO_2 concentration (Arevalo, Bhatti, Chang, Jassal, & Sidders, 2010;

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Schlesinger & Andrews, 2000). The role of land use, or terrestrial ecosystems, is critical in the global C cycle as it releases CO_2 into the atmosphere, which accounts for 90% of the total ecosystem respiration that is predominantly facilitated by soil CO_2 efflux (Hanson, Edwards, Garten, & Andrews, 2000). Soil CO_2 efflux refers to the instantaneous CO_2 transports via soil ground surface into the atmosphere, and *vice versa*, which includes rhizosphere, microbes, and soil fauna respiration (Raich & Schlesinger, 1992; Maher, Asbjornsen, Kolka, Cambardella, & Raich, 2010). Besides that, soil CO_2 efflux varies significantly among plant biomes, indicating environmental changes in vegetation via land use conversion which potentially alters the soil CO_2 emissions into the atmosphere, and *vice versa* (Raich & Tufekcioglu, 2000). However, the impacts of anthropogenic activities on soil CO_2 efflux from land use modification differ from one site to another, and are still poorly documented (Nazaries et al., 2015; Raich & Schlesinger, 1992; Veldkamp, Purbopuspito, Corre, Brumme, & Murdiyarso, 2008).

Studies on soil CO_2 efflux in Malaysia have been conducted and quantified to improve the understanding of soil CO_2 efflux at various levels. However, these studies focused on tropical forests (Mande, Abdullah, Aris, & Nuruddin, 2014a; Mande, Abdullah, Zaharin, & Ainuddin, 2014b), peat soils (Choo, Nuriati, & Ahmed, 2014), and single plantation species (Firdaus & Husni, 2011; Choo et al., 2014). In addition, soil CO_2 efflux studies in Malaysia generally explored a single ecosystem age (Mande et al., 2014a). Thus, little to no information is available on the chronosequence associations, which also remain unclear. Therefore, there is a knowledge gap on soil CO_2 efflux dynamics with the association of chronosequence between different ecosystem types despite the acknowledgement of the importance of soil CO_2 efflux in terrestrial-atmosphere balance, especially in Malaysia. A comprehensive understanding of soil CO_2 efflux, particularly on its impact on environmental factors from the types of forest management, is important as it will enhance our knowledge of the fundamental ecological processes controlling soil CO_2 efflux (Fan, Yang, & Han, 2015; Liu et al., 2016). Therefore, the present study was undertaken to explore the association of oil palm and rubber plantations at different ecosystem age stands and its influence on soil CO_2 efflux.

MATERIALS AND METHODS

Study Site

The study was conducted at 6- and 22-year-old oil palm (*Elaeis guineensis*) and rubber (*Hevea brasiliensis*) plantations at Universiti Putra Malaysia, Serdang, Selangor, from April to June, 2016. The experimental plots are located at about 2°59′ N, 101°43 ′E, with the mean annual temperature and mean annual rainfall of 27°C and 2,215.7 mm, respectively. One month (March 2016) prior to commencement of the study, rainfall was 89.5 mm and the area received 134.6, 355.2, and 218.4 mm during the

study months of April, May, and June, respectively. The soil series is classified as Haplic Nitisols. Important physical and edaphic characteristics of the stand ages are presented in Table 1.

Table 1
Site characteristics of oil palm and rubber plantation trees on sample plots, each with 6- and 22-year-old ecosystem ages

		Physical attributes		Soil characteristics				
Species	Age	Diameter (cm diameter at breast height)	Height (m)	C (%) (N = 3)	N (%) (N = 3)	Soil organic content (kg C m^{-2}) (N = 3)	pH (N = 3)	Bulk density (g cm^{-3}) (N = 3)
Oil palm	6	45.21 ± 0.21 (N = 36)	2.47 ± 0.11 (N = 36)	2.26 ± 0.09	0.16 ± 0.01	7.57 ± 0.13	3.39 ± 0.07	1.31 ± 0.12
	22	58.12 ± 0.43 (N = 32)	9.5 ± 0.31 (N = 32)	1.93 ± 0.06	0.26 ± 0.06	7.80 ± 0.13	3.38 ± 0.06	1.3 ±0.11
Rubber	6	14.12 ± 0.70 (N = 95)	17.32 ± 0.56 (N = 95)	2.21 ± 0.07	0.28 ± 0.03	7.55 ± 0.14	3.43 ± 0.08	1.34 ± 0.10
	22	33.76 ± 1.38 (s = 70)	23.23 ± 1.43 (N = 70)	2.18 ± 0.06	0.24 ± 0.05	6.49 ± 0.12	3.30 ± 0.07	1.35 ± 0.09

The data are mean ± SE

For oil palm maintenance, the fertilizer types used were Blue NPK (13:2:14) and organic, which were applied at the recommended amounts of 6.0 and 10.0 kg per tree per year, respectively, in three split applications per year. Meanwhile, the fertilizers for the rubber plantation were MPOB F1 (10:5.4:16.2), Blue NPK, and organic, which were applied at 8.0, 6.0, and 10.0 kg per tree per year, respectively, in three split applications per year. Oil palm stands were typically established on 8.8 m × 8.8 m × 8.8 m spacing and rubber stands on 7 m × 3.5 m, and averaging approximately 150 and 408 trees per hectare, respectively. The chronosequence technique in the present study presents an integration of a forest type with different ages as a unit, replacing space for time (Wellock, Rafique, LaPerle, Peichl, & Kiely, 2014). The condition of sites is approximately identical so as to reduce variability besides the age stand features. There was no fertilization and maintenance executed on the stands during the research.

Experimental Design

The experiment was done in the field for three consecutive months by using random sampling method. Forest types (oil palm and rubber) and age stand (6- and 22-year-old) were used as factors influencing soil CO_2 efflux. Three replications for each stand

age, measuring 50 × 50 m plots for oil palm and 25 × 50 m plots for rubber plantations, were randomly selected for soil CO_2 efflux measurements. The replications represented by 12 stands were distributed across four geographical blocks. Subplots were established in selected plots, each measuring 25 × 25 m for the oil palm plantation, and 25 × 50 m for the rubber plantation. The chosen subplots were then specified to 1 m^2 plot grids through random number generator for the soil collar installation to account for within-stand variability.

Measurements of Soil CO_2 Efflux and Environmental Factors

Soil CO_2 efflux (g CO_2 m^{-2} h^{-1}) was measured using a portable LI-8100A Automated Soil CO_2 Flux System (Li-Cor Inc., Lincoln, NE) with a 20-cm-sized chamber connected to an infrared gas analyzer (IRGA, LI-8100A, Li-Cor Inc., Lincoln, NE). Soil collars (measuring 100 mm high and 200 mm diameter) were installed approximately at 80 mm soil surface depth 2 weeks prior to soil respiration measurements to avoid biases due to soil disturbance during collar installation. Litterfall of approximately 1 m radius, around and within the soil collar, was manually removed from the soil surface. Soil CO_2 effluxes were measured hourly at intervals of 2 min from 0900-1700 h. Soil CO_2 efflux measurements were taken on a bi-monthly basis for 3 months from April to July at each forest type. LI-8100A instrument software program was used to analyze the primary data of soil CO_2 efflux measurements.

Soil temperature and soil relative humidity (RH) were simultaneously measured using two probes connected to the LI-8100A gas analyzer recorder at approximately 80 mm soil depth. Air temperature and RH were recorded hourly throughout the study period using a data logger weather station (WatchDog Model 2475 Plant Growth Station, Spectrum Technologies) placed within 50 m of the study sites. All the measurements were taken in triplicates. Soil sampling was conducted to determine soil pH, bulk density (BD), soil organic content (SOC), carbon (C), and nitrogen (N). Using a soil auger, three samples were collected randomly from each plot to yield composite samples of soil at 0–30 cm depths. The soil samples were air dried for 3 days, ground, sieved through a 2-mm sieve, and stored in sealed plastic bags before further laboratory analysis. SOM was determined using a conversion factor of 1.72, where organic matter was assumed to contain 58% organic carbon using equation:

$$\text{Organic matter (\%)} = \text{Total organic carbon (\%)} \times 1.72 \quad (1)$$

Total C and N concentration were measured using the CN-element analyzer (PE 2400 II CHN elemental analyzer; Perkin-Elmer, Boston, MA). Soil pH was determined in salt solution 1:2.5 dilution of potassium chloride (KCl). BD was determined using the soil analysis standard method (Blake, 1965).

Statistical Analysis

All the statistical analyses were conducted using SPSS software 23 (SPSS Inc., Chicago, IL, USA). Normality and homoscedasticity data were tested with the Kolmogorov–Smirnov and Levene's tests, respectively, and no significant deviations from normality or homoscedasticity were found. A two-way analysis of variance (ANOVA) and Tukey's honestly significant (HSD) tests were used to examine the effects of land use types and chronosequence on soil CO_2 efflux. Meanwhile, one-way ANOVA and Tukey's HSD tests were used to examine the effects of temporal variations and plot on soil CO_2 efflux, with $P < 0.05$ being at significant level. In order to examine soil CO_2 efflux–soil temperature relations, regression analysis was conducted using a classic parametric exponential model (Lloyd & Taylor, 1994):

$$\text{Soil } CO_2 \text{ efflux} = \alpha e^{\beta T} \quad (2)$$

where T = soil temperature (°C) at 80 mm depth, α and β = regression coefficients.

The temperature sensitivity of soil respiration on soil temperature, expressed by Q_{10} which is the difference in respiration rates over a 10°C interval, was calculated using the equation (Boone, Nadelhoffer, Canary, & Kaye, 1998):

$$Q_{10} = e^{10\beta} \quad (3)$$

A linear function model (Han, Huang, Liu, Zhou, & Xiao, 2015) was used to describe the relationship between soil CO_2 efflux and soil RH:

$$\text{Soil } CO_2 \text{ efflux} = \alpha_1 RH + \beta_1 \quad (4)$$

where RH = soil RH, and α_1 and β_1 are the fitted parameters.

RESULTS AND DISCUSSION

Soil CO_2 Efflux Spatial Variations

A two-way ANOVA assessed the effects of two land uses, which were oil palm plantation and rubber plantation at the age stands of 6 years and 22 years, respectively, on soil CO_2 efflux. There was a statistically significant interaction between the forest types and chronosequence on soil CO_2 efflux, $F(1, 104) = 241$, $P < 0.05$. It was found that forest types had a statistically significant result, indicating the differences in the mean values between oil palm (M = 0.74; SE = 0.01 g CO_2 m^{-2} h^{-1}) and rubber (M = 0.71; SE = 0.01 g CO_2 m^{-2} h^{-1}), $F(1, 104) = 4.15$, $P < 0.05$, which influenced soil CO_2 efflux. The age effect was also shown to be statistically significant on soil CO_2 efflux, $F(1, 104) = 364.09$, $P < 0.05$ (Figure 1a). The 22-year-old ecosystem age stand (M = 0.91; SE = 0.01 g CO_2 m^{-2} h^{-1}) had significantly higher soil CO_2 efflux than the 6-year-old ecosystem age stand (M = 0.54; SE = 0.01 g CO_2 m^{-2} h^{-1}), as shown in Figure 1(b).

The present study contradicts with that of Zhao et al. (2016), where no significant effect of forest types on soil CO_2 efflux

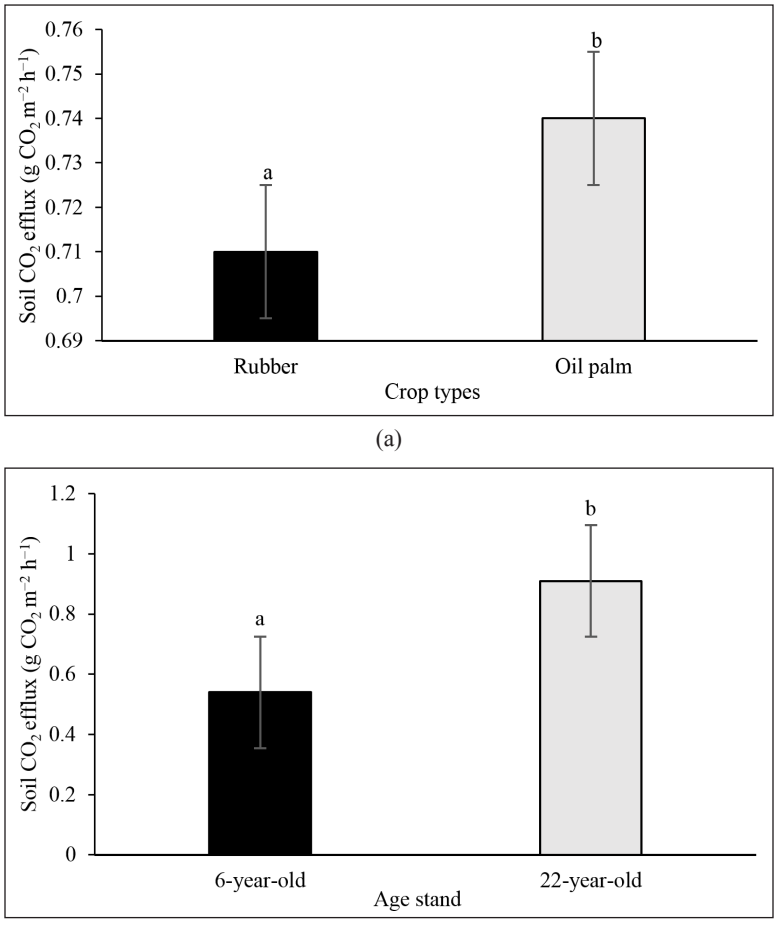

Figure 1. Soil CO_2 efflux between: (a) crop types; and (b) age stand; means with different letters indicate significant differences ($P < 0.05$)

was found within a tropical region. The difference in the findings of soil CO_2 efflux is most likely caused by the tree species at different regions (Wang et al., 2016). Both oil palm and rubber plantations had a similar pattern, whereby soil CO_2 efflux increases with the age of stand. Older stands have higher soil CO_2 efflux compared with younger stands (Yan, Zhang, Zhou, & Liu, 2009). The increase of soil CO_2 efflux with age was associated with the differences in canopy density, fine root biomass, and soil substrates (Zhao et al., 2016). However, the present findings contradicted with other studies, where younger stands revealed higher soil CO_2 efflux due to the abundance of fine root biomass in younger stands, or forest carbon input, and canopy density (Mande et al., 2014a; Wang et al., 2017).

Younger oil palms in the present study (0.70 g CO_2 m^{-2} h^{-1}) had an approximately similar range of soil CO_2 efflux to that

of Mande et al. (2014a) with 0.52 g CO_2 m^{-2} h^{-1} recorded at Pasoh Reserve Forest. Meanwhile, the soil CO_2 efflux of the 22-year-old stand (0.77 g CO_2 m^{-2} h^{-1}) at the oil palm plantation was only slightly higher than the younger stand in the present study. Adachi, Bekku, Rashidah, Okuda and Koizumi (2006) reported a higher soil CO_2 efflux with 0.97 g CO_2 m^{-2} h^{-1} at the 28-year-old oil palm plantation, which was also recorded at Pasoh Forest Reserve. As for the rubber plantation, the present study recorded lower soil CO_2 efflux (0.35 g CO_2 m^{-2} h^{-1}) compared with readings from other locations reported by Mande et al. (2014a) with 0.74 g CO_2 m^{-2} h^{-1}. Another reason for the lower and variation in soil CO_2 emissions from the younger stand was the accumulation of recalcitrant C in the soil (Table 1) as a result of higher stability in soil mineral particles (Zhao et al., 2016). Besides, forests at different developmental stages have varied changes in terms of the aboveground and belowground quantity and quality of litterfall affecting soil CO_2 efflux (Tedeschi et al., 2006).

In the present study, the values of soil CO_2 efflux recorded were <1, which are similar to the primary and secondary tropical forest types. The highest soil CO_2 efflux recorded in the 22-year-old rubber plantation was 1.05 g CO_2 m^{-2} h^{-1}. Soil CO_2 efflux is one of the fundamental terrestrial C cycling components that is controlled by various environmental factors, and varies spatially that sometimes result in contradictory findings due to the complex interactions between factors (Martin & Bolstad, 2009). One of the main reasons is the difference between the morphological structure of oil palm and rubber trees. Oil palms are single stemmed with pinnate leaves, while rubber trees are tall several-stemmed deciduous trees with three leaflets and spirally arranged leaves with inflorescences. Younger stands had less soil CO_2 efflux caused by the canopy photosynthesis reduction from less litter inputs which eventually reduce C substrates supply to microbes (Gong et al., 2014). Gong et al. (2014) explained that litter quality modifies utilization pattern of soil microbes affecting the soil characteristics such as the BD. The morphological difference and low-density influence root biomass, soil microbial biomass, and belowground C inputs such as roots, exudates, and also litter into the soil (Yan et al., 2009). Meanwhile, spatial and temporal variations of soil CO_2 efflux are attributed to the total aboveground and belowground biomass and forest carbon stock resulting from forest disturbance and land conversion (Mande et al., 2014a). Over time, changes take place in the species composition, soil functions, and processes such as biology, soil organic matter, and biogeochemical cycles (Yan et al., 2009). In the present study, mature stands had higher biomass from litterfall received from larger morphological characteristics (leaves, branches, stems, and roots) and subsequently triggered decomposition on the soil surface that increased microbial activity which eventually released soil CO_2 efflux. On the chronosequence, there was incremental growth in tree biomass that

caused changes in the total ecosystem C (Wellock et al., 2014). The results indicated that the older the stand age, the higher the release of CO_2 from the soil into the atmosphere.

Temporal Variability of Soil CO_2 Efflux and Environmental Factors

Temporal variations of soil CO_2 efflux revealed a significant effect ($P < 0.05$) at both oil palm and rubber plantations throughout the investigation. During the period from April to June 2016, soil CO_2 efflux was the highest in April (0.33–1.12 g CO_2 m^{-2} h^{-1}) for all stand ages, except in June for the 22-year-old oil palm, as illustrated in Figure 2(a). Meanwhile, significant temporal variations were also recorded between the monthly variations of soil temperature and soil RH in older oil palm stand and younger rubber plantation, as shown in Figures 2(b) and 2(c). Soil temperature and soil RH

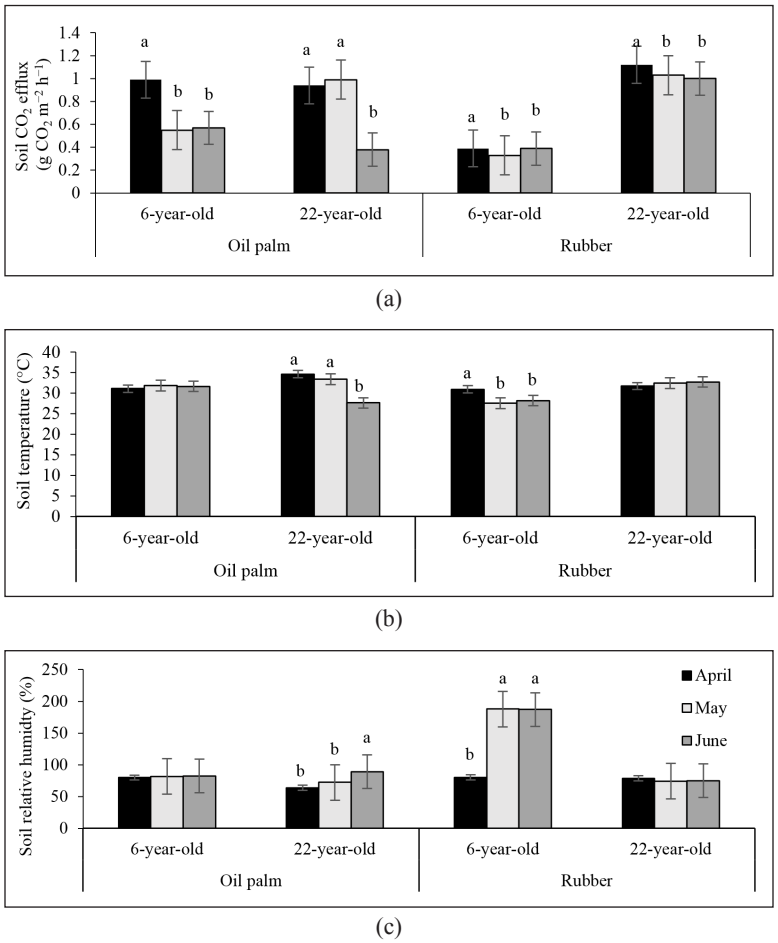

Figure 2. Mean monthly values (±SE) of (a) soil CO_2 efflux in relation to (b) soil temperature, and (c) soil RH, for four study plots; different letters indicate significant differences between months across oil palm and rubber plantation at both 6- and 22-year-old ($P < 0.05$)

had contradictory patterns every month. Meanwhile, air temperature and air RH were not significant throughout the months. Soil temperatures were the highest during April and the lowest during June, ranging from 25-35°C, whereas soil RH produced the opposite results from 50-190%. The overall temporal parameters also did not portray any association and no discernible trends with each other throughout the investigation.

These findings are in contradiction with the previous results reported by Wellock et al. (2016) who found a similar pattern of soil CO_2 efflux changes between 1–2 years old, 4–6 years old, 8–12 years old, and 20–25 years old of *Pinus taeda* (loblolly pine) throughout the year. This rather contradictory result might be due to the differences in soil characteristics and climatic factors on seasonal changes between two different regions leading to soil CO_2 efflux variation. A number of studies on soil CO_2 efflux have documented a distinct temporal dependency governed by abiotic factors such as soil temperature and soil water content (Davidson, Verchot, Cattânio, Ackerman, & Carvalho, 2000; Wang et al., 2016). Hourly observation was also done on the trends in soil CO_2 efflux from 0900 h until 1700 h at all stand ages (2°59′ N, 101°43′ E). Soil CO_2 efflux pattern was similar for all the plots, whereby from 0900 h, the efflux was found to be consistent until it reached the peak at 1300 h and fluctuated until 1400 h, after which the effluxes remained consistent. The 22-year-old rubber plantation had significantly higher rates of soil CO_2 efflux compared to other stands (Figure 3).

The rubber stands also portrayed slightly higher soil CO_2 efflux emission than oil palm stands. Rubber and oil palm possess similar growth requirements such as deep soils, high and stable temperature, as well as constant moisture (Verheye, 2010). The higher soil CO_2 efflux in rubber are most

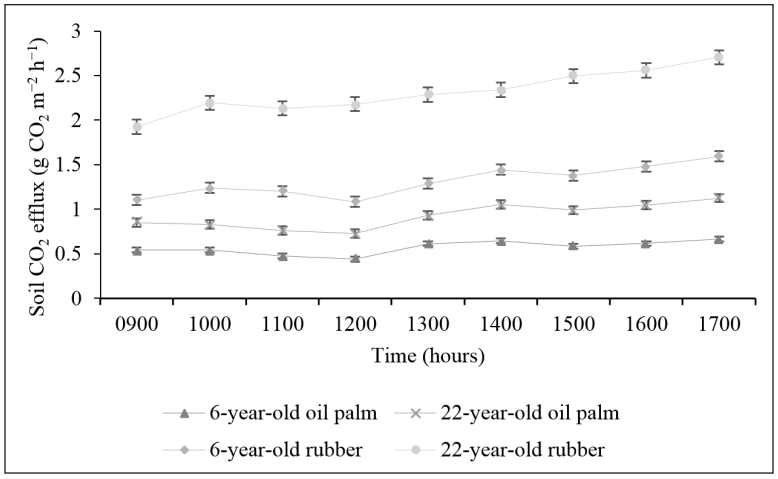

Figure 3. Soil CO_2 efflux trends during the measurement period of study (mean ± SE)

likely caused by the presence of flowers, fruits, and seeds that fall off after certain period of time. Thus, resulting in better quality and quantity of litterfall for vigorous microbial decomposition. The gradual increase of soil CO_2 efflux from morning to afternoon (at 1400 h) was due to the rise in soil temperature when photosynthesis and other plant metabolic activities started to take place. The physiological and metabolic processes reached the optimum state at 1400 h, and then slowed down, indicating the decrease of soil CO_2 efflux. Temporally, soil temperature and soil RH were the main factors controlling the variation in soil CO_2 efflux, suggesting the marginal impact differences in mature oil palm and younger rubber plantations in the present study. Meanwhile, Figure 4 shows a more detailed trend of soil CO_2 efflux every month at each plantation (bi-monthly soil CO_2 efflux measurements). There is an inconsistent variation in the temporal pattern of soil CO_2 efflux reflecting the nature of the field phenomena. Therefore, other factors that could be affecting the temporal trends of soil CO_2 efflux need to be studied, preferably on a longer period.

Relationship between Soil CO_2 Efflux and Soil Temperature and Soil RH

Q_{10} is commonly applied to describe the dependence of soil CO_2 efflux with soil temperature as soil temperature is also accounted as one of the most important drivers of soil CO_2 efflux (Tang et al., 2015). Besides that, the relationship between soil CO_2 efflux and soil temperature is often described exponentially in many different types of forest (Wang et al., 2017). There are several studies investigating Q_{10} values at different soil depths and soil temperatures, with results ranging from 1 to 4 (Davidson & Janssens, 2006; Shi et al., 2014).

Even though Q_{10} indicates an important relationship portraying temperature sensitivity towards soil CO_2 efflux (Zhao et al., 2016), that is not the case in the present study. Soil temperature dependence at all sites had weak positive relationships with soil temperature and this is similar with other published findings (see Yi et al., 2007; Wang et al., 2016) in the tropical and subtropical forests compared to temperate forests (Davidson et al., 2000; Yi et al., 2007). In the present study, oil palm and rubber plantations at both young and mature stands had Q_{10} values in the range of 1 (less sensitive to temperature changes). Meanwhile, the Q_{10} values of soil CO_2 efflux in an oriental arborvitae forest and bare land were 1.97 and 1.43 at a semiarid ecosystem in China (Shi, Yan, Zhang, Guan, & Du, 2014), which were slightly higher than the present study conducted in a tropical ecosystem at 1.05–1.17. Q_{10} having the value of 1 indicates that soil temperature is less sensitive due to water stress that reduces the substrate supply from less organic matter decomposition process (Davidson & Janssens, 2006).

The main reason for the weak relationship was the temporal scale executed in the study. The soil CO_2 efflux measurements (3–5 months) may have confounded the relationship between soil

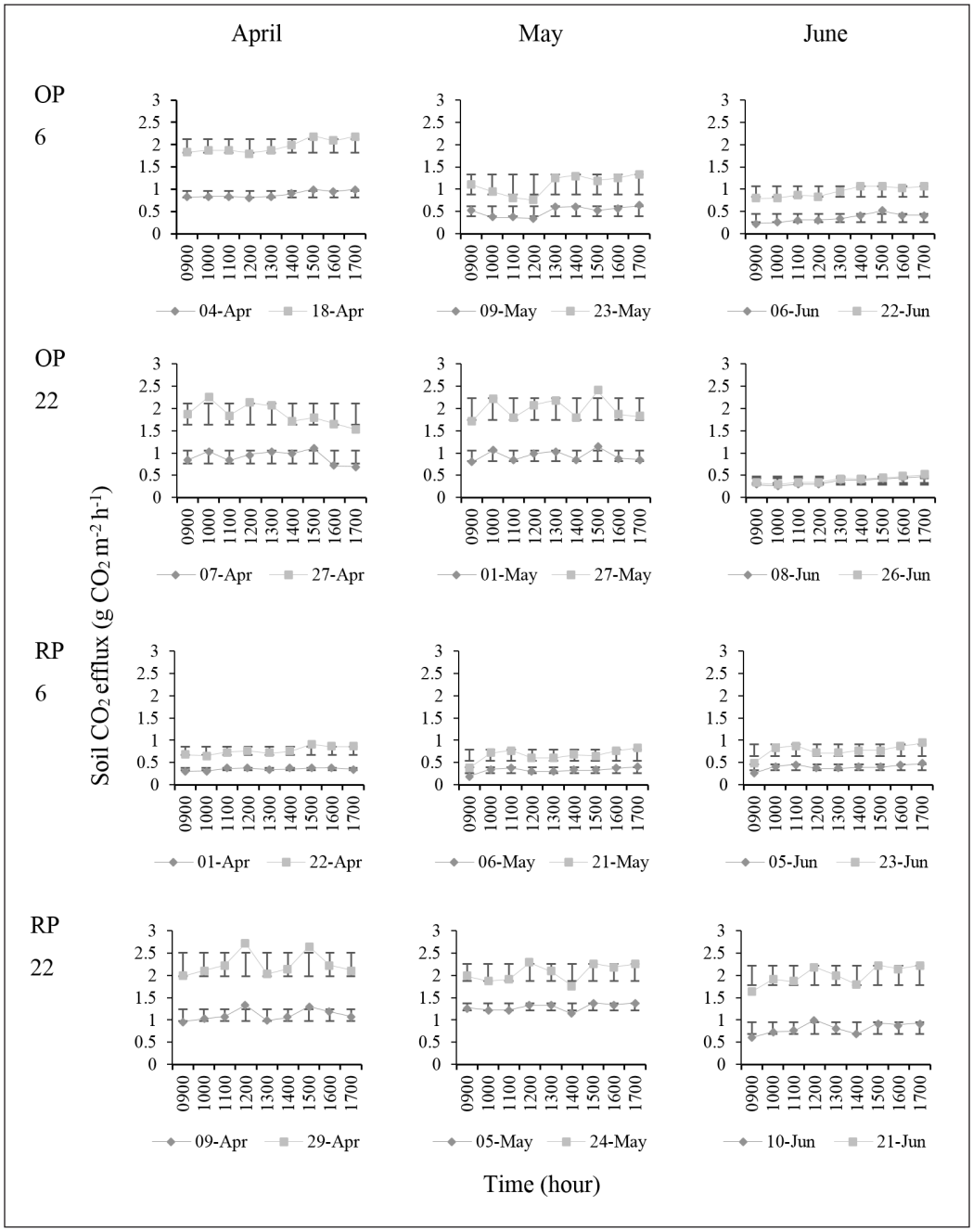

Figure 4. Soil CO_2 efflux trends during the measurement period of study per month at OP 6 (6-year-old oil palm), OP 22 (22-year-old oil palm), RP 6 (6-year-old rubber), and RP 22 (22-year-old rubber) plantation (mean ± SD)

CO_2 efflux and soil temperature such as root growth/mortality, seasonality, and litter inputs, resulting in Q_{10} not suitable for the measurement of temperature sensitivity (Adachi et al., 2006; Yuste, Janssens, Carrara, Meiresonne, & Ceulemans, 2003). Lower Q_{10} was caused by high temperatures that reduced the enzymatic and substrates activities for respiration (Zhao et al., 2016). Studies have shown that in humid and semi-humid regions, effects of soil RH on soil CO_2 efflux are weak due to sufficient water available in the soil pore spaces and microbial activities (Liu et al., 2006). The present findings showed soil temperature played non-significant role that affected soil CO_2 efflux compared with soil RH. Nonetheless, soil RH only significantly affected soil CO_2 efflux at younger stands spatially. The results in present study are similar to that reported by Gong et al. (2014), where soil RH is the primary constraint, and soil temperature plays a secondary role in influencing soil CO_2 efflux.

Nonetheless, the relationship between soil CO_2 efflux and soil RH is varied and complex, and often regulated by site specific. In fact, soil CO_2 efflux varies according to time and space where soil temperature and soil RH are the necessary environmental drivers for the variation (Adachi et al., 2006). The chronosequence studied in the present experiment should be expanded for the next few years, assessing for the repetition of younger sites or older ones for further insight on soil CO_2 efflux from forest establishment and management (Wellock et al., 2014).

CONCLUSION

The present study has shown that different types of land use had significant influence on soil CO_2 efflux. Age factor also played an important role in controlling soil CO_2 efflux, where older stands emit higher CO_2 efflux from soil. The significant differences on soil CO_2 efflux were associated with the changes in land structure leading to the evolution in soil CO_2 efflux variations, especially the morphological and physiological aspects which eventually contributed to the litterfall quantity and quality for decomposition and microbial biomass. Besides that, major environmental influences on soil CO_2 efflux were soil temperature and soil RH, which reacted differently in different forest types and age stand, as well either spatially or temporally. The non-discernible trends of temporal soil CO_2 efflux, soil temperature and RH indicated other significant factors could be the catalysts and thus, further research is required for justification.

However, no harm or degrading effects from soil CO_2 emissions resulted from the establishment of forest plantations recorded in the study. Proper decision making for an establishment of a large-scale plantation area is essential to reduce deteriorating impacts towards maintaining a sustainable ecosystem. The findings obtained from this study provide invaluable information to better understand the effects of human interferences, especially from the establishment of forest plantations on soil CO_2 efflux due to its close association with C cycle and climate change. Nonetheless,

there is still a need for continuous and improved measurement of soil CO_2 efflux for future research.

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Foliar Application of Potassium and Gibberellic Acid to Improve Fruit Storability and Quality of Parthenocarpic Cucumber

Priyanka Pal[1], Kuldeep Yadav[1], Satender Yadav[2] and Narender Singh[1]*

[1]*Department of Botany, Kurukshetra University, Kurukshetra, Haryana, India*
[2]*Centre of Excellence for Vegetables Indo-Israel, Gharaunda (Karnal), Haryana, India*

ABSTRACT

The study was carried out during the 2013–2014, 2014–2015, and 2015–2016 seasons in an insect-proof nethouse at the Centre of Excellence for Vegetables, an Indo-Israel project, at Gharaunda (Karnal), India. The aim was to examine the effect of foliar application of potassium at 1.0 (K_1), 2.5 (K_2), or 5.0 g/L (K_3), and gibberellic acid (GA_3) at 0.005 (G_1), 0.010 (G_2), or 0.015 g/L (G_3), used alone and in combinations on fruit quality and storability of the parthenocarpic cucumbers (*Cucumis sativus* L.) 'KUK 9' and 'Sevenstar' stored at high (27°C) and low (10°C) temperatures. Among individual treatments, foliar application of K_2 alone resulted in least weight loss, electrolyte leakage, and fruit decay percent. The fruit from the treatment combination of $G_2 + K_2$ was best in total soluble solids, with reduced weight loss, electrolyte leakage, and less decay compared to fruit from other treatments or the control. Fruit of 'KUK 9' exhibited better shelf-life than did 'Sevenstar'. Storability of fruit from plants treated with K and GA_3, either alone, or in combination, was found to be better, as it minimized fruit weight loss and decayed fruit, and extended the storage life of parthenocarpic cucumber.

Keywords: Cucumis sativus, F_1 hybrid, fruit quality, fruit storability, total soluble solids

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E-mail addresses:
nsheorankukbot11@gmail.com/nsheorankuk@yahoo.com (Narender Singh)
palpriyanka247@gmail.com (Priyanka Pal)
kuldeep0608@gmail.com (Kuldeep Yadav)
satender.yadav@rediffmail.com (Satender Yadav)
* Corresponding author

INTRODUCTION

Most harvested vegetables are lost during postharvest handling due to fungal decay, chilling injury, and rapid maturation that leads to enhanced senescence process (Chan & Tian, 2006). Up to 50% losses of total harvested vegetables occur during postharvest storage in developing countries, including India, seriously affecting

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availability of vegetables to consumers (Sudheer & Indira, 2007). Storage of vegetables at an appropriate temperature is generally the most effective way to maintain quality. Certain reactive oxygen species are produced during postharvest which lead to senescence and short shelf-life of vegetables. Storage temperature and humidity generally control the amount of water loss. Stored vegetables continue to respire and transpire causing water and weight loss (Thompson, 2003). In horticultural crops, several preharvest aspects including irrigation, growing temperature, pest management, light conditions, maturity, mineral nutrition, and growth substance affect produce quality and storability (Wang, 1997). A physiological and pathological disorder of harvested vegetables tends to occur more frequently on softer senescent tissues (Ladaniya, 1997).

Plant growth regulators (PGRs) play an important role in delaying senescence and promoting postharvest life. Exogenous supplies of growth regulators at different stage of developing vegetables, as well as endogenous level, are reflected in vegetable development and quality. High yields would not be achieved without nutrient use efficiency that affected quality and postharvest storability of vegetables (Srivastava & Handa, 2005).

Foliar application is an economical way of supplementing plant growth substances and fertilizers and reduces the amount of nutrient usage (Jamal, Hamayun, Ahmad, & Chaudhary, 2006). Potassium plays an important role in improving the fruit shelf-life of many horticultural crops (Lester, Jifon, & Stewart, 2007). Potassium foliar feeding promotes firmness, an important indicator of shipping quality; texture; and shelf-life of horticultural crops. The effect of potassium on shelf-life is favorable through slowing of senescence and decrease of numerous physiological diseases (Harker, Redgwell, Hallett, Murray, & Carter, 1997).

Cucumber (*Cucumis sativus* L.) contains water (95%), carbohydrate (3%), protein (1%), total fat (0.5%), dietary fiber (1%), vitamins A, C, K, E, and potassium, manganese, calcium, zinc, and phosphorus (USDA, National Nutrient Data Base, 2014).

The present study was carried out to evaluate the effects of foliar application of concentrations of potassium and gibberellic acid applied alone and in combination on fruit storability of F1 hybrid parthenocarpic cucumber.

MATERIAL AND METHODS

A field experiment was carried out in the growing seasons during three successive seasons (September to December 2013–2014, 2014–2015, and 2015–2016) in an insect-proof nethouse at the Centre of Excellence for Vegetables, an Indo-Israel project, at Gharaunda (Karnal), India, located at 29-32°N latitude and 76-59°E longitude at temperatures of 32–34°C (day) and 17–27°C (night). The F_1 hybrid cucumbers 'KUK 9' and 'Sevenstar' were used and seeds were procured from the Centre of Excellence for Vegetables. The soil in the field plots was sandy loam in

texture (sand, silt, and clay content was 82.20, 6.11, and 11.19%, respectively), slightly alkaline (pH 7.70), low to medium in electrical conductivity (EC = 0.27 M/m), with low levels of organic carbon (0.16%) and medium levels of phosphorus (15.23 kg/ha), potassium (146.50 kg/ha), and sulfur (52.39 ppm), and 12.3% moisture availability.

The experiment was carried out in a randomized complete block design, with three replicates per treatment, with concentration of potassium at 1.0 (K_1), 2.5 (K_2), or 5.0 g/L (K_3), and gibberellic acid at 0.005 (G_1), 0.01 (G_2), or 0.015 g/L (G_3) applied alone or in the combinations of G_1K_1, G_2K_2, and G_3K_3 for a total of nine treatments and one control. Stock solutions were prepared fresh in distilled water at the time of each application and dilutions to required concentrations were made from the stock solutions.

Seeds were sown on raised beds, 6 m × 80 cm × 30 cm (length × width × height), separated at a distance of 45 cm from each other and with 40 cm spacing between plants on the same bed. Nitrogen from urea, and potassium from muriate of potash, at 13:00:45 kg/ha was applied with a drip irrigation system for all treatments twice a week. The first foliar application of potassium, from muriate of potash, was at 20 days after sowing and then twice weekly until maturity. The GA_3 (Gibberellic acid) foliar application was on 21, 30, and 60 days after sowing using a power pump sprayer. Other agriculture practices, that is, irrigation, hoeing, and weeding were carried out throughout the growing season. At fruit maturity, uniform size fruit from each treatment and the control were randomly selected for storage at high (27°C) and low (10°C) temperatures. Fruits were harvested at their commercial maturity stage (45–50 days). Fruits were put in a polythene bags each having 24 holes, 1–2 mm diameter, and stored at the desired temperature. Samples were taken at a 3-day interval for analysis until all fruit were unmarketable.

Weight loss of fruit was determined at the regular intervals according to the Association of Official Analytical Chemists (AOAC, 1994). The total soluble solids (TSS) content of fruit was determined using a hand refractrometer (0-32° Brix). Electrolyte leakage, as percent of total electrical conductivity was determined according to Lutts, Kinet, & Bouharmont (1995). Decay percent of fruit was calculated using the formula of El-Anany, Hassan and Rehab (2009).

The experimental data are presented as the mean and standard error of the mean (SEM) of different parameters studied in the present investigation. Statistical analysis was done using the Statistical Packages for Social Sciences (SPSS) version 8.0.

RESULTS

Weight loss, percent fruits decay, TSS%, and electrolyte leakage increased at 27°C compared to 10°C as time in storage increased. Higher concentrations of gibberellic acid negatively affected postharvest parameters. Fruit of 'KUK 9' had better shelf-life than 'Sevenstar'.

Cultivar KUK 9 better delayed weight loss than fruit of 'Sevenstar' (Figures 1 and 2). During storage, the highest percent weight loss was in the control. Among treatments, the lowest percent weight loss was in 0.01 g/L GA_3 + 2.5 g/L K, followed by 0.005 g/L GA_3 + 1.0 g/L K. The maximum fruit weight loss was for 0.015 g/L GA_3 + 5.0 g/L K at 10°C. Other treatments lost >80% of their weight by the 18th day of storage and were not marketable.

There was a progressive increase in TSS content of cucumber fruits with storage time up to the 12th day and thereafter a decline in TSS% occurred. Decrease in TSS in both cultivars was higher at 27°C (Figures 3 and 4). Fruit of 'KUK 9' maintained higher TSS accumulation at the end of the storage. In control fruit, TSS content declined from 9-18 days of storage; there was a progressive increase in TSS content up to 9 days of storage and then declined. The lowest TSS content occurred from 0.015 g/L GA_3 + 5.0 g/L K on the 9th day of storage with the maximum decrease on the 18th day of storage, followed by 0.005 g/L GA_3 + 1.0 g/L K. The highest TSS content was in 'KUK 9' treated with 0.01 g/L GA_3 + 2.5 g/L K on the 9th day of storage.

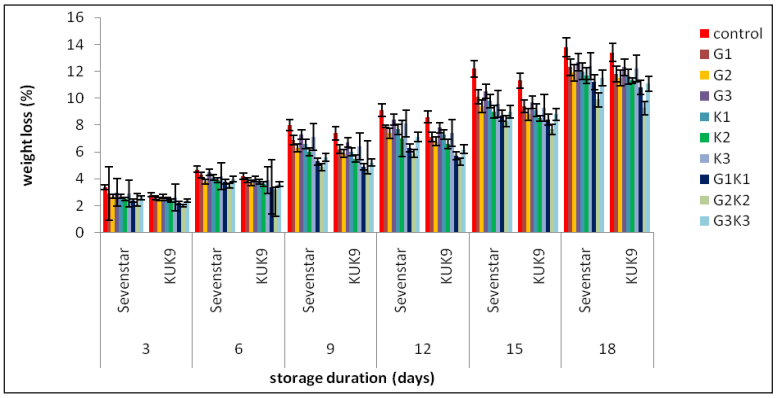

Figure 1. Weight losses (%) during storage of cucumber fruits at 27°C

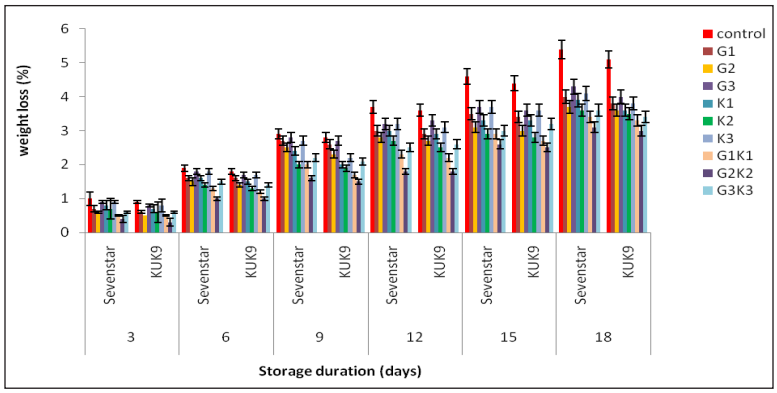

Figure 2. Weight losses (%) during storage of cucumber at 10°C

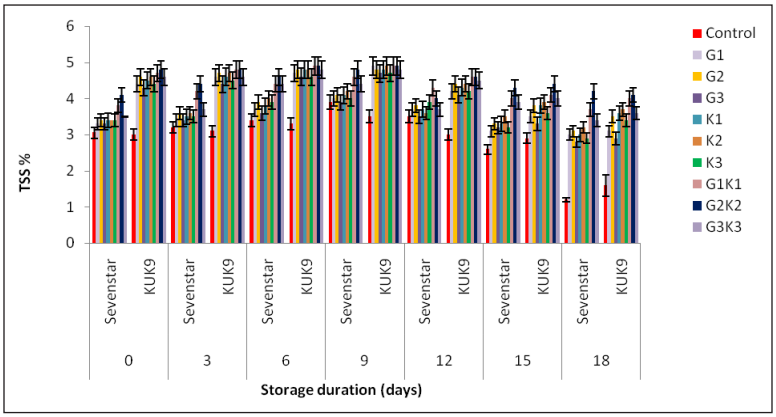

Figure 3. Total soluble solids (%) during storage of cucumber fruits at 27°C

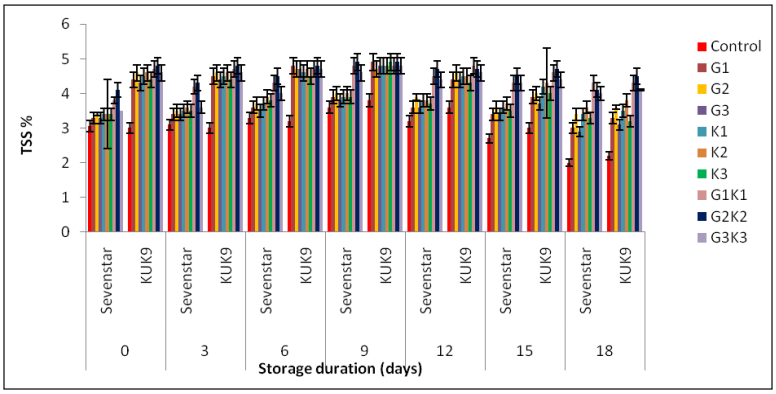

Figure 4. Total soluble solids (%) during storage of cucumber fruits at 10°C

Electrolyte leakage of fruit increased gradually from the 3rd day to the 18th day in storage for both cultivars and all treatments. Storage at 10°C for 18 days caused less decrease in electrolyte leakage of cucumber fruit compared to fruit stored at 27°C (Figures 5 and 6). Fruits of 'KUK 9' maintained the electrolytes better compared to 'Sevenstar'. During storage the highest percent electrolyte leakage was for the control. Individual application of gibberellic acid and potassium influenced electrolyte leakage percent in fruit. The maximum percent electrolyte leakage was found in the 0.015 g/L GA_3 treatment which had higher electrolyte leakage. The maximum percent electrolyte leakage of fruits was found for the 5.0 g/L K treatment at 10°C in 'KUK 9'. Fruit from the combined treatments maintained electrolytes after 18 days of storage compared to the individual treatments including the control. The 0.015 g/L GA_3 + 5.0 g/L K treatment caused increases in electrolyte leakage. Fruit from the 2.5 g/L K + 0.01 g/L GA_3 treatments had lower electrolyte leakage followed by the 0.005 g/L GA_3 + 1.0 g/L K at 10°C for 'KUK 9'. In our study, 2.5 g/L K + 0.01 g/L GA_3 treatment was most effective in maintaining electrolytes.

Percent decay of cucumber fruits increased with length of storage. There was no visible sign of decay in fruit from treatments up to 6 days of storage at 27°C and 9 days of storage at 10°C in both cultivars (Figures 7 and 8). Percent decay was less when fruit were stored at 10°C compared to 27°C. The 'KUK 9' was more effective in maintaining low fruit percent decay. Control fruit had the maximum percent fruit decay over all other treatments. Less percent decay occurred with the 0.01 g/L GA_3 + 2.5 g/L K, followed by the 0.005 g/L GA_3 + 1.0 g/L K treatment. The maximum percent decay was for the 0.015 g/L GA_3 + 5.0 g/L K treatment. Control fruit started spoiling after nine days of storage and almost 90.3% decay occurred by the 15th day of storage; the least percent decay (14.23%) was due to the 0.01 g/L GA_3 + 2.5 g/L K treatment which was more effective in reducing percent decay compared to other treatments.

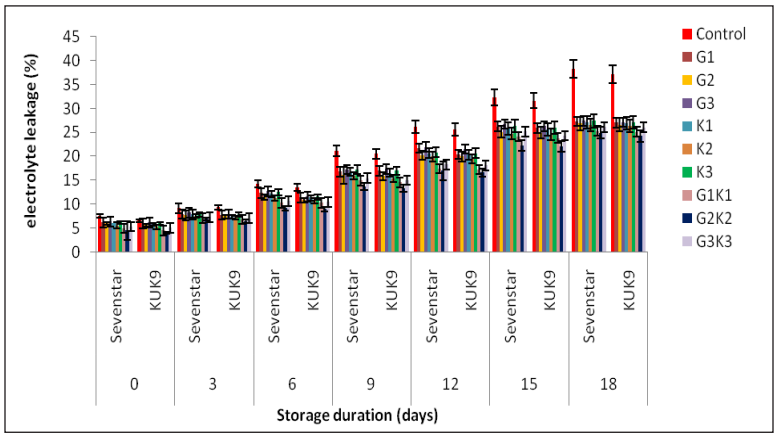

Figure 5. Electrolyte leakage (%) during storage of cucumber fruits at 27°C

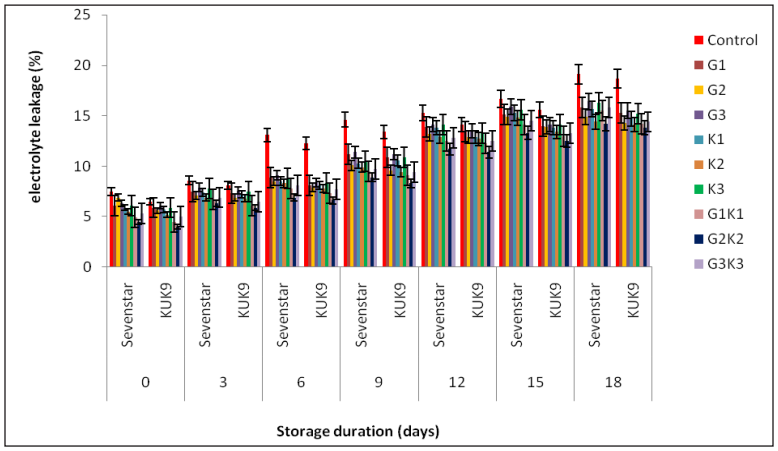

Figure 6. Electrolyte leakage (%) during storage of cucumber fruits at 10°C

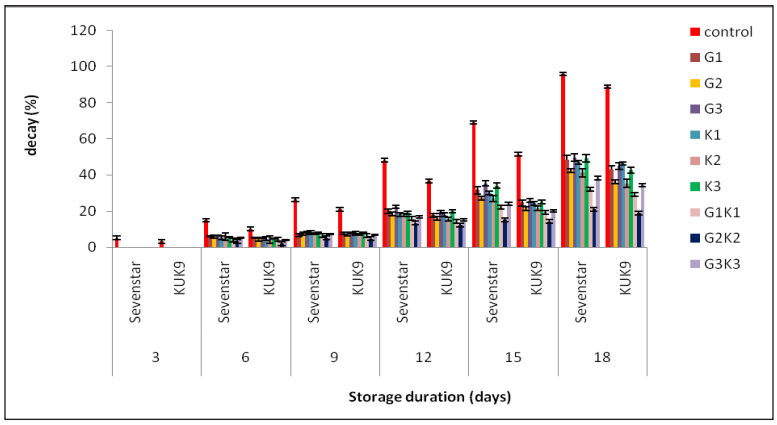

Figure 7. Decay (%) during storage of cucumber fruits at 27°C

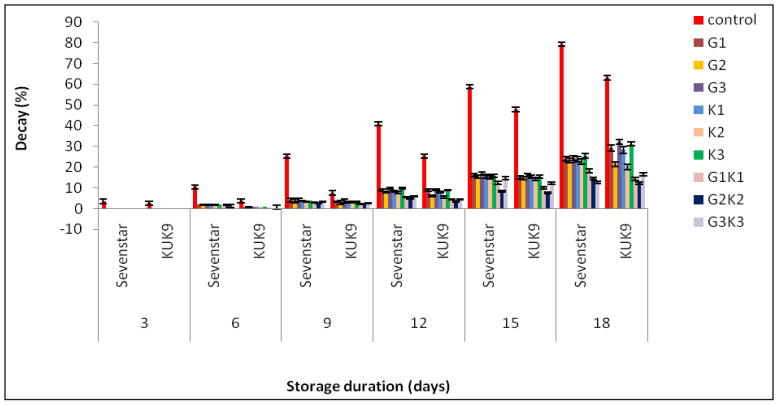

Figure 8. Decay (%) during storage of cucumber fruits at 10°C

DISCUSSION

Kim, Luo and Gross (2004) also observed an increase in weight loss of fruits with storage time in white and violet salad savoy plant. Low storage temperature is the main postharvest way to improve storage life of perishable products due to lowered ethylene production, fruit respiration, and metabolism. Homin and Kuenwoo (1999) reported that packaging produce in perforated polyethylene and storage at 10°C prolonged storage life of fruit and retains fresh weight and firmness.

Weight loss of vegetables is mainly depending on the control of internal gas composition (Park, 2000). After harvesting, a continuous water loss due to respiration leads to shrinkage and weight loss (Mahajan, Oliveira, & Macedo, 2008). In fresh vegetables weight loss might be ascribed to cellular breakdown, deterioration of membrane integrity and respiration, and carbohydrate degradation to yield carbon dioxide and water (Aquero, Ponce, Moreira, & Raura, 2011). Treatment with gibberellic acid resulted in decreased weight loss

because of antisenescent action of GA_3. In a general way, it was evident that cucumber fruit need a high amount of water and carbon; water availability is very much affected by endogenous level of gibberellins (Sudha et al., 2007). The exogenous application of PGRs at flowering and fruit setting stage tends to increase fruit water content and their positive effect on fruit quality is evaluated immediately after harvest (Khalid, Malik, Khan, & Jamil, 2012). Physiological weight loss can be reduced through a decrease in tissue permeability by gibberellic acid treatment in *Solanum lycopersicum* L. (Choudhary & Dhruve, 2014). Potassium accumulates in cell vacuoles, with sugars, where it contributes to osmotic pressure, turgor potential, and water uptake in plants (Waraich, Ahmad, Saifullah, & Ehsanullah, 2011). Potassium application stimulates total solids, increases firmness, and reduces Ca availability and lowers physiological weight loss during storage (Voogt & Sonneveld, 1997).

The decay of fruit increased as storage lengthened. Gibberellic acid results in lowered decay rate in tomato fruit (Pila, Gol, & Rao, 2010). Fruit decay due to fungi was the major contributor to loss of fruit quality. Decay incidence greatly increased in fully ripe fruit during storage (Nunes & Morais, 2002). Preharvest gibberellic acid treatment reduced postharvest decay of cucumber and extended shelf-life. There have been relatively few reports on the effect of preharvest application of gibberellic acid. Decrease in peel senescence and increase in peel puncture resistance could reduce decay of fruit, prolong the storage life, and decrease the unmarketable fruit (Siddiqui & Dhua, 2010). Gibberellic acid enhances ultrastructural morphogenesis of plastids which stimulates retention of chlorophyll and delays senescence (Arteca, 1997; Ben-Arie, Mignani, Greve, Huysamer, & Labavitch, 1995). Application of potassium at an optimum level resulted in improved fruit quality, higher doses cause an imbalance of the sugar/acid ratio making more fruit more susceptible to fungal decay (Javaria, Qasim, Rahman, & Bakhsh, 2012).

Bahnasawy and Khater (2014) reported an increased TSS with increased storage temperature. As storage time increases accumulation of TSS increased and then decreased during storage was also noticed in mandarin (Bhardwaj, Sen, & Mukherjee, 2005), guava (Mahajan, 2004), strawberry (Singh, Sharma, & Tyagi, 2007), sapota fruit (Pawar, Patil, & Joshi, 2011). The interaction of gibberellic acid and potassium was best in keeping the level of TSS at an optimum level. Gibberellic acid application maintained the higher TSS% level in papaya fruit during storage (Rajkumar, Karuppaiah, & Kandasamy, 2005). Abd El-Razek, Abd-Allah and Saleh (2013) found that TSS level was influenced by potassium concentration. A possible reason for TSS maintenance under storage may be because of slowed respiration that lowers changes of insoluble sugar into soluble sugar and least utilization of organic acid in respiration (Choudhary & Dhruve, 2014; Pila et al., 2010). The TSS increases as maturity progresses during postharvest storage and is reduced

due to utilization of sugar in respiration (Miaruddin, Chowdhury, Rahman, Khan, & Mozahid-E-Rahman, 2011; Salamat, Ghassemzadeh, Heris, & Hajilou, 2013). Kittur, Saroja and Tharanathan (2001) reported that percentage of TSS is correlated with hydrolytic changes in starch and conversion to sugar further reduces TSS during storage. During storage, TSS% level was affected by gibberellic acid because it reduces the ethylene level that stimulates starch synthesis (Abu-El-Ez, Behairy, & Ahmed, 2002). Preharvest foliar application of gibberellic acid caused increased fruit soluble solids in sweet cherry as recorded by Clayton, Biasi, Agar, Southwick and Mitcham (2006). Increased potassium concentration resulted in better firmness and increased TSS (Cakmak, 2005).

Increased electrolyte leakage occurred with increased storage temperature (Sharom, Willemot, & Thompson, 1994). Ion leakage is effective to determine the relative health of cell plasma membranes because it is expressed in rate of change in membrane permeability (Knowles, Trimble, & Knowles, 2000). Electrolyte leakage was indicative of quality loss in fruit. Gibberellic acid and potassium combination treatments were more effective in reducing electrolyte leakage. Potassium nitrate reduces membrane permeability in pepper (Kaya & Higgs, 2003). The effects of potassium on electrolyte leakage agree with Williumsen, Petersen and Kaack (1996) who reported a role for potassium in influencing calcium availability and permeability of cell membranes. Gibberellic acid was effective in reducing electrolyte leakage and K^+ efflux and reducing tissue permeability because of the preservative effects on the solute efflux capacity from intact tissue and survival of protoplasts (Choudhary & Dhruve, 2014; Pila et al., 2010).

CONCLUSION

Foliar spray of gibberlic acid and potassium either alone or in combination at appropriate concentration proved to be beneficial in controlling the fruit weight loss and maintaining TSS during storage. However, the excessive concentration of GA_3 and K imparts negative effect on shelf-life. Fruit of 'KUK 9' had better shelf-life than 'Sevenstar'.

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Annotated Checklist of Orchids Found in Merapoh Trail (Gunung Tahan, Malaysia)

Siti Fatimah Md. Isa[1], Jamilah Mohd. Salim@Halim[2], Christina Seok Yien Yong[1], Janna Ong Abdullah[3] and Rusea Go[1]*

[1]Department of Biology, Faculty Science, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[2]School of Marine and Environmental Sciences, Universiti Malaysia Terengganu (UMT), 21300 Kuala Nerus, Kuala Terengganu, Terengganu, Malaysia
[3]Department of Cell and Molecular Biology, Faculty of Biotechnology and Molecular Sciences, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia

ABSTRACT

Gunung Tahan is the highest mountain peak in Peninsular Malaysia (2187 m a.s.l.) and is a part of Taman Negara Pahang that houses many rare and endemic species of flora and fauna. This account is conducted to evaluate, compile, and update the orchids collections from Gunung Tahan after almost 126 years, since the first botanical exploration was conducted by H.N. Ridley and other foreign botanist. The compilation presented in this study resulted in 294 species obtained from the selected herbarium, published record, and current expedition. A total of 57 species were recorded for recent work, out of which 18 species were new discoveries for Merapoh Trail and thus reported as new records for Gunung Tahan. IUCN Red List of Threatened Species version 2017.3 listed two species; *Claderia viridiflora* Hook.f. and *Spiranthes sinensis* (Pers.) Ames as least concern species and *Paphiopedilum bullenianum* (Rchb.f.) Pfitzer as endangered species. The list of the compiled species from herbarium and published records for Gunung Tahan is also included.

Keywords: Botanical collections, herbarium, H. C. Robinson, H. N. Ridley, Orchidaceae, Pahang

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E-mail addresses:
sitifatimahmdisa@gmail.com (Siti Fatimah Md. Isa)
jamilah@umt.edu.my (Jamilah Mohd. Salim@Halim)
chrisyong@upm.edu.my (Christina Seok Yien Yong)
janna@upm.edu.my (Janna Ong Abdullah)
rusea@upm.edu.my (Rusea Go)
* Corresponding author

INTRODUCTION

Taman Negara Pahang covers an area of about 4343 km^2 in three protected areas of three states of Pahang, Kelantan, and Terengganu. It is estimated to be 130-million-year- old. Gunung Tahan is one of the mountainous peak zones located in Taman Negara Pahang which covers about

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57% of the total area (Pakhriazad, Hasmadi, Aida & Jusoff, 2009).

The mountain was named after a Malay word "Tahan" meaning though, to reflect the toughness associated with the trekking around it. At present it can be accessed through two main trails of Kuala Tahan (old and traditional trail) and Merapoh Trail. The later trail was developed in 1993 as an alternative trail. Other less popular trail through which the mountainous region can be accessed is Kuala Koh Trail, which is located in the state of Kelantan but rarely used by the climbers due to its remote location, long, and exhausting journey which takes at least 16 days to reach the summit.

Generally, the forest formation up to the summit of Gunung Tahan consists of lowland dipterocap forest (<350 m a.s.l.) followed by hill dipterocarp forest (>350 m a.s.l.); upper hill dipterocarp forest (>750 m a.s.l.); oak-laurel forest (>1,200 m a.s.l.); and montane ericaceous forest (>1,500 m a.s.l.). The plants and flora communities gradually changes as one reach each of the forest elevations. The most significant changes were observed along the ridges approaching Kem Bonsai at the elevations of 1705 m a.s.l. where most of the plants were dwarf (about 1 m tall) and growing on and among the white sandstone and quartz-fragments rocks. The most abundant plant in the area is *Leptospermum javanicum* Blume which is mixed with *Baeckea frutescens* L. and *Rhododendron elegans* Ridl. (Ridley 1892, 1915). The plants in this area are typically adapted to xerophytic life. Along the trail from Kem Bonsai to Bukit Botak towards the Tahan summit, there are few damp areas with many hygrophytic plants and low herbaceous shrubs (Robinson, 1908).

One of the noteworthy plants on Gunung Tahan is *Johannesteijsmannia altifrons* (Rchb.f. & Zoll.) H.E. Moore with a huge oar-shaped leaves, and a leaf blade that can extend to over 6 ft long, found growing at the elevation up to about 1000 m. Another notable and rarely seen species only found on the two highest mountains in the Malay Peninsular; Gunung Tahan, and Gunung Korbu is *Utricularia vitellina* Ridl. (Chew & Haron, 2011). It is a small, usually <10 cm tall, terrestrial carnivorous plant. This species grows in a nutrient poor environment with low pH along with the highlands plateau just below the Tahan summit.

The previous expeditions to Gunung Tahan were usually financed by the Trustees of the British Museum in England assisted by the participation of local communities, especially guides, coolies, and local headman. The first expedition to Gunung Tahan was led by H. N. Ridley in 1892, however, the first group that successfully found the trails to reach the Gunung Tahan peak was led by H. C. Robinson in 1908 which resulted in 250 botanical collections that were significantly important in enhancing the knowledge of the flora in Malay Peninsular (Ridley, 1908). However, after almost 126 years from Ridley and Robinson's explorations, there is no inclusive published report on botanical

collections, particularly on orchids even though numerous botanical expeditions were conducted on Gunung Tahan. Additionally, due to the grant agreement made between the Trustees of the British Museum and Robinson, the whole collections made during the expeditions had been deposited at the Natural History Museum; and the first set had become the property of the Trustees. Therefore, most of the collections can only be accessed by visiting the herbaria or through online herbarium catalog.

This study was conducted to evaluate the species richness of Orchidaceae in Gunung Tahan, focusing on Merapoh Trail to the summit of Gunung Tahan. Most of the specimens collected from Gunung Tahan in the eighteenth century were mostly gathered from Kuala Tahan Trail. Therefore, collections from Kuala Tahan Trail compiled by Ridley, I. M. Turner and herbarium catalog were also provided for comparison. List of orchids from both trails were compiled and evaluated to determine the richness of orchids species in the whole area of Gunung Tahan. Thus, compilation of both data from current and previous studies will represent the most updated checklist for the orchids of Gunung Tahan from the two trials. However, the checklist should be used or quoted with caution. It is expected that more information could be discovered on the orchids if a more comprehensive work could be done in this area apart from the two main trails of Kuala Tahan Trail and Merapoh Trail.

MATERIALS AND METHODS

Orchids documentation was conducted through expedition from September 1–7, 2013 using Merapoh Trail to reach the peak of Gunung Tahan. The expedition covered a floristic survey through four main rivers and tributaries of Kuala Luis, Lata Luis, Kem Kor, Kem Permatang, Kem Kubang, Kem Belumut, Kem Bonsai, and Kem Botak before reaching the summit of the mountain. Annotations were made starting from Kuala Juram all the way to the summit. Photographic images were taken for all the species, respectively; with or without flowers and their details reported in the field notebook.

Collections from the above expedition were then combined and compared with collections published by Ridley in 1908 and 1915 as well as from the herbarium collections in selected herbaria. List of herbarium collections from the Forest Research Institute Malaysia (FRIM) was made possible by visiting the herbarium. While, list of herbarium collections from the Singapore Botanical Garden (SING) and the Royal Botanic Garden (KEW) were accessed online through their herbarium catalog. List of species recorded by Turner (1995) was also included in the compilations.

All of the identified species and compilations from the herbarium specimens were then validated with the World Checklist of Selected Plant Families (WCSP) to check their current accepted names and general distribution.

RESULTS

The expedition along the Merapoh Trail up to the summit has contributed a total of 57 species of orchids, which comprise 35 genera of four subfamilies: Apostasioideae, Cypripedioideae, Epidendroideae, and Orchidoideae (Table 1). No species from subfamily Vanilloideae was collected from this expedition. The highest numbers of species collected were from subfamily Epidendroideae with 48 species, followed by subfamily Orchidoideae with seven species; Apostasioideae and Cypripedioideae with one species for each subfamily. Species collected with flowers were shown in Figures 1 to 3.

Table 1 indicated that *Paphiopedilum bullenianum* was listed as endangered species by the IUCN Red List of Threatened Species version 2017.3 and can only be found in Gunung Tahan. While, *Claderia viridiflora* and *Spiranthes sinensis* were listed globally as least concern species. Other 55 species were not yet evaluated by IUCN Red List of Threatened Species version 2017.3.

In total, Gunung Tahan harbors 294 species of orchids which were compiled and updated through this expedition as well as herbarium and published records. Species that were published or preserved in the selected herbaria were listed in Table 2, with most of the specimens collected from Kuala Tahan Trail prior to the establishment and opening of the Merapoh Trail. The 18 species that were collected from Merapoh Trail were documented as new record for Gunung Tahan, since they were never recorded before in Kuala Tahan Trail (Table 1). Therefore, the current checklist could be considered as a new and updated orchids collection for Gunung Tahan.

Table 1
Collection of orchids species along Merapoh Trail to the summit of Gunung Tahan and conservation status based on The International List of Threatened Species (IUCN)

2013 Collections (Merapoh Trail)			
Taxon		Collector	Status IUCN
Subfamily Apostasioideae Genera *Apostasia*	*Apostasia nuda* R. Br.	SFMI et al. 25 [UPM]	NE
Subfamily Cypripedioideae			
Genera *Paphiopedilum*	*Paphiopedilum bullenianum* (Rchb.f.) Pfitzer.	SFMI et al. 11 [UPM]	E
Subfamily Epidendroideae			
Genera *Agrostophyllum*	*Agrostophyllum majus* Hook.f.	SFMI et al. 84 [UPM]	NE
Appendicula	**Appendicula cornuta* Blume	SFMI et al. 91 [UPM]	NE
Aphyllorchis	**Aphyllorchis montana* Rchb.	SFMI et al. 57 [UPM]	NE
Arundina	*Arundina graminifolia* (D.Don) Hochr.	SFMI et al. 82 [UPM]	NE

Table 1 *(continue)*

2013 Collections (Merapoh Trail)			
Taxon		Collector	Status IUCN
Ascidieria	*Ascidieria longifolia* (Hook.f.) Seidenf.	SFMI et al. 89 [UPM]	NE
Bromheadia	*Bromheadia rupestris* Ridl.	SFMI et al. 43 [UPM]	NE
	**Bromheadia aporoides* Rchb.f.	SFMI et al. 92 [UPM]	NE
Bryobium	*Bryobium hyacinthoides* (Blume) Y.P.Ng & P.J.Cribb	SFMI et al. 80 [UPM]	NE
Bulbophyllum	*Bulbophyllum coniferum* Ridl.	SFMI et al. 54 [UPM]	NE
	Bulbophyllum limbatum Lindl.	SFMI et al. 27 [UPM]	NE
	Bulbophyllum microglossum Ridl.	SFMI et al. 45 [UPM]	NE
	**Bulbophyllum pileatum* Lindl.	SFMI et al. 26 [UPM]	NE
	Bulbophyllum tahanense Carr.	SFMI et al. 49 [UPM]	NE
	Bulbophyllum uniflorum (Blume) Hassk.	SFMI et al. 07 [UPM]	NE
	Bulbophyllum viridescens Ridl.	SFMI et al. 42 [UPM]	NE
Calanthe	**Calanthe pulchra* (Blume) Lindl.	SFMI et al. 09 [UPM]	NE
Campanulorchis	*Campanulorchis pellipes* (Rchb.f. ex Hook.f.) Y.P.Ng & P.J.Cribb	SFMI et al. 44 [UPM]	NE
Chelonistele	**Chelonistele sulphurea* (Blume) Pfitzer	SFMI et al. 93 [UPM]	NE
Claderia	*Claderia viridiflora* Hook.f.	SFMI et al. 18 [UPM]	LC
Coelogyne	*Coelogyne prasina* Ridl.	SFMI et al. 24 [UPM]	NE
	**Coelogyne radicosa* Ridl.	SFMI et al. 40 [UPM]	NE
Cymbidium	*Cymbidium roseum* J.J.Sm.	SFMI et al. 32 [UPM]	NE
	Cymbidium finlaysonianum Lindl.	SFMI et al. 76 [UPM]	NE
Dendrobium	*Dendrobium angustifolium* (Blume) Lindl.	SFMI et al. 41 [UPM[	NE
	**Dendrobium foxii* Ridl.	SFMI et al. 78 [UPM]	NE
	Dendrobium hughii Rchb.f.	SFMI et al. 90 [UPM]	NE
	Dendrobium longipes Hook.f.	SFMI et al. 17 [UPM]	NE
	Dendrobium uniflorum Griff.	SFMI et al. 51 [UPM]	NE
Dendrochilum	*Dendrochilum gracile* (Hook.f.) J.J.Sm.	SFMI et al. 19 [UPM]	NE
Dilochia	*Dilochia cantleyi* (Hook.f.) Ridl.	SFMI et al. 38 [UPM]	NE
	Dilochia wallichii Lindl.	SFMI et al. 05 [UPM]	NE
Eria	**Eria neglecta* Lindl.	SFMI et al. 30 [UPM]	NE
	Eria robusta (Blume) Lindl.	SFMI et al. 79 [UPM]	NE

Table 1 *(continue)*

2013 Collections (Merapoh Trail)			
Taxon		Collector	Status IUCN
Oxystophyllum	*Oxystophyllum carnosum* Blume	SFMI et al. 28 [UPM]	NE
	**Oxystophyllum excavatum* Blume	SFMI et al. 29 [UPM]	NE
	Oxystophyllum sinuatum (Lindl.) M.A.Clem.	SFMI et al. 94 [UPM]	NE
Pholidota	*Pholidota carnea* var. *carnea*	SFMI et al. 21 [UPM]	NE
	Pholidota carnea var. *pumila* (Ridl.) de Vogel	SFMI et al. 53 [UPM]	NE
Phreatia	*Phreatia crassifolia* Ridl.	SFMI et al. 20 [UPM]	NE
Podochilus	**Podochilus microphyllus* Lindl.	SFMI et al. 10 [UPM]	NE
Spathoglottis	*Spathoglottis aurea* Lindl.	SFMI et al. 04 [UPM]	NE
	**Spathoglottis plicata* Blume	SFMI et al. 81 [UPM]	NE
Tainia	*Tainia speciosa* Blume	SFMI et al. 36 [UPM]	NE
Thecostele	**Thecostele alata* (Roxb.) Par. & Reichb.f.	SFMI et al. 95 [UPM]	NE
Thrixspermum	**Thrixspermum pauciflorum* (Hook.f.) Kuntze	SFMI et al. 52 [UPM]	NE
	Thrixspermum tahanense Carr.	SFMI et al. 22 [UPM]	NE
Trichotosia	*Trichotosia ferox* Blume	SFMI et al. 03 [UPM]	NE
	**Trichotosia gracilis* (Hook.f.) Kraenzl.	SFMI et al. 23 [UPM]	NE
Subfamily Orchidoideae			
Genera *Corybas*	*Corybas holttumii* J.Dransf. & G.Sm.	SFMI et al. 16 [UPM]	NE
	**Corybas villosus* J.Dransf. & G.Sm.	SFMI et al. 15 [UPM]	NE
Cryptostylis	*Cryptostylis arachnites* (Blume) Hassk.	SFMI et al. 33 [UPM]	NE
Cystorchis	*Cystorchis variegata* Blume	SFMI et al. 08 [UPM]	NE
Pelatantheria	*Pelatantheria angustata* (Ridl.) Ridl.	SFMI et al. 34 [UPM]	NE
Spiranthes	**Spiranthes sinensis* (Pers.) Ames	SFMI et al. 83 [UPM]	LC
Pinalia	**Pinalia densa* (Ridl.) W.Suarez & Cootes	SFMI et al. 37 [UPM]	NE

Note: E = endangered, LC = least concern, NE = not evaluated. Collector: SFMI et al. = Siti Fatimah Md. Isa, Rusea Go, Suhaima Md. Suki, Wong Wee Nee, Nur'izzati Shaipuddin, Nur Adilah Auyob, Yoh Kok Hon, Azizul Aziz, Norazlan, and Helmi Rozario.
* = new record for Gunung Tahan

Table 2
List of published or preserved orchids species collected from Gunung Tahan

Genera	Published/Preserved at				
	FRIM	SING	KEW	Ridley (1908, 1915)	Turner (1995)
Acriopsis		*A. indica*		*A. lilifolia* var. *lilifolia*	
Agrostophyllum	*A. stipulatum*	*A. elongatum* *A. glumaceum* *A. stipulatum* subsp. *bicuspidatum*	*A. elongatum* *A. glumaceum* *A. majus*		
Aphyllorchis		*A. pallida* *A. strata*			
Apostasia		*A. nuda* *A. wallichii*	*A. nuda*	*A. nuda*	
Apostasia		*A. cornuta* *A. densifolia* *A. pendula* *A. reflexa* *A. rupestris* *A. undulata*	*A. rupestris*	*A. cornuta* *A. rupestris*	
Arundina	*A. graminifolia*	*A. graminifolia*		*A. graminifolia*	
Ascidieria				*A. longifolia*	
Ascochilopsis		*A. myosurus*	*A. myosurus*		
Biermannia		*B. laciniata*			
Bromheadia		*B. alticola* *B. pungens* *B. scirpoidea*	*B. pungens*	*B. alticola* *B. pungens* *B. rupestris*	*B. pungens* *B. rupestris*
Bryobium		*B. hyacinthoides*			
Bulbophyllum	*B. gusforfii* *B. apiferum*	*B. abbrevilabium* *B. apodum* *B. biseriale* *B. brevipes* *B. clandestinum* *B. corolliferum* *B.dischidiifolium* subsp. *aberrans* *B. dryas* *B. flavenscens* *B. lilacinum* *B. lumbriciforme* *B. mahakamense* *B. medusae* *B. microglossum* *B. nematocaulon* *B. obtusum* *B. odoratum* *B. ovalifolium* *B. papilosofilum* *B. praetervisum* *B. purpurascens* *B. rugosum* *B. serratotruncatum* *B. skeatianum* *B. sulcatum* *B. tahanense* *B. titanea*	*B. apodum* *B. coniferum* *B. dryas* *B. flabellum-veneris* *B. nematocaulon* *B. octorhopalon* *B. odoratum* *B. pan* *B. purpurascens* *B. skeatianum* *B. stormii* *B. tahanense* *B. tortuosum*	*B. capitatum* *B.coniferum* *B. dryas* *B. microglossum* *B. pan* *B. skeatianum* *B.uniflorum* *B.viridescens*	*B.biseriale* *B.foetidoles* *B. holttumii* *B. ignevenosum* *B. lumbriciforme* *B. pan* *B. papillosofilum* *B.retusiusculum* *B. tahanense* *B. tekuense* *B. titanea* *B.viridescens*

Table 2 *(continue)*

Genera	Published/Preserved at				
	FRIM	SING	KEW	Ridley (1908, 1915)	Turner (1995)
		B. tortuosum *B. uniflorum* *B. virescens* *B. viridescens* *B. sulcatum*			
Calanthe	*C. angustifolia* *C. aurantiaca*	*C. angustifolia* *C. aurantiaca* *C. carrii* *C. ceciliae* *C. monophylla* *C. rigida*	*C. aurantiaca* *C. carrii* *C. ceciliae* *C. ventilabrum*	*C. ceciliae* *C. triplicate*	*C. carrii*
Callostylis		*C. puchella*		*C. pulchella*	
Campanulorchis		*C. pellipes*		*C. pellipes*	
Ceratostylis	*C. subulata*	*C. ampullacea* *C. eria* *C. lancifolia* *C. pendula* *C. subulata*	*C. eria*	*C. gracilis*	
Chroniochilus		*C. minimus*	*C. minimus*		
Claderia	*C. viridiflora*				
Cleisostoma		*C. halophilum* *C. scortechinii* *C. suffusum*	*C. scortechinii* *C. teretifolium*		
Coelogyne	*C. anceps* *C. prasina* *C. radicosa* *C. stenochila*	*C. cuminggii* *C. foerstermannii* *C. prasina* *C. radicosa* *C. rochussenii* *C. septemcostata* *C. stenochila* *C. testaceae* *C. tomentosa* *C. xyrekes*	*C. xyrekes*	*C. cumingii* *C. radicosa* *C. stenochila* *C. tomentosa* *C. xyrekes*	*C. xanthoglossa*
Cordiglottis			*C. pulverulenta*		*C. pulverulenta*
Corybas		*C. holttumii*	*C. pictus*		*C. caudatus* *C. holttumii*
Corymborkis		*C. veratrifolia*	*C. veratrifolia*		
Crepidium		*C. micranthum*	*C. micranthum*		
Cryptostylis	*C. arachnites*	*C. arachnites* *C. conspicua*		*C. arachnites*	
Cymbidium		*C. atropurpureum* *C. bicolor* subsp. *bicolor* *C. chloranthum* *C. roseum*	*C. chloranthum* *C. chloranthum* subsp. *chloranthum* *C. roseum*		*C. chloranthum*
Cystorchis		*C. aphylla* *C. variegata*	*C. aphylla*		
Dendrobium	*D. cornutum* *D. geminatum* *D. hasseltii* *D. hughii* *D. longipes*	*D. aloifolium* *D. angustifolium* *D. bancanum* *D. cornatum* *D. crocatum*	*D. acerosum* *D. aloifolium* *D. crumenatum* *D. farmeri* *D. fugax*	*D. angustifolium* *D. geminatum* *D. hasseltii* *D. hymenopterum* *D. kingie*	*D.acinaciforme* *D.luxurians* *D.pahangense* *D.rupicolum* *D.striatellum*

Table 2 *(continue)*

Genera	Published/Preserved at				
	FRIM	SING	KEW	Ridley (1908, 1915)	Turner (1995)
		D. farmeri *D. geminatum* *D. grande* *D. hendersonii* *D. hosei* *D. hughii* *D. indivisum* *D. indragiriense* *D. leonis* *D. linguella* *D. longipes* *D. luxurians* *D. macropodum* *D. pachyphyllum* *D. pensile* *D. rhodostele* *D. rupicola* *D. salaccense* *D. subflavidum* *D. uniflorum* *D. zebrinum*	*D. geminatum* *D. hosei* *D. hughii* *D. indragiriense* *D. longipes* *D. luxurians* *D. macropodum* *D. quadrilobatum* *D. rupicola* *D. zebrinum*	*D. longipes* *D. macropodum* *D. rupicola* *D. subflavidum* *D. uniflorum*	
Dendrochilum	*D. angustifolium* *D. carnosum* *D. linearifolium* *D. pallidiflavens* var. *pallidiflavens*	*D. angustifolium* *D. carnosum* *D. gracile* *D. kingii* *D. linearifolium* *D. longifolium* *D. pallidiflavens* var. *pallidiflavens*	*D. linearifolium* *D. longifolium*	*D. gracile* *D. linearifolium*	
Didymoplexiella		*D. ornata*			
Dilochia	*D. cantleyi*	*D. cantleyi* *D. wallichii*	*D. cantleyi*	*D. cantleyi*	
Dilochiopsis	*D. scortechinii*	*D. scortechinii*	*D. scortechinii*	*D. scortechinii*	
Dipodium		*D. pictum*			
Eria	*E. crassipes* *E. robusta*	*E. crassipes* *E. earine* *E. javanica* *E. mucronata* *E. nutans* *E. robusta*	*E. crassipes* *E. javanica*	*E. crassipes* *E. earine* *E. nutans* *E. robusta*	*E. earine*
Eulophia		*E. spectabilis*	*E. spectabilis*		
Gastrochilus	*G. calceolaris*	*G. calceolaris* *G. patinatus*		*G. obliquus* var. *obliquus*	
Geodorum		*G. densiflorum*			
Goodyera					*G. colorata*
Grammatophyllum		*G. speciosum*	*G. speciosum*		
Grosourdya		*G. appendiculata* *G. incurvicalcar* *G. minutiflora*	*G. appendiculata* *G. incurvicalcar* *G. muscosa*		*G. minutiflora*
Habenaria		*H. kingii*			
Hetaeria		*H. elegans*		*H. elegans*	*H. elegans*
Hippeophyllum		*H. scortechinii*	*H. scortechinii*		

Table 2 *(continue)*

Genera	Published/Preserved at				
	FRIM	SING	KEW	Ridley (1908, 1915)	Turner (1995)
		D. farmeri *D. geminatum* *D. grande* *D. hendersonii* *D. hosei* *D. hughii* *D. indivisum* *D. indragiriense* *D. leonis* *D. linguella* *D. longipes* *D. luxurians* *D. macropodum* *D. pachyphyllum* *D. pensile* *D. rhodostele* *D. rupicola* *D. salaccense* *D. subflavidum* *D. uniflorum* *D. zebrinum*	*D. geminatum* *D. hosei* *D. hughii* *D. indragiriense* *D. longipes* *D. luxurians* *D. macropodum* *D. quadrilobatum* *D. rupicola* *D. zebrinum*	*D. longipes* *D. macropodum* *D. rupicola* *D. subflavidum* *D. uniflorum*	
Dendrochilum	*D. angustifolium* *D. carnosum* *D. linearifolium* *D. pallidiflavens* var. *pallidiflavens*	*D. angustifolium* *D. carnosum* *D. gracile* *D. kingii* *D. linearifolium* *D. longifolium* *D. pallidiflavens* var. *pallidiflavens*	*D. linearifolium* *D. longifolium*	*D. gracile* *D. linearifolium*	
Didymoplexiella		*D. ornata*			
Dilochia	*D. cantleyi*	*D. cantleyi* *D. wallichii*	*D. cantleyi*	*D. cantleyi*	
Dilochiopsis	*D. scortechinii*	*D. scortechinii*	*D. scortechinii*	*D. scortechinii*	
Dipodium		*D. pictum*			
Eria	*E. crassipes* *E. robusta*	*E. crassipes* *E. earine* *E. javanica* *E. mucronata* *E. nutans* *E. robusta*	*E. crassipes* *E. javanica*	*E. crassipes* *E. earine* *E. nutans* *E. robusta*	*E. earine*
Eulophia		*E. spectabilis*	*E. spectabilis*		
Gastrochilus	*G. calceolaris*	*G. calceolaris* *G. patinatus*		*G. obliquus* var. *obliquus*	
Geodorum		*G. densiflorum*			
Goodyera					*G. colorata*
Grammatophyllum		*G. speciosum*	*G. speciosum*		
Grosourdya		*G. appendiculata* *G. incurvicalcar* *G. minutiflora*	*G. appendiculata* *G. incurvicalcar* *G. muscosa*		*G. minutiflora*
Habenaria		*H. kingii*			
Hetaeria		*H. elegans*		*H. elegans*	*H. elegans*
Hippeophyllum		*H. scortechinii*	*H. scortechinii*		

Table 2 *(continue)*

Genera	Published/Preserved at				
	FRIM	SING	KEW	Ridley (1908, 1915)	Turner (1995)
Lecanorchis		*L. malaccensis* *L. multiflora*			
Liparis		*L. parviflora* *L. parvula* *L. rhombea*	*L. parviflora* *L. rhombea*		*L. purpureoviridis* *L. rhombea*
Luisia		*L. antennifera*			
Malleola		*M. dentifera* *M. penangiana*	*M. penangiana* *M. sylvestris*		
Mycranthes	*M. oblitterata*	*M. oblitterata* *M. pannea*	*M. oblitterata*	*M. oblitterata*	
Nephelaphyllum	*N. pulchrum*	*N. pulchrum* *N. tenuiflorum*			
Nervilla		*N. concolor* *N. punctata*			
Neuwiedia		*N. griffithii*			
Oberonia		*O. dissitiflora* *O. insectifera* *O. lunata* *O. lucopodioides* *O. miniata*	*O. fungumolens* *O. lunata* *O. suborbicularis*		*O.semifimbriata* *O.suborbicularis*
Octarrhena		*O. angraecoides* *O. parvula*		*O. angraecoides*	
Oxystophyllum		*O. atrorubens* *O. sinuatum*	*O. carnosum*	*O. sinuatum*	
Paphiopedilum		*P. bullenianum* *P. bullenianum* var. *bullenianum*	*P. bullenianum*	*P. barbatum* *P. bulleninaum* var. *bullenianum*	*P. bullenianum*
Pelatanthera		*P. cristata*	*P. cristata*	*P. angustata*	
Pennilabium		*P. angraecum* *P. struthio*	*P. struthio*		
Peristylus		*P. gracilis*	*P. gracilis*		
Phalaenopsis		*P. appendiculata* *P. fuscata* *P. maculate*	*P. appendiculata* *P. fuscata*		*P. appendiculata* *P. fuscata* *P. maculata*
Pholidota	*P. carnea* var. *pumila*	*P. carnea* var. *carnea P.carnea* var. *parviflora* *P. carnea* var. *pumila*		*P. carnea* var. *carnea* *P. carnea* var. *pumila*	
Phreatia		*P. crassifolia*	*P. crassifolia* *P. listrophora*	*P. crassifolia* *P. listrophora*	*P. linearia*
Pinalia		*P. appendicula* *P. bicristata* *P. floribunda* *P. lancifolia* *P. pachystachya* *P. punctate*		*P. appendicula*	
Podochilus				*P. sciuroides* *P. tenuis*	
Pomatocalpa			*P. simalurense* *P. spicatum*		

Table 2 *(continue)*

Genera	Published/Preserved at				
	FRIM	SING	KEW	Ridley (1908, 1915)	Turner (1995)
Porpax					*P. elwesii*
Pteroceras			*P. biserratum* *P. pallidum* *P. violaceum*		*P. violaceum*
Renanthera			*R. elongata*		
Spathoglottis	*S. aurea*		*S. aurea*	*S. aurea*	
Taeniophyllum			*T. glandulosum* *T. gracillimum* *T. palliflorum* *T. rostratum* *T. stella*		*T.pallidiflorum* *T.rostratum*
Tainia	*T. speciosa*			*T. speciosa* *T. vegetissima*	*T. vegetissima*
Thrixspermum			*T. acuminatissimum* *T. ridleyanum* *T. sarcophyllum* *T. trichoglottis*	*T. sarcophyllum* *T. scortechinii*	*T. sarcophyllum* *T. tahanense*
Trichoglottis			*T. retusa*		
Trichotosia	*T. ferox* *T. poculata*		*T. pauciflora* *T. poculata*	*T. ferox* *T. pauciflora* *T. poculata*	
Zeuxine					*Z. gracilis* *Z. purpurascens*

Figure 1. A. *Aphyllorchis montana* Rchb. B. *Apostasia nuda* R.Br. C. *Arundina graminifolia* (D.Don) Hochr. D. *Ascidieria longifolia* (Hook.f.) Seidenf. E. *Bulbophyllum pileatum* Lindl. F. *Campanulorchis pellipes* (Rchb.f. ex Hook.f.) Y.P.Ng & P.J.Cribb G. *Chelonistele sulphurea* (Blume) Pfitzer H. *Claderia viridiflora* Hook.f. I. *Coelogyne radicosa* Ridl. Photos by SFMI

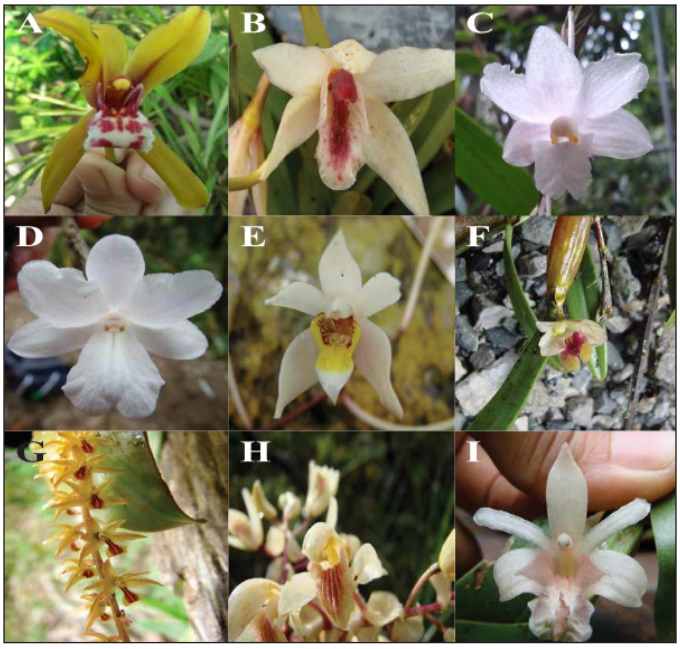

Figure 2. A. *Cymbidium finlaysonianum* Lindl. B. *Cymbidium roseum* J.J.Sm. C. *Dendrobium foxii* Ridl. D. *Dendrobium hughii* Rchb.f. E. *Dendrobium longipes* Hook.f. F. *Dendrobium angustifolium* (Blume) Lindl. G. *Dendrochilum gracile* (Hook.f.) J.J.Sm. H. *Dilochia cantleyi* (Hook.f.) Ridl. I. *Eria neglecta* Lindl. Photos by SFMI

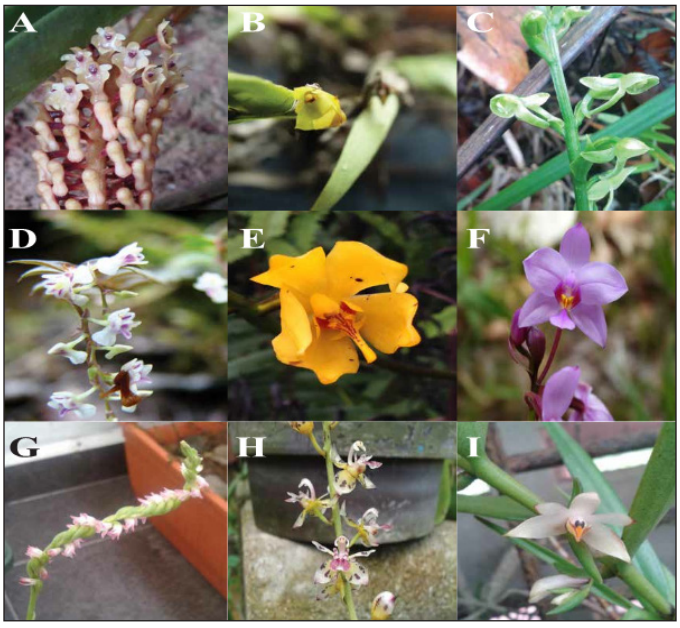

Figure 3. A. *Eria robusta* (Blume) Lindl. B. *Oxystophyllum carnosum* Blume C. *Pelatantheria angustata* (Ridl.) Ridl. D. *Podochilus microphyllus* Lindl. E. *Spathoglottis aurea* Lindl. F. *Spathoglottis plicata* Blume G. *Spiranthes sinensis* (Pers.) Ames H. *Thecostele alata* (Roxb.) Par. & Reichb.f. I. *Trichotosia gracilis* (Hook.f.) Kraenzl. Photos by SFMI

DISCUSSION

The information presented here could serve as a new insight for orchids recorded in Gunung Tahan, particularly from the habitat along the later established Merapoh Trail which may enhance the value of Taman Negara Pahang as the center of flora diversity in Malaysia. More undiscovered or new species could be found if a more comprehensive study from the southern, northern, and western side of Gunung Tahan could be undertaken. This will possibly reveal a habitat specific species of orchids in Gunung Tahan, which may be driven by site-specific preferences.

There were no representative species collected for the subfamily Vanilloideae in this study. Only one genus *Lecanorchis* was preserved in the SING which was collected from Kuala Tahan Trail. *Lecanorchis* is one of the leafless myco-heterotrophs or formerly called as saprophytes species in subfamily Vanilloideae. Their leafless, thin, and brittle dark stem, which bear few-to-many small flowers characters make it difficult for the climbers to find while climbing the mountain. However, one leafless saprophytic species that was characterized under subfamily Epidendroideae, *Aphyllorchis montana* Rchb.f. was found from Merapoh Trail.

Based on the current study two species; *C. viridiflora* and *S. sinensis* were listed globally as least concern species, while *P. bullenianum* was listed as endangered species by IUCN Red List of Threatened Species version 2017.3. According to the IUCN the population of *P. bullenianum* is decreasing which give alarming sign to the conservation of the species in Gunung Tahan. In addition to the 18 new records for Gunung Tahan, the presence of the endangered species highlights the need for conservation effort in Gunung Tahan even though the area has been gazetted as forest reserve and is one of the Malaysia's premier national park.

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Effect of Planting Dates on Growth, Yield, and Phenology of Different Soybean Lines Grown Under Tidal Swamp Land

Heru Kuswantoro

Indonesian Legume and Tuber Crops Research Institute, Indonesian Agency for Agricultural Research and Development, 12540 Malang, Indonesia

ABSTRACT

The water availability in tidal swamp land type C is similar to the rain-fed area. However, waterlogging may occur if the irrigation system is not good. This experiment aimed to study the effect of different planting dates in growth, yield, and phenology of different soybean lines grown under tidal swamp land. A randomized complete block design was repeated four times, each planting date differed 46 days apart. Interaction of genotype and planting date were demonstrated by days to flowering and days to maturity. Different response on days to flowering and days to maturity showed that the tested genotypes had phenological adaptation on a particular planting date. No interaction was shown by other agronomical traits, even though the genotypes were significantly different. Most of the lines had similar seed yield to the control varieties. The genotype of Menyapa (G12) had the largest number of filled pods, but the seed size was the smallest. Consequently, the seed yield of G12 was lower than the genotypes with the slightly lower number of filled pods and larger seed size. The shortest maturity genotypes of Tgm/Brg-584 (G10) also showed similar seed yield to the control varieties. Seed size is substantially responsible for the performance of seed yield. The most promising lines was Snb/1087-148-2-1 (G4), because this line had high yield and large seed size. Interaction in phenological response reflecting in days to flowering and days to maturity was not followed by agronomical traits suggesting that the effect of phenological traits to agronomical traits is weak. Therefore, the soybean promising lines can be grown at those two planting dates.

Keywords: Growth, phenology, planting date, soybean, tidal swamp land, yield

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herukusw@gmail.com

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INTRODUCTION

Soybean is a staple food and is the third after rice and corn in Indonesia. By 2015, Indonesia's consumption of soybean-based products reached 6.12 kg/capita/year (Pusat Data dan Sistem Informasi Pertanian, 2016). Usually, soybean is used in food products such as tempeh, tofu, soy sauce, and soymilk. The requirement for soybean for food consumption cannot be fulfilled from domestic production. In 2015, soybean production reached 9,63,183 tons, but the soybean imports were much larger at 2,256,931 tons (Pusat Data dan Sistem Informasi Pertanian, 2016). This condition is worsened by the shrinkage of harvested area, whereby the area of soybean harvest in 2016 was about 589 thousand ha (Pusat Data dan Sistem Informasi Pertanian, 2016), while it was about 614 thousand ha in 2015 (Badan Pusat Statistik, 2017a) which meant a decrease of about 4%. However, even though the harvested area decreases, soybean production can still be maintained if the productivity increases. In 2015, there was an increase of soybean productivity to 15.68 ku/ha (Badan Pusat Statistik, 2017b) of which the production was maintained in that year although the harvested area declined.

The progressive of non-agricultural sector development in Indonesia causes a decrease in the availability of arable land. This condition forced agricultural land to shift to less fertile land. Many suboptimal lands in Indonesia, including tidal swamp land covers 20,192 million ha (Alihamsyah et al., 2003). There are four types of tidal swamp land, namely type A, B, C, and D. Types A and B face excess water stress. Type A is logged at large and small tidal period, while type B is logged only at large tidal period. Types C and D are not logged by the water. Type C is the tidal land where the ground water limit is <50 cm below the ground surface, while type D has ground water limit >50 cm below the ground surface. However, in a bad irrigation system, the type C can also face water excess that leads to rhizospheric hypoxia which affect the root morphological traits (Jitsuyama, 2015, 2017), hinder root development and plant growth at the seedling stage (Suematsu, Abiko, Nguyen, & Mochizuki, 2017), and finally decreases seed yield (Kuswantoro, 2015a; Nguyen et al., 2012). Besides the excess water, the low pH is also a problem in this soil. Deficiency in macronutrients and toxicity in micronutrients can be experienced by plants in this soil (Fageria & Nascente, 2014), such as a deficiency in nitrogen (Thomas, Ayarza, & Lopes, 2000), phosphorus (Zheng, 2010), and aluminum toxicity (Zheng, 2010).

Generally, in subtropics, planting dates are associated with maturity groups (Nyagumbo, Mkuhlani, Mupangwa, & Rodriguez, 2017; Salmerón et al., 2016). In the tropics like Indonesia, the maturity group is relatively unaffected, since the length of the day is somewhat similar. This planting date is closely related to temperature and light that can affect the growth and yield of soybeans (Arslanoglu & Aytac, 2010). The main problem of planting date in the tropics is the availability of water,

such as the rain-fed area. It is also a problem in soybeans grown on land with uncertain water availability, where in some cases a puddle occurs when water shortages occur. Soybean cultivation in tidal swamp area is done in type C. Soybean cultivation is carried out during the rainy season whereby soybeans can receive water from the rain for growth and development. Although there is water in the dry season, the water is so acidic that it cannot be used for soybean watering. In the rainy season, it is still dependent on the occurrence of waterlogging within the land with poor drainage problems. Lack of water is more influential on soybean growth as compared to soil acidity (Kuswantoro & Zen, 2013) because water is the essential for plant growth.

Soybean cultivation in tidal swamp land type C is generally grown during the rainy season because the tidal condition is unable to rise through the soil because the water table is about 50 cm below the soil surface. Different rainfall during the plant growth and development will affect plant performance expressed in phenological and agronomical respones. Therefore, planting date can influence phenological and agronomical traits. This study was to investigate the effect of different planting dates in growth, yield, and phenology of different soybean lines grown under tidal swamp land.

MATERIALS AND METHOD

Experimental Site

The research site was in the village of Sari Makmur and Dadahup district, Kuala Kapuas regency, Central Kalimantan province, Indonesia. This site had a latitude of 2°39′33″ N, and a longitude of 114°28′16″ E.

Experimental Design

The experimental design used in each planting date was a randomized complete block design that was repeated four times. There were two factors in this study. The first factor was the planting date consisted of two planting dates, D1 and D2, where D1 was planted on the 6th of April 2014 and D2 was planted on the 22nd of May 2014, with 46 days from the day when D1 was planted. The second factor was the genotype which consisted of 12 genotypes as described in plant materials.

Plant Materials

The research material consisted of ten soybean promising lines, that is, Snb/1087-147-2-2 (G1), Snb/1087-147-2-7 (G2), Snb/1087-148-1-5 (G3), Snb/1087-148-2-1 (G4), Snb/1087-148-2-10 (G5), Snb/1087-148-2-3 (G6), Snb/1087-210-1-1 (G7), Sby/Pdm-651 (G8), Snb/1087-210-4-12 (G9), Tgm/Brg-584 (G10), and two control varieties of Lawit (G11) and Menyapa (G12). The promising lines of Snb/1087 were derived from the crossing of Sinabung variety and the genotype of MLGG 1087. Sinabung is a variety with high agronomical traits in an optimal land and MLGG 1087 is a genotype that is tolerant to acid tidal-swamp land. Sby/Pdm-651 was derived from the selection of Sibayak and Panderman crossing. Sibayak is an acid dry land tolerant

variety, and Panderman is a large seeded optimal land variety. Tgm/Brg-584 was obtained from the selection of Tanggamus and Burangrang crossing. Tanggamus is also acid dry land tolerant variety and Burangrang is an optimal land variety with large seed and early maturity. The two control varieties are the varieties for acid tidal swamp land (Balitkabi, 2009).

Soil Properties

The soil type in this experimental site was organosols with pH of 4.4. This soil pH includes in extremely acidic. Exchangeable Al and H were 12.9 and 10.7 me (100 g)$^{-1}$, respectively. The other soil properties were presented in Table 1.

Table 1
Soil properties of the experimental site

Soil properties	Value
pH	4.4
N (%)	0.29
Fe (ppm)	2679
Mn (ppm)	0.27
Cu (ppm)	20.02
Zn (ppm)	51.81
K (me (100 g)$^{-1}$)	0.53
Na (me (100 g)$^{-1}$)	0.44
Ca (me (100 g)$^{-1}$)	0.74
Mg (me (100 g)$^{-1}$)	0.40
CEC (me (100 g)$^{-1}$)	26.03
Al_{ex} (me (100 g)$^{-1}$)	12.9
H_{ex} (me (100 g)$^{-1}$)	10.7

Planting

Before planting, the soil was ploughed and then flattened. Drainage canals were made every 4.5 m with 20 cm depth and 40 cm width. The planting space was 40 cm × 15 cm, two plants per hill. Every soybean line was grown on 2.4 m × 4.5 m.

Cultural Practice

Fertilizer of 250 kg/ha Phonska, 100 kg/ha SP36, and 1 ton/ha organic fertilizers were provided throughout the planting time. Weed control was done manually at ages 2 and 4 weeks after planting. Watering was carried out based on the rainfall as stated in Table 2.

Table 2
Weather data of the experimental site from April to September 2014

Month	Minimum temperature (°C)	Maximum temperature (°C)	Average temperature (°C)	Relative humidity (%)	Rainfall (mm)
April	24.1	32.8	27.5	84.6	575.2
May	24.3	32.6	27.8	84.7	223.0
June	24.1	32.5	27.5	86.2	207.5
July	23.3	32.7	27.4	82.9	41.0
August	23.1	32.7	27.1	81.0	62.3
September	23.0	33.4	27.6	77.0	120.9

Weather Data

The average temperature of this site was around 27°C, while the humidity ranged between 77.0% and 86.2%, whereas the minimum and maximum humidity was in September and June 2014, respectively. The rainfall in April was the highest (575.2 mm), while the rainfall in July was the lowest (41.0 mm). Weather data of the experimental site were presented in Table 2.

Data Collection

Observations were carried out for the days to flowering, days to maturity, weight of 100 seeds, and seed yield. These four traits were observed based on the population of plants per plot. Besides these four traits, plant height, number of reproductive nodes, and number of filled and unfilled pods were observed. These traits were recorded based on ten sample plants.

Statistical Analysis

The data were analyzed using statistical software of PKBT STAT 1.0 for the analysis of variance. When the analysis of variance for a trait was significant, multiple comparisons were performed with least significant difference at 5% (LSD 5%) significance level using the same software.

RESULTS AND DISCUSSION

Analysis of variance revealed that interaction between genotype and planting date was shown on days to flowering and days to maturity. The other agronomical traits had no interaction, but they showed differences in the genotypes. However, the agronomical traits with no interaction were also not affected by planting date (Table 3). Genotype × planting date interaction on days to flowering and maturity indicated that a genotype has a different response when grown on a different planting date. The interaction on days to flowering and maturity reflects the phenological adaptation of a genotype on a certain planting date. The differences of plant phenological development phase as well as genotype × environment interaction can result in the variability of plant development (Junior et al., 2015).

Table 3
Mean square of agronomical traits

Agronomical trait	Date	Rep. × Date	Genotype	G × Date
Days to flowering (days)	102.09**	1.25	14.58**	5.80**
Days to maturity (days)	228.17**	2.02*	31.25**	6.37**
Plant height (cm)	0.13	255.41	496.51**	2.98
Number of branches per plant	0.00	0.49*	1.19**	0.00
Number of reproductive nodes per plant	0.01	19.79**	55.47**	0.00
Number of filled pods per plant	0.84	66.08**	131.65**	1.36
Weight of 100 grains (g)	0.20	0.70	8.38**	0.16
Seed yield (t/ha)	0.66	0.32**	0.11**	0.01

The interaction between genotype and planting date on days to flowering revealed that G1, G2, G7, and G9 had longer days to flowering on D1 than D2. These four genotypes were not significantly different to the two control varieties (G11 and G12) (Figure 1). Days to flowering at D1 were longer than that of D2. The shortest days to flowering was shown by G8 and G10 at D2. The longer duration of days to flowering was due to the high level of the rainfall (Table 2) at D1 than that for D2. At high rainfall, days to flowering is longer. This is related to crop adaptation, in which the plant develops a good vegetative period when water is fully available. The development of vegetative traits, especially leaves, is needed as it assimilates source in the development of generative organs. Candoğan and Yazgan (2016) also reported a similar case in which there was an increase in days to flowering when there was a rise in rainfall. Solar radiation also plays a major role in days to flowering due to the lack of solar radiation which resulted in prolonged days to flowering (Yin, Olesen, Wang, Öztürk, & Chen, 2016). In the subtropics region, the development of vegetative organs was reported due to the differences in photoperiods (Dogra, Kaur, & Srivastava, 2015; Spehar Francisco, & Pereira, 2015). Benlahbil, Zahidi, Bani-Aameur and El Mousadik (2015) stated that extended days to flowering might be as a strategy in minimizing reproductive failure. However, the two control varieties showed consistent values between the two planting dates. The response of these control varieties was different than the other promising lines. It may be due to the lower phenotypic plasticity of these two control varieties. Plasticity is needed in plant adaptation, whereby plants with higher adaptive plasticity may be able to survive better in a new environment (Gratani, 2014).

Days to maturity of all tested genotypes were longer at D2 than that for D1, except G10 showed no significant difference at

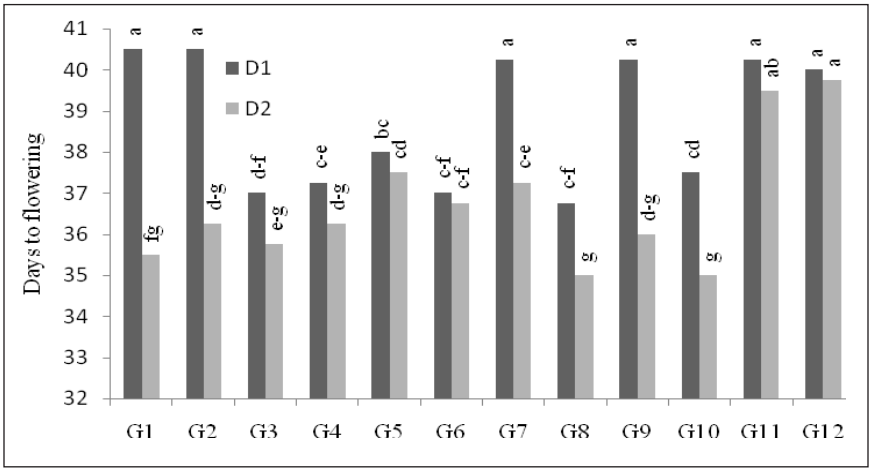


Figure 1. Days to flowering of twelve soybean genotypes at different planting dates

the two planting dates. The longest days to maturity were demonstrated by the two control varieties at D2, while the shortest was shown in G10 at the two planting dates (Figure 2). The longer days to maturity is due to the high rainfall during the reproductive phase. In this study, the rainfall during the reproductive phase at D1 was lower as compared to D2 (Table 2). Kuswantoro and Zen (2013) also reported similar results for the acidic dryland. The highest responses were shown in the control varieties of which these two varieties had 5 days difference of maturity between D1 and D2. The response of ten promising lines was lower than the control varieties, reflecting that promising lines had lower plasticity and more stable in days to maturity. Phenotypic plasticity in days to maturity is lower than days to flowering. This may be related to the duration of filling pods triggered by solar radiation. Iqbal et al. (2010) reported no differences in the two different growing seasons, with the number of different rainfall in days to flowering and days to maturity. This is in contrast to Zhang et al. (2015) in a study of the soybeans grown in subtropics, where narrow differences were found on days to flowering trait, but wide differences were observed on days to maturity trait. The duration of a vegetative and reproductive period can be influenced by photoperiod, temperature, and rainfall (Hu et al., 2012).

There was no difference between D1 and D2 on plant height trait. The highest plant height was shown in G12 (Figure 3). Plant height is important in soybean because it is the main trait that can indicate a plant growing in normal or stress conditions. Plant height in acid soil is lower than that in normal condition. It is because the low availability of nitrogen reduces the plant growth in acid soil (Thomas et al., 2000). However, plant height is greatly affected by the water availability than the acidity (Kuswantoro & Zen, 2013). Even

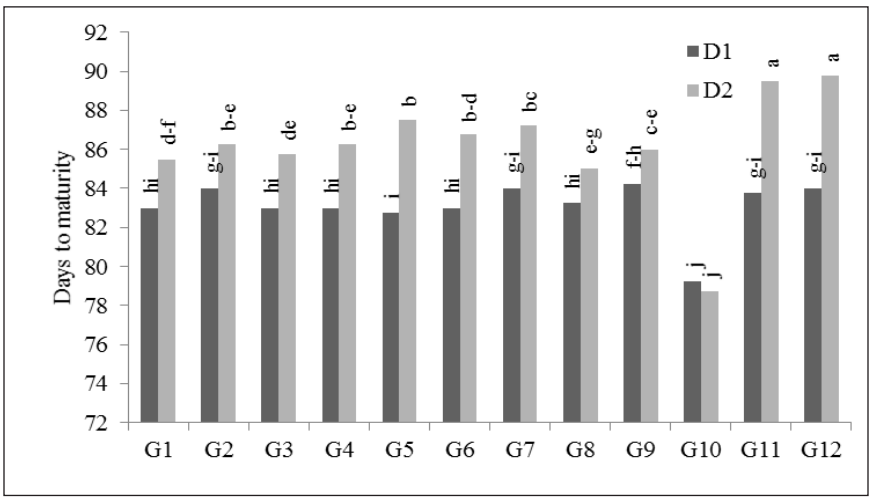
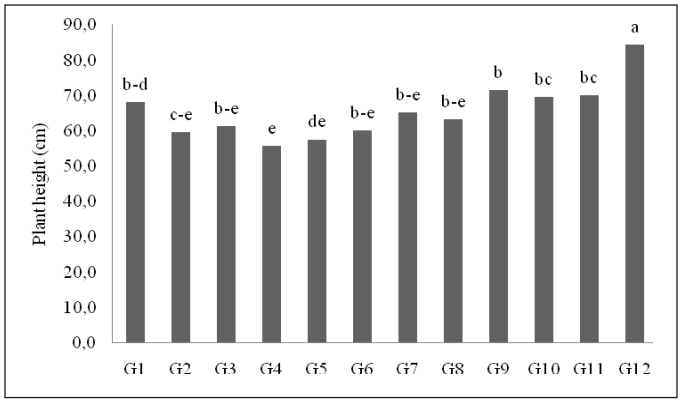
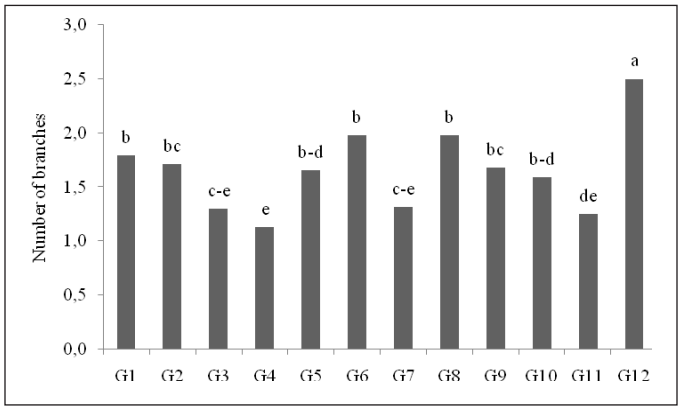


Figure 2. Days to maturity of twelve soybean genotypes at different planting dates



(a)



(b)

Figure 3. Means of : (a) plant height; and (b) number of branches of twelve soybean genotypes at different planting dates

though it is grown in normal condition, the plant height decreases more than that in acidic soil when there is a lack of water (Kuswantoro, Zubaidah, & Sulisetijono, 2014; Kuswantoro, 2015b). The increase in plant height during rainy season is also due to the ultraviolet radiation exclusion, which lengthens the soybean internodes (Zhang et al., 2014).

Number of branches also did not differ between the two planting dates. The number of branches in this study were <2 branches per plant. This is lower than Kuswantoro (2017), which reported a mean of 2.3 branches per plant on another tidal land. It indicates that the number of branches is more affected by the characteristic of the genotypes and not dependent on the maturity group (Junior et al., 2015). However, number of branches is influenced by planting space, where wider planting space leads to a higher number of branches (Güllüoğlu, Bakal, & Arioğlu, 2016). The branches are located on the plant stem. Therefore, plant height and the number of branches mostly have a similar pattern of

which the highest number of branches per plant was also shown in G12, while the lowest was shown in G4 (Figure 3). This similar pattern confirms the important role of plant height. Jain, Srivastava, Singh, Indapurkar and Singh (2015) reported that plant height and the number of branches had a significant correlation. The higher internodes may provide a higher chance for the plants to form more branches.

A reproductive node is any node with one or more pods. This node is important because it supports the yield through the number of pods. Different planting dates did not affect the number of reproductive nodes, but number of reproductive nodes was more affected by the genotypes (Table 3; Figure 4). The highest number of reproductive nodes were achieved by G12 followed by four similar lines of G1, G5, G6 and G8, while the lowest was reached by four similar lines of G3, G4, G7, and G11 (Figure 4). A similar pattern was shown by the number of filled pods. However, G4, one of lines with the lowest number of reproductive nodes, showed the higher number of filled pods. It

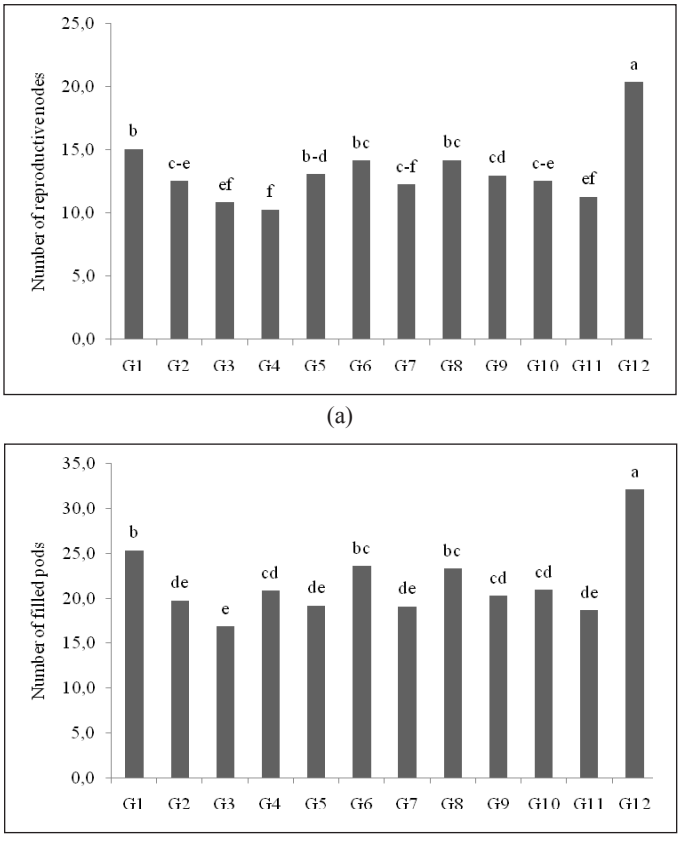
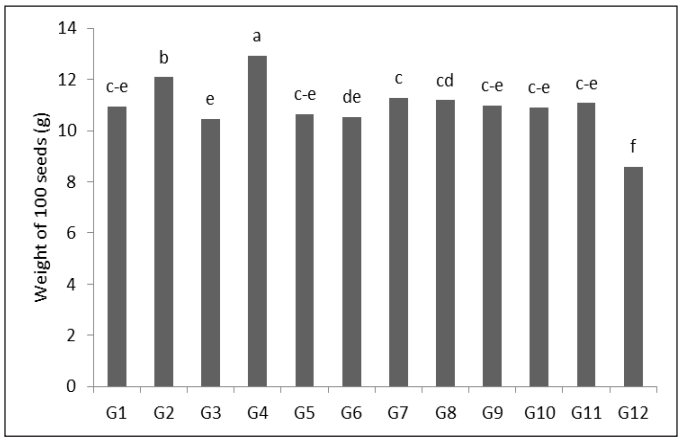


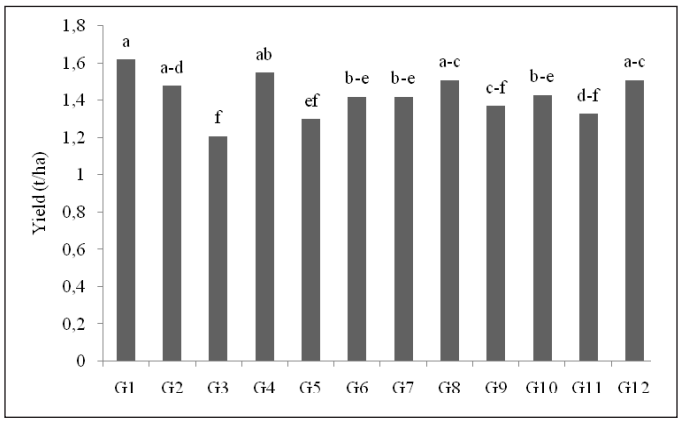
Figure 4. Means of number of reproductive nodes and number of filled pods of twelve soybean genotypes at different planting dates

may be due to this genotype of which there were more filled pods per reproductive nodes than other genotypes. Also, the similar pattern was shown by plant height. The relationship between plant height, number of reproductive nodes, and number of nodes may be due to the effect of planting date on the number of nodes, especially on nodes forming at V9 growth stage and the flowers decreasing in the R2 growth stage (Junior et al., 2015). The decrease in flowers may be due to the increase in abscission on the flower attributes and due to the pollination and seed development failures (Hoque, Hassan, Khan, Khatun, & Baten, 2015).

Seed size was measured from weight of 100 seeds. Seed size was not significantly different between the planting dates but differed significantly between genotypes (Table 3). The line of G4 had the largest seed size, while the G12 line had the smallest seed size (Figure 5). Differences



(a)



(b)

Figure 5. Means of weight of 100 seeds and seed yield of twelve soybean genotypes at different planting dates

in seed size are influenced by environmental conditions such as water availability (Hu & Wiatrak, 2012), soil acidity (Kuswantoro et al., 2014; Kuswantoro, 2015b) and air temperature (Hoque et al., 2015). In this study, there was no difference in soil acidity between planting dates because soybeans were grown in the same location. Air temperature also did not differ between planting dates, but the amount of rainfall was different. Heritability of seed size is high (Berger-Doyle, Zhang, Smith, & Chen, 2014; Kuswantoro, 2017), this means that seed size is not much influenced by the environmental factor. However, under very different environmental conditions, seed size will change (Kuswantoro et al., 2014; Kuswantoro, 2015b). In this study, the environmental changes were not extreme, so the size of the seeds on both planting dates was not significantly different. Differences in rainfall have no effect on seed size change. It may be implied from the amount of rainfalls being received in the pods filling period in the two planting dates that were not significantly different (Table 2).

Seed yields were not significantly different between the planting dates. The environmental differences in these planting dates had no effect on seed yield. Many reports suggested that the heritability of seed yields is low (Berger-Doyle et al., 2014, Kuswantoro, 2017) since the yield of the seeds is controlled by many genes. In this study, seed yield was not influenced by planting date × environment interaction whereby the differences in the environment might be less distinctive. In the subtropical region, the effect of these seed yields is reported because of the difference in photoperiod (Spehar et al., 2015). Furthermore, the differences in planting dates are generally a combined effect of photoperiod, temperature, and rainfall (Hu & Wiatrak, 2012). However, the rainfall volume in reproductive phase is not always directly related to the yield in a location (Dogra et al., 2015). Nevertheless, there were seed yield differences between the soybean lines. The highest seed yield was achieved by G1, G2, G4, G8, and G12. The similar seed yield of G12 than G1 was due to the smallest seed size of G12 (Figure 5) even though G12 had the largest number of filled pods (Figure 4). It appears that seed size plays a significant role in the seed yield than the number of filled pods (Kuswantoro et al., 2014; Kuswantoro, 2015b). A soybean line that can maintain a large seed size will produce a greater seed yield.

CONCLUSION

Genotype × planting date interaction was presented in the days to flowering and days to maturity. The response of a genotype on those two traits differ when grown in diverse planting dates. There was a genotypic adaptation by changing phenological traits on a different planting date. The different phenological development due to genotype × environment interaction did not result in the differences of other plant development, whereby the six agronomical traits were more affected by the genotype rather than the environmental factor. The presence of interaction in phenological traits and the

absence of interaction on agronomical traits suggest that phenological traits had no effect on agronomical traits. Therefore, the tested soybean promising lines can be grown at those two planting dates.

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Deciphering the Stability and Association of Ear Leaves Elements with Nutrients Applied to Grain Yield of Maize

Abdulwahab Saliu Shaibu[1]*, Jibrin Mohammed Jibrin[2,3], Bello Muhammad Shehu[3], Bassam Abdulrahman Lawan[3] and Adnan Aminu Adnan[1]

[1]Department of Agronomy, Bayero University, Kano, Nigeria
[2]Centre for Dryland Agriculture, Bayero University, Kano, Nigeria
[3]Department of Soil Science, Bayero University, Kano, Nigeria

ABSTRACT

Ear leaf as a vegetative part has proven to be useful for evaluation of nutritional indices in maize and for making predictions about yield. This study was conducted to determine the stability of ear leaves nutrient under varying fertilizer applications and their relationship with grain yield. Thirty-five (35) nutrient omission trials were established in four locations using two maize varieties; IWD (OPV) and Oba Super-9 (hybrid) making a total of eight environments in 2015 wet season across the Guinea Savannas of Nigeria. Ten ear leaves were sampled in the period between tasseling and silking immediately when the position of the ear was identified and analyzed for macro and micro elements. The results showed that environment contributed to most of the variability observed in all the elements rather than the treatments. The GGEbiplot showed that Mg, Mn, and Cu are positively associated with grain yield and are the most stable elements. The confirmatory analysis also showed the importance of these elements in predicting grain yield. The environment has demonstrated to be a major determinant of ear leaves elements in maize. Therefore, accurate envirotyping of maize producing regions in Nigeria is important for better classification of maize-growing regions.

Keywords: Ear leaf, enviryotyping, grain yield, nutrients, stability

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E-mail addresses:
asshuaibu.agr@buk.edu.ng (Abdulwahab Saliu Shaibu)
jibrinnj@buk.edu.ng (Jibrin Mohammed Jibrin)
abdulshaks@yahoo.com (Bello Muhammad Shehu)
balawan.ssc@buk.edu.ng (Bassam Abdulrahman Lawan)
aaadnan.agr@buk.edu.ng (Adnan Aminu Adnan)
* Corresponding author

INTRODUCTION

Fertilizer research is usually conducted to determine the yield responses of crops to fertilizer and to make better predictions for several soil and management conditions.

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Availability of nutrients in the soil is majorly determined from the nutrient composition of the plant. The plant is also used to determine key nutrient and their levels required for growth and development of the plant were further increase will have low or no yield response, and a decrease will result in low yield. In other to make accurate predictions of this yield response using plants, knowledge of the interrelationships among different nutrient omissions and compositions of the plants with yield responses are required. Ear leaf; the leaf covering the upper cob of a maize plant, is a vegetative part of the plant and have proved to be useful for evaluation of nutritional indices in maize and for making predictions about yield. It is the most sensitive stage that usually affects kernel formation on a cob (Jones, Schreiber, & Roessler, 1996). The ear leaf can be greatly exploited to predict grain yield which is an advantage of ear leaf analysis (Soltanpour, Malakouti, & Ronaghi, 1995). However, a major disadvantage is the low practical applicability of obtained models using ear leaf. The lack of knowledge about the stability of the ear leaf elements under different nutrient management and environment sometimes make the applicability of the model low. The ear leaf stage is close to flowering and marks the end of the vegetative stage. So for nutrient diagnostic purposes, the ear leaves should be sampled at the earliest stages, just before the inflorescences set up. It has been reported that maize plants reach their highest rate of absolute growth and are significantly affected by nitrogen supply and other elements at this stage (Grzebisz, Baer, Barłóg, Szczepaniak, & Potarzycki, 2010). Several studies have shown the importance of the nutritional status of maize ear leaves to grain yield (Grzebisz, Wrońska, Diatta, & Dullin, 2008; Mallarino & Higashi, 2009). Based on the importance of the ear leaf nutrient composition in predicting yield, the present study was conducted to determine the stability of ear leaf nutrient composition under varying fertilizer applications and the relationship of these nutrients with grain yield with a view of increasing the applicability of the model in predicting grain yield.

MATERIALS AND METHODS

Thirty-five (35) nutrient omission trials (NOTs) were established in four locations, Zango Kataf (5), Karu (10), Kokona (10), and Tsafe (10) in 2015 wet season across the Guinea Savannas of Nigeria (Supplementary Table 1). The NOTs consisted of six treatments (Table 1) with plots sizes of 5 m by 6 m used and two maize varieties were used, IWD (OPV) and Oba Super-9 (hybrid), which were considered as environments. Nitrogen was applied in three split application (planting, 3 weeks, and 6 weeks after planting).

Ten ear leaves were sampled in the period between tasseling and silking (male and female flowering, respectively) immediately when the position of the ear was identified. Ear leaf is removed by plucking downwards (at roughly an adjacent angle of <30°) with moderate force as this allows the leaf to cut at the collar, leaving

Table 1
Treatment structure of NOT in Guinea Savanna of Nigeria

Treatment code	Treatment	Nitrogen (kg N/ha)	Phosphorus (kg P/ha)	Potassium (kg K/ha)	Secondary and micronutrients
1	Control	0	0	0	0
2	NK	140	0	50	0
3	NP	140	50	0	0
4	NPK	140	50	50	0
5	NPK (S-Ca-Mg-Zn-B)	140	50	50	24-10-10-5-5, respectively
6	PK	0	50	50	0

behind the leaf base that circles the stem. A total of ten plants in the two rows next to the net plot (5 in each row) were sampled, taking representative samples. The leaf samples were placed into clearly labelled large khaki paper sample bags and carefully sealed. The samples were washed with distilled water to remove contaminants and then oven-dried at 60^{o}C for 48 h.

Total nitrogen in the ear leaves samples was determined using the Micro-Kjeldahl digestion method (Bremner & Mulvaney, 1982). While P, K, Mg, Ca, Cu, B, and Zn were digested with hot nitric acid (HNO_3) and their concentration was determined using inductively coupled plasma optical emission spectroscopy (ICP-OES).

Surface soil samples were collected at 0–20 cm depth from each field. Soil pH, total soil organic carbon, total nitrogen, available phosphorus, and exchangeable cations (Ca, Mg, and K) were analyzed using the methods of Gee and Or (2002), Heanes (1984), Bremner (1996), and Mehlich (1984), respectively.

Grain yield data were collected from the field. The data collected for grain yield and nutrient concentration in the ear leaves element were analyzed using JMP 10.1.2. Variance components of each of the factors were estimated to determine the contribution of each factor to grain yield. The treatments were treated as fixed effect while the replication, environment, and interaction of treatments with the environment were considered as random effects. The GGE biplot analysis was done using R GGE biplot graphical user interface package. Partial least square regression was done to determine the important variables contributing to grain yield.

RESULTS

Sand was the dominant soil textural fraction in all the study locations with a median value of >60% (Table 2). Kokona and Tsafe have a moderately acid soil pH, while Zango and Karu have strongly acidic and moderately acidic reactions, respectively. All the four locations have low organic carbon (<1%), low N (<0.1%), and low available P, respectively, according to the Esu (1991) soil fertility classification. Calcium is low in Karu, Kokona, and Zango (<2 cmol/kg) and moderate in Tsafe (2–5

Table 2
Physical and chemical properties of the soil

Locations	Sand (%)	Silt (%)	Clay (%)	pH	OC (%)
Karu	76 (71.80)	9 (4.12)	16 (12.20)	6.1 (5.7,6.2)	0.59 (0.44,1.24)
Kokona	71 (65.77)	12 (7.15)	16 (14.22)	5.9 (5.5,6.1)	0.63 (0.42,1.22)
Tsafe	61 (54.66)	25 (19.25)	15 (14.21)	6 (5.8,6.6)	0.48 (0.38,0.81)
Zango	60 (53.67)	13 (12.16)	26 (20.34)	5.1(4.9,6.1)	0.75 (0.41,1.06)
Locations	N (%)	Meh_P (mg/kg)	Ca (cmol/kg)	Mg (cmol/kg)	K (cmol/kg)
Karu	0.05 (0.03,0.07)	3.08 (2.06,4.11)	1 (0.19,2.46)	0.59 (0.41,0.83)	0.11 (0.09,0.12)
Kokona	0.04 (0.03,0.04)	2.26 (2.06,4.93)	1.31 (0.38,3)	0.6 (0.23,0.66)	0.15 (0.11,0.26)
Tsafe	0.03 (0.02,0.04)	3.49 (2.88,5.69)	2.44 (0.56,4.31)	0.85 (0.46,0.96)	0.15 (0.11,0.17)
Zango	0.04 (0.04,0.05)	2.06 (1.65,2.67)	0.56 (0.38,1.69)	0.26 (0.19,0.26)	0.17 (0.12,0.26)

Numbers in parenthesis "( )" are minimum and maximum values, respectively

cmol/kg). Soil Mg concentration was in moderate condition (0.3–1.0 cmol/kg) in all the four locations. Soil K concentration were low (<0.15 cmol/kg) in Karu and moderate (0.15–0.30 cmol/kg) in Kokona, Zango, and Tsafe, respectively.

The variance components and percent contribution of each of the factors to macro elements in maize grain yield are presented in Table 3. The minimum percent variance contribution was from the treatment by environment interactions (TEI) for yield and all the macro elements of ear leaf. The TEI contribution ranges between 0.04% for grain yield to 11.96% for Ca. The unexplained variability for grain yield as a result of noise was 31% this was followed by the variability due to treatment effect (28%) and variability as a result of the environment (24%). The unexplained noise effect for N and P were 35% and 39%, respectively, followed the effect of environment (32% and 24%, respectively). The treatment effect also explained some percentage of the variability observed in the amount of N and P in the ear leaves. The variability in K for the ear leaves was majorly from the environment (56%) followed by the replication within environment effect (35%). The treatment effect was 0.32% which is an indication that any difference observed in K was not the result of the treatment but majorly due to the environment (combined effect with replication was about 90%). The addition or omission of K from the treatments did not affect the response of maize ear leaves to K. About 39% and 33% of the variability in Ca was accounted for by the unexplained noise and replication within environment effects. While for Mg, the replication within environment contributed 51% of the variability observed followed by the residual effect. The treatments only explained 0.7% of the variability while the environment explained just 0.2% of the variability in Mg content of the ear leaves.

The contribution of the treatment and the TEI to the variability observed in the micro nutrients were very low except for Mn where they accounted for 9% and 20% of the

variability, respectively (Table 4). The noise variance was 31% of the total variability observed for Mn while the environmental variance was 27.84%. The environmental variance for Zn, Cu, and B were 24%, 40%, and 35%, respectively. The noise variance for Fe was very high (93%) followed by that of Zn (64%) and for Cu and B, the environmental variance was higher than the noise variance.

The principal axis explained about 78% of the treatment and treatment by ear leaves element interaction (Figure 1). Basically, in GGE biplot, the smaller the angle between two variables the closer the association. Copper has the highest association with grain yield while other elements in close association with grain yield in decreasing order are Mg, Mn, and N. In the polygon view (Figure 2), the vertex treatment in each sector represents the highest yielding treatment for the ear leaves element that falls within that sector. Five sectors were identified in the biplot. The response of Fe contents in the ear leaves was closely associated with treatment 2 (NK) followed by K and Zn. None of the element showed a response to the control (treatment 1) and B

Table 3
Variance components and percent contribution of factors to yield and macro elements in ear leaves of maize

Random effect	Grain yield	N	P	K	Ca	Mg
Replication (environment)	452806.52 (17.17)	0.02 (7.94)	0.00087 (18.13)	0.3169 (35.31)	0.0049 (33.01)	0.00243 (50.90)
Environment (E)	639461.22 (24.25)	0.09 (31.92)	0.00116 (24.16)	0.5022 (55.97)	0.0011 (7.10)	0.00001 (0.21)
Treatment (T)	727618.72 (27.59)	0.06 (19.55)	0.00073 (15.22)	0.0029 (0.32)	0.0013 (8.67)	0.00003 (0.70)
T × E	1169.28 (0.04)	0.01 (3.14)	0.00017 (3.57)	0.0029 (0.32)	0.0018 (11.96)	0.00014 (2.93)
Residual	816244.04 (30.95)	0.11 (37.46)	0.00187 (38.92)	0.0725 (8.07)	0.0058 (39.27)	0.00216 (45.26)
Total	2637299.79	0.28	0.00480	0.8974	0.0149	0.00476

Number in parenthesis "( )" are percentage contributions

Table 4
Variance components and percent contribution of factors to yield and micro elements in ear leaves of maize

Random effect	Mn	Fe	Zn	Cu	Br
Replication (environment)	164.15 (11.42)	976.68 (3.30)	7.89 (8.40)	10.10 (33.21)	115.04 (31.31)
Environment (E)	400.00 (27.84)	582.80 (1.97)	22.41 (23.86)	12.22 (40.18)	126.19 (34.35)
Treatment (T)	136.60 (9.51)	616.37 (2.08)	−0.18 (0.00)	−0.10 (0.00)	12.11 (3.30)
T × E	291.49 (20.28)	−579.68 (0.00)	3.47 (3.70)	0.88 (2.89)	20.64 (5.62)
Residual	444.81 (30.95)	27444.21 (92.65)	60.12 (64.04)	7.21 (23.72)	93.39 (25.42)
Total	1437.05	29620.06	93.89	30.41	367.38

Number in parenthesis "( )" are percentage contributions

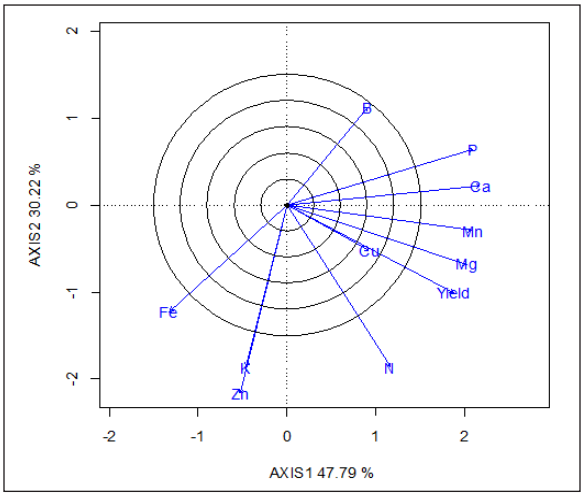

Figure 1. Relationship between the ear leaves element and grain yield

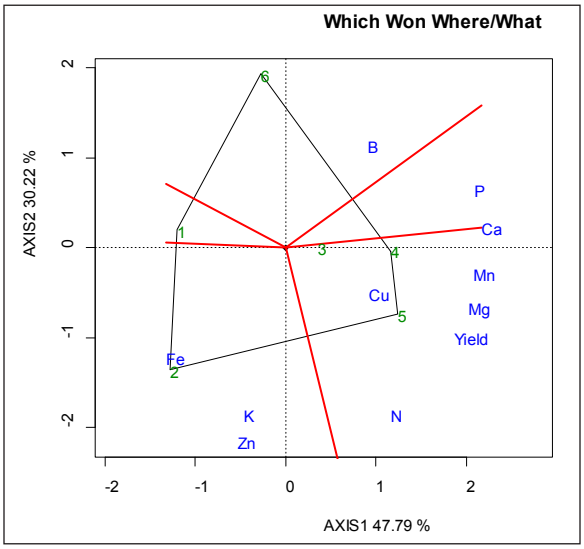

Figure 2. Which won where/what Biplot of NOTs and ear leaves of maize

slightly showed a response to PK (treatment 6). Grain yield per se was more influenced by treatment 5 (NPK + S-Ca-Mg-Zn-B) and 4 (NPK) while the response of Cu and Mg was more affected by treatment 5.

The length of the vector of elements describes its discriminating power, whereas the angle between an element and the thick horizontal axis measures its representativeness. Most of the elements had long vectors indicating that they are able to discriminate among the treatments but only Mg and Mn concentrations had smaller angles with the horizontal axis describing their representativeness. Also, grain yield had a good discriminating and representative power (Figure 3).

The most stable nutrient omission treatment was treatment 5 followed by treatment 3 while treatment 2 and 6 were highly unstable (Figure 4). Copper was the most stable among the ear leaf elements followed by grain yield.

Six factors were identified to explain the variability in grain yield and factor 1 was loaded more with Mg, Mn, Ca, P, and N (Figure 5[a]). Among all the six factors, factor 1 accounted for about 90% of the variability observed (Figure 5[b]). A variable important plot was plotted using partial least square regression to identify most important elements in predicting grain yield (Figure 6). From the plot, Mn, Mg, N, Ca, and P were the most critical elements in determining grain yield of maize.

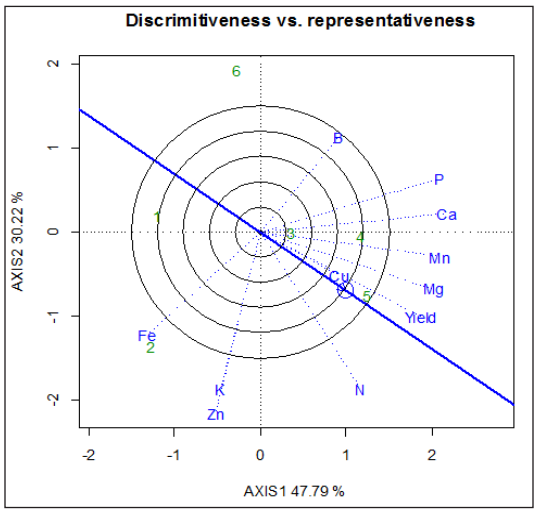

Figure 3. Discrimitiveness and representativeness of ear leaves element

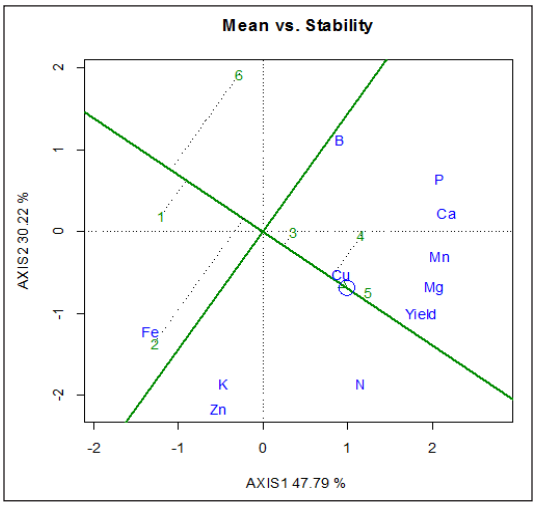

Figure 4. Stability of NOTs and ear leaves element of maize

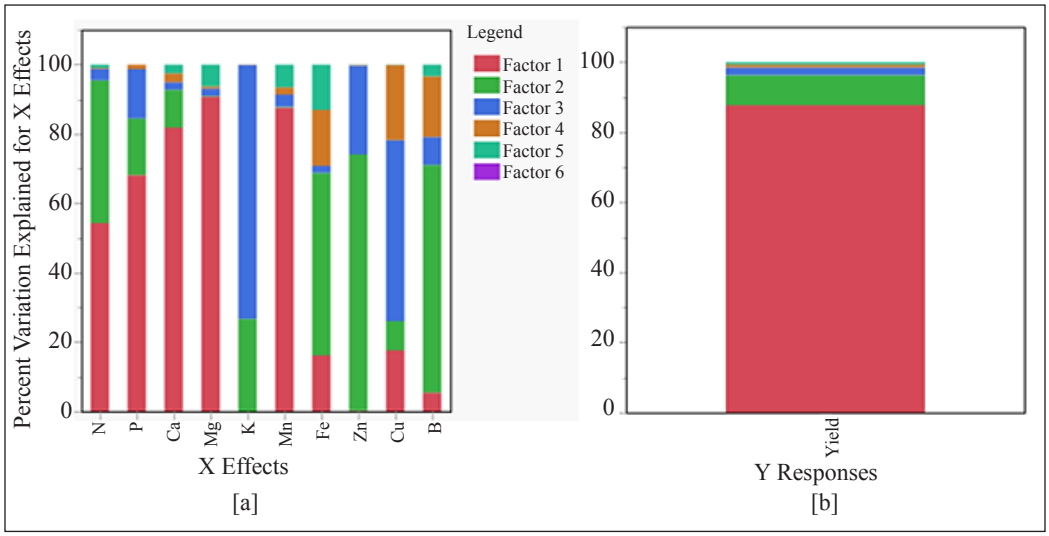

Figure 5. Variation of grain yield explained by the ear leaves element of maize

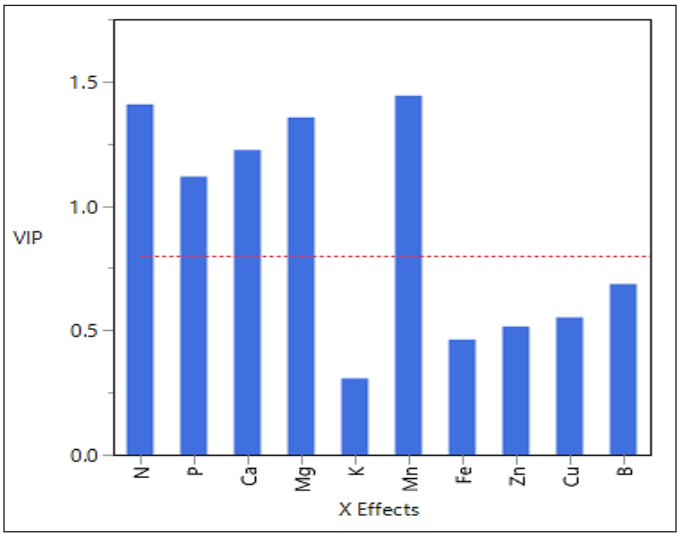

Figure 6. Variable important plot for ear leaves element in relation to yield

DISCUSSION

The high sand content in the locations is attributed to the parent material as the soils were developed largely on deeply pre-Cambrian basement complex rocks such as sandstone. The low O, C, N, and P could be related to the inherent low status as the parent materials where dominated by a low activity clays such as Kaolinite and complete removal of plant residue materials by the farmers in the study locations (Manu, Bationo, & Geiger, 1991; Shehu, Jibrin, & Samndi, 2015). Low Ca in Karu, Kokona, and Zango indicates the potential

development of Ca deficiency. Moderate K in Kokona, Zango, and Tsafe have been attributed to the appreciable amount of K-bearing feldspar minerals in the sand and silt fractions in the northern Nigerian Savanna soils (Møberg & Esu, 1991) and/or residual effect of past K application through NPK fertilizers.

The variability in grain yield was majorly explained by the treatment structure because of the high signal/noise ratio. The signal here includes the combined effect of variance component in the model with the exception of residual variance which is the noise component. Also, the signal/noise ratio was high for N and P and the environment contributed a higher percentage. For K, the environment accounted for most of the variability observed not the treatment effect per se. This is an indication that there is a high level of K variability in the environment and the environment, not the treatment was responsible for observed differences of K in the ear leaves. The environment can be further inferred to be highly variable for N, P, and K and it is responsible for the differences in these elements in the ear leaves. For Ca and Mg, high signal/noise ratio was observed with a larger proportion of the environment contributing the signal effect.

The signal/noise ratio in the micro elements of maize ear leaf was also high except for Fe and Zn which were having a very low signal/noise ratio and may be as a result of high insolubility of Fe and Zn. For Cu and B, the environment contributed majorly to the variability because plants respond to Cu deficiency or limitation by increasing Cu uptake and, where possible, switching to non-Cu-requiring protein.

GGE biplot was used in this study to determine the association of the ear leaves element with grain yield of maize, identifying the elements that could represent and discriminate grain yield, determine possible treatments that could increase grain yield and finally understand the stability of the nutrient omission treatments. Some elements such as Cu, Mg, Mn, and N showed a positive association with grain yield. Among these elements, Cu and Mg were majorly associated with the application of NPK + S-Ca-Mg-Zn-B followed by NPK which also have a high association with Mn. Application of NP had shown positive association to Cu, Mg, and Mn but not as high as the two NPK treatments. Kayode and Agboola (1985) reported that in addition to NPK, Mg and Cu were necessary for high yield of maize and in some rare cases the inclusion of Fe and Zn to NPK might be required. However, in this study, the high response of Fe and Zn was majorly from the control plots and both elements together with the control treatments were highly unstable and show no association with grain yield. Cu was the most stable elements while Mg and Mn were found to be the highly representative elements of ear leaves for all the treatments and they could discriminate among the treatments. The most stable treatment across the environments and in terms of ear leaves element was NPK + S-Ca-Mg-Zn-B followed by NP.

As a confirmatory analysis, the partial least square regression was done and six factors were identified as important factors. Factor 1 accounted for about 90% of the variability observed and can be used alone to explain the variability in grain yield. The most important elements in factor 1 were Mn, Mg, N, Ca, and P. These components of the ear leaves can be used in predicting grain yield of maize. Mn is very important for photosynthesis, pollen germination, and pollen tube growth. It is also an activator or cofactor for more than 30 other enzymes in plants (Millaleo, Reyes-Diaz, Ivanov, Mora, & Alberdi, 2010). Mg deficiency directly limits photosynthesis and causes leaf chlorosis that is aggravated by high light due to the production of reactive oxygen species (Shaul, 2002).

Generally, from the study, the environment has demonstrated to be a major determinant of ear leaves elements in maize. There is, therefore, a need for accurate envirotyping of maize producing regions in Nigeria in other to classify the environments base on the availability of nutrients that have demonstrated a strong association with grain yield through ear leaves and are major determinants of yield. Phenomics will always depend on the accurate envirotyping and without accurate envirotyping, phenomics will be meaningless. Enviryotypic data can also be used in environmental characterization, genotype by environment interaction analysis, predicting plant phenotype under variable environments, construction of near-iso-environment, precision agriculture, and breeding (Xu, 2016). In some parts of Africa, selection of the trial sites that are best suitable for different stresses such as drought, low nitrogen, low pH, stem borer, and Striga has been done (Xu, 2016). Bänziger, Setimela, Hodson and Vivek (2006) identified eight maize mega-environments in South Africa using maximum temperature, season precipitation, and subsoil pH. For the US corn-belt target population of environments, Cooper et al. (2014) identified typical temporal modes of environmental variation for the soil–plant water balance.

CONCLUSION

As much as we are interested in increasing grain yield on maize through the nutrient application, this study demonstrated that the environment should be greatly put into consideration as the phenotype is the sum total of genotype + environment. If the environmental effect is high, the observed phenotype will be only a reflection of the environment rather than the genotype (treatment). To increase the applicability of ear leaves in predicting grain yield, the environment needs to be well understood.

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APPENDIX

Supplementary Table 1
Coordinates of sites used for the experiments

S/N	State	LGA	Longitude (N)	Latitude (E)
1	Kaduna	Zango Kataf	9.74969	8.39246
2	Kaduna	Zango Kataf	9.75623	8.37620
3	Kaduna	Zango Kataf	9.75573	8.37736
4	Kaduna	Zango Kataf	9.75487	8.37769
5	Kaduna	Zango Kataf	9.75802	8.36775
6	Nasarawa	Karu	9.17998	7.87595
7	Nasarawa	Karu	9.18196	7.87068
8	Nasarawa	Karu	9.17766	7.91596
9	Nasarawa	Karu	9.17055	7.86985
10	Nasarawa	Karu	9.12460	7.94194
11	Nasarawa	Karu	9.11256	07.9358
12	Nasarawa	Karu	9.13739	7.94287
13	Nasarawa	Karu	9.14620	7.94759
14	Nasarawa	Karu	9.03674	7.91573
15	Nasarawa	Karu	9.03813	7.91309
16	Nasarawa	Kokona	8.8401	8.00863
17	Nasarawa	Kokona	8.85228	7.99768
18	Nasarawa	Kokona	8.84577	7.99223
19	Nasarawa	Kokona	8.84382	7.99171
20	Nasarawa	Kokona	8.83967	7.98729
21	Nasarawa	Kokona	8.83965	8.00084
22	Nasarawa	Kokona	8.83650	8.00208
23	Nasarawa	Kokona	8.83571	8.00152
24	Nasarawa	Kokona	8.83821	7.98671
25	Nasarawa	Kokona	8.83911	8.01819
26	Zamfara	Tsafe	12.02667	6.88916
27	Zamfara	Tsafe	12.02775	6.88679
28	Zamfara	Tsafe	12.02656	6.88577
29	Zamfara	Tsafe	12.02842	6.89183
30	Zamfara	Tsafe	12.04519	6.88357
31	Zamfara	Tsafe	12.04295	6.88122
32	Zamfara	Tsafe	12.03496	6.87785
33	Zamfara	Tsafe	12.03779	6.87315
34	Zamfara	Tsafe	12.04519	6.88357
35	Zamfara	Tsafe	12.03632	6.87102

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In Vitro Mass Multiplication of *Artocarpus heterophyllus* Lam var. Tekam Yellow

Nurul Husna Mustafa Kamal[1]*, Maheran Abd Aziz[1,2], Saleh Kadzimin[3] and Azmi Abdul Rashid[1]

[1]Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[2]Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[3]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia

ABSTRACT

A protocol for rapid micropropagation of *Artocarpus heterophyllus* from seeds of a single fruit was established. The seeds were successfully sterilised using 40% Clorox (20 min) + 20% Clorox (15 min) and 50% Clorox (20 min) + 20% Clorox (15 min). The survivability percentage was 44.44%, while the contamination percentage was 14.81%. Experiments to assess the effect of shoot tip and different node positions on shoot induction, and to test the effect of decapitation on shoot proliferation were performed. The explants used for both experiments were derived from 8-week-old seedlings grown in half-strength MS basal media supplemented with 2.5 mg/L BAP. There was no significant difference in terms of percentage of explants regenerating shoots and mean shoot number produced per explants. However, node 2 significantly produced the highest mean shoot length (2.53 cm). In the decapitation experiment, there was no significant difference in terms of percentage of explants regenerating shoots and mean shoot length. Nevertheless, decapitated shoots significantly produced the highest mean shoot number per explant (18.33). 2.5 mg/L BAP was chosen as the best treatment for shoot induction from seed with a mean shoot number of 7.33 and mean shoot length of 2.95 cm. For shoot multiplication, 1.0 mg/L BAP significantly produced the highest mean shoot number (17.13), while 5.0 mg/L BAP significantly produced the

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E-mail addresses:
nhusna.mkamal@gmail.com (Nurul Husna Mustafa Kamal)
maherandmr@gmail.com (Maheran Abd Aziz)
salehkd@upm.edu.my (Saleh Kadzimin)
azmi_ar@upm.edu.my (Azmi Abdul Rashid)
* Corresponding author

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highest shoot length (2.95 cm). For rooting, IBA at 2.5 mg/L and 5.0 mg/L produced the highest mean root number at 18.73 and 17.27 respectively. The highest mean root length (3.37 cm) was significantly obtained in the control treatment. The plantlets were successfully acclimatised in a potting mixture consisting of top soil and organic soil (1:1) with 88.89% survival rate.

Keywords: Acclimatisation, *Artocarpus heterophyllus* Lam, decapitation, micropropagation, shoot induction, shoot multiplication

INTRODUCTION

Artocarpus heterophyllus Lam. is a tropical tree that belongs to the mulberry family, Moraceae. Also known as jackfruit in English or *nangka* in Malay, it is considered a health food and a rich source of carbohydrate, protein, fat, minerals, vitamins, starch, calcium and thiamine (Baliga, Shivashankara, Haniadka, Dsouza, & Bhat, 2011; Burkill, 1997). In addition, all parts of the plant, in different forms, can be used for timber, fuel, fodder, and medicinal and industrial products, all of which have varying importance in economic values (Hasan, Ahmed, & Miah, 2008).

Jackfruit can be propagated through seeds and vegetative parts. There are, however, some problems still faced by growers. For propagation from seeds, the main problem is the recalcitrant nature of the seeds whereby the seeds cannot be stored outside for a long period of time. Moreover, seedlings from germinated seeds take longer time to reach fruit-bearing age than those trees propagated by vegetative methods. Apart from that, seedling-propagated trees grow unnecessarily taller than those propagated by vegetative methods, which is a constraint in management and harvesting process. The trees, however, can also be dwarfs and tend to produce branches at low levels which in turn results in low quality timber with a short trunk (Hossain & Haq, 2006).

Therefore, culture technique of cells, tissues, organs or whole plant under controlled environment and nutrition is generally applied to produce clones or true-to type plants of a selected genotype (Hussain, Qarshi, Nazir, & Ullah, 2012; Thorpe, 2007). It is a very quick propagation process which can lead to the production of pathogen-free plants (García-Gonzáles, Quiroz, Carrasco, & Caligari, 2010).

However, non-woody species are usually studied for tissue culture. Woody species are often labelled difficult to propagate species. According to Mc Cown (1986), some woody plant species might take up to a year to complete their establishment and initiation stage as they contained less juvenile cells than herbs. Moreover, woody species contain high phenolic compound content which could be toxic for *in vitro* plant. This was confirmed by Dobránszki and Teixeira da Silva (2010) who reported oxidation of phenolic compounds mainly caused browning and necrosis in tissue culture explants of woody species. Additionally, obtaining tap root is also a problem for woody species due to recalcitrant rooting in woody species (Mohan Jain & Häggman, 2007), thus making the tissue

culture technique for woody species more adventurous. Nunes et al. (2007) found the recalcitrance in a number of woody species to form their adventitious roots had restricted micropropagation.

Research in *in vitro* culture techniques for *A. heterophyllus* has been reported for potential mass propagation in order to produce planting materials on a large scale. There is, however, a dearth of studies on using seeds as the initial explants to produce aseptic culture for mass propagation of *A. heterophyllus* as well as to determine the best explant to provide high rejuvenation rate for *in vitro* mass propagation of *A. heterophyllus*. The position of nodes as well as the presence of shoot tips actually make a big difference in the rejuvenation rate of explants. Specifically, Shefakandeh and Khosh-Khui (2008) reported that shoot tip and the nodes from different parts of a plant shoot responded differently for growth and shoots proliferation. Usually, shoot tips are related to apical dominance. Apical dominance often plays a crucial role in shoot proliferation as it is termed as the control that the shoot tip has over axillary bud outgrowth (Cline, 1997). Apical dominance is best related to decapitation, which refers to the removal of the shoot tips (Dun, Ferguson, & Beveridge, 2006). Decapitation is normally executed as a way of testing the importance of the apical bud in controlling growth of lateral buds (Beveridge, Symons, & Turnbull, 2000).

This study investigated the possibility of micro propagating *A. heterophyllus* from seeds for mass production as they can be easily and abundantly obtained all year round which make them ideal for micropropagation. Specifically, the effects of shoot tips and different node positions on shoot induction were tested followed by the testing of the effects of decapitation on shoot proliferation in order to determine the best explants to be used for multiplication. Additionally, the effects of different plant growth regulators at varying concentrations on shoot multiplication and rooting in order to determine the optimum concentration for multiplication and rooting were tested, followed by assessment of different potting mixtures for successful acclimatisation of the plantlets.

MATERIALS AND METHODS

Six experiments were performed using seeds of *Atrocarpus heterophyllus*: (1) Effects of different concentrations of BAP and KIN on shoot proliferation from seeds; (2) Shoot induction and using shoot tips and different node positions; (3) Effects of decapitation on shoot proliferation; (4) Shoot multiplication; (5) Root induction; and (6) Acclimatisation.

Procurement of Plant Materials, Seed Surface Sterilisation and Seed Germination

Whole seeds were procured from 8-month fruits of *A. heterophyllus* trees grown at Malaysian Department of Agriculture plot in Seremban, Negeri Sembilan. Seed coats were removed, and seeds were surface-sterilised in 70% ethanol for 1 minute, followed by 20 minutes of sterilisation in

three different concentrations (30%, 40% and 50%) of Clorox® (5.25% of sodium hypochlorite). Seeds were then rinsed once with sterile distilled water, followed by 15 minutes of sterilisation in three other concentrations (10%, 20% and 30%) of Clorox®, Tween 20 was added to Clorox® as surfactant. Seeds were later rinsed 5 times with sterile distilled water. Following this, seeds were germinated on a medium consisting of half-strength MS basal salts (Murashige & Skong, 1962), supplemented with 30 g/L sucrose, 3.0 g/L Gelrite (Duchefa, Haarlem, The Netherlands) without plant growth stimulators (half-strength MSO). The medium was adjusted to pH 5.7 prior to adding agar and autoclaved at 121°C at 103 kPa for 20 minutes. The cultures were incubated at 25 ± 2ºC with a 16-h photoperiod of 35 - 40 µmol.m-2.s-1 provided by cool white fluorescent lights. The cultures were observed for 4 weeks. Two parameters were recorded: percentage of contamination and percentage of explant survival.

Effects of Different Concentrations of BAP and KIN on Shoot Proliferation from Seeds

Sterilised seeds were cultured in individual jars containing half-strength MS medium supplemented with 30.0 g/L sucrose, 3.0 g/L Gelrite™ and at different concentrations (0, 1.0, 2.5, 5.0, 7.5 and 10.0 mg/L) of BAP and KIN separately. Seed response in term of shoot induction under two cytokinin types at different concentrations was observed. Following establishment of surface sterilisation protocol obtained from this study, aseptic seeds were cultured in individual jars on half-strength MS medium supplemented with 30.0 g/L sucrose, 3.0 g/L Gelrite™ and different concentrations (0, 1.0, 2.5, 5.0, 7.5 and 10.0 mg/L) of BAP and KIN separately. The cultures were maintained on the same media for 8 weeks with sub-culturing at 4-week intervals. Eleven treatments were done with 3 replications per treatment. Three parameters were recorded: percentage of explants regenerating shoots, mean number of shoots per seed and mean length of shoots (cm).

Shoot Induction Using Shoot Tip and Different Node Positions

Shoot tip, node 1 and node 2 excised from 8-week-old seedlings were cultured on half-strength MS medium supplemented with 30.0 g/L sucrose, 3.0 g/L Gelrite™ and 2.5 mg/L BAP (the best cytokinin concentration determined from previous experiment). This study was conducted to determine the part of the shoot that is most suitable for shoot induction. The cultures were observed for 4 weeks. Three treatments with 3 replications per treatment were done. The parameters recorded were the percentage of explants regenerating shoots, mean number of shoots per explant and mean length of shoots (cm).

Effects of Decapitation on Shoot Proliferation

Shoots were excised from 8-week old seedlings and cultured on half-strength MS medium supplemented with 30.0 g/L

sucrose, 3.0 g/L Gelrite™ and 2.5 mg/L BAP (the best cytokinin concentration determined from previous experiment). For decapitated explants, the tip of the shoot was removed while for the non-decapitated explants, the tip of the shoot was left alone. This was to determine whether the removal of the apical bud would have significant effect on the growth and proliferation of lateral buds. Two treatments were done with 3 replications per treatment. The parameters recorded were percentage of explants regenerating shoots, mean number of shoots per explant and mean length of shoots (cm).

Shoot Multiplication

Nodes of shoots derived from the seeds were cultured on half-strength MS medium supplemented with 3 different cytokinin, each cytokinin administered at 5 different concentrations, namely BAP (1.0, 2.5, 5.0, 7.5 and 10.0 mg/L), KIN (1.0, 2.5, 5.0, 7.5 and 10.0 mg/L) and TDZ (0.05, 0.1, 0.5, 1.0 and 2.0 mg/L). The cultures were maintained 12 weeks in the same media with sub culturing 4-week interval. Sixteen treatments were tested with 3 replications per treatment. The parameters recorded were percentage of shoot formation, mean number of shoots and mean length of shoot (cm).

Root Induction

Shoots derived from the multiplication step were cultured on medium without hormones for 2 weeks to eliminate the effects of hormones applied in the multiplication stage. The shoots which were 5 – 6 cm long were then separated individually and placed on half-strength MS rooting medium containing concentrations of IBA and NAA at 0, 1.0, 2.5 and 5.0 mg/L for 8 weeks. Seven treatments were tested with 3 replications per treatment. The parameters recorded were percentage of root formation, mean number of roots and mean length of roots (cm).

Acclimatisation

After rinsing the completely rooted plantlet under tap water to remove the agar, the plantlets were next rinsed with sterile distilled water and soaked for 5 minutes in 0.4% fungicide (Benocide 50WT®) (Hextar Chemicals Pte. Ltd., Klang, Malaysia). The plantlets were then transferred to medium sized pots (10 x 8 cm) (W x H) containing the following potting mixtures; organic soil + topsoil (1:1), perlite + sand (1:1), peat moss + sand (1:1) and organic matter + topsoil + sand (1:1:1). Each pot was maintained at 80% relative humidity by covering it with polythene bag. The pots were then incubated for 4 weeks in a misting chamber at 25°C to 30°C with 16/8-h photoperiod (light/dark) (21 μmol $m^{-2}s^{-1}$). Four treatments were tested with 3 replications per treatment. The parameter recorded was the percentage of survived plants.

Experimental Design and Statistical Analysis

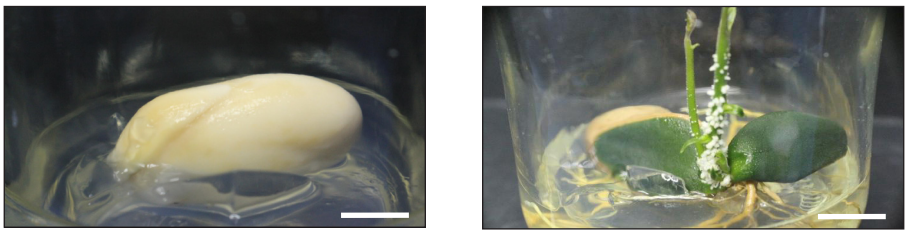
Randomized Completely Block Design (RCBD) was used in all experiments except for "Shoot Induction Using Shoot Tip and

Different Node Positions" which employed the Completely Randomized Design (CRD). Data was analysed using ANOVA except for the experiment "Effect of Decapitation on Shoot Regeneration", [t-test analysis was used]. All data was subjected to normality test, and data transformation was done if data was abnormal or the coefficient of variation (CV) values was high. Also, for data in the form of percentages, Arcsin transformation was applied. Data was analysed using SAS program version 9.3.

RESULTS

Optimising Culture Sterilisation

In this experiment, contamination of seeds by bacteria was observed around five days after sterilisation while contamination by fungi was observed a little earlier on the third day and lasted until fifth day after the sterilisation process. Figure 1(a) shows the seedlings on the first day before contamination sets in. Figure 1(b) shows the survived seedlings.



(a) (b)

Figure 1. Seed culture of *A. heterophyllus*: (a) Seed at day 1 (Bar = 0.9 cm); (b) Survived and germinated seed explant (Bar = 1.0 cm)

Table 1
Mean contamination level (%) and mean survival rate (%) of A. heterophyllus *seeds 4 weeks after exposure to different sterilisation treatments*

Clorox (%)		Mean percentage of contamination (%)*	Mean percentage of survival (%)*
(20 min)	(15 min)		
30% Clorox	+10% Clorox	33.33 ± 5.56abc	37.03 ± 3.70abc
30% Clorox	+ 20% Clorox	40.74 ± 4.90ab	33.33 ± 5.56abc
30% Clorox	+ 30% Clorox	25.92 ± 4.90abc	22.22 ± 7.86c
40% Clorox	+ 10% Clorox	51.85 ± 5.86a	51.85 ± 9.80ab
40% Clorox	+ 20% Clorox	14.81 ± 5.86cd	29.63 ± 8.69abc
40% Clorox	+ 30% Clorox	3.70 ± 3.70d	22.22 ± 5.56bc
50% Clorox	+ 10% Clorox	22.22 ± 9.62bcd	40.74 ± 10.80abc
50% Clorox	+ 20% Clorox	14.81 ± 5.86cd	44.44 ± 7.86abc
50% Clorox	+ 30% Clorox	29.63 ± 6.68abc	51.85 ± 8.07a

Note: *Means followed by the same letter(s) within each column are not significantly different ($p \leq 0.05$) using DMRT. (Data has been transformed using arcsine). In each treatment, seeds were subjected to 35 minutes of sterilisation with Clorox, split into two sterilisation stages, at 20-minute stage at a Clorox concentration followed by a 15-minute one at another Clorox concentration.

Empirical data for this part of the study are shown in Table 1 below. The table shows mean contamination level (%) and mean survival rate (%) of *A. heterophyllus* seeds 4 weeks after exposure to different sterilization treatments. The results indicated significant differences among some of the treatments.

The highest mean seed survival rates were from the sterilisation treatments consisting of 40% Clorox (20 min) + 10% Clorox (15 min) and 50% Clorox (20 min) + 30% Clorox (15 min), both giving a mean survival rate of 51.85% respectively at standard error of ±9.80% and ±8.07%. These two mean treatments did not differ significantly from each other, although each of them may or may not differ significantly from other treatments. The lowest mean survival rates were from sterilisation treatments consisting 30% Clorox (20 min) + 30% Clorox (15 min) and 40% Clorox (20 min) + 30% Clorox (15 min), both giving a mean survival rate of 22.22%, with respective standard error of ±7.86 and ±5.56. The means of the two treatments did not significantly differ from each other although each of them may or may not differ significantly from means of other treatments. Mean survival rates for other treatments range from 29.63% ±8.69 [for a sterilisation treatment consisting of 40% Clorox (20 mins) + 20% Clorox (15 mins)] to 44.44% ±7.86 [for a sterilisation treatment consisting of 50% Clorox (20 mins) + 20% Clorox (15 mins).

From Table 1, based on ANOVA and DMRT results, it can be seen that mean survival rates for some treatments are not significantly different from each other ($p>.05$) [treatments: 30% Clorox (20 mins) +10% Clorox (15 mins) ; 30% Clorox (20 mins) + 20% Clorox (15 mins); 40% Clorox (20 mins) + 20% Clorox (15 mins); 50% Clorox (20 mins) + 10% Clorox (15 mins) and 50% Clorox (20 mins) + 20% Clorox (15 mins)] while mean survival rates for other treatments are significantly different from each other ($p<.05$) and from the above-mentioned means [treatments: 30% Clorox (20 mins) + 30% Clorox (15 mins); 40% Clorox (20 mins) + 10% Clorox (15 mins); 40% Clorox (20 mins) + 30% Clorox (15 mins); and 50% Clorox (20 mins) + 30% Clorox (15 mins)].

Effects of Different Concentrations of Cytokinin (BAP and KIN) on Shoot Proliferation from Seeds

In this experiment, seeds of *A. heterophyllus* were cultured on half-strength MS media supplemented with two types of cytokinin, BAP and KIN, at different concentrations. BAP was administered at the following concentrations: 1.0 mg/L, 2.5 mg/L, 5.0 mg/L and 7.5 mg/L. KIN was administered at 1.0 mg/L, 5.0 mg/L, and 10.0 mg/L. A control treatment was set up with neither BAP nor KIN (0 mg/L). The upper seed leaf started to bend backwards after four to five days of culture and the plumule could already be seen by this time. After around 4 weeks, the shoot length was about 1.0 cm. Multiple shoots were produced after 8 weeks of culture. Results from statistical analyses showed significant differences among the treatments on percentage of explants regenerating shoots (Table 2).

Table 2
Effects of different concentrations of BAP and KIN on shoot proliferation from seeds of A. heterophyllus after 8 weeks of culture

Treatment (mg/L)	Quantity (%) of explants able to regenerate shoots*	Mean no of shoots per explant*	Mean length of shoots (cm)*
0 (control)	100.00 ± 0.00a	6.08 ± 2.12abc	2.60 ± 0.02b
1.0 BAP	100.00 ± 0.00a	3.75 ± 0.88abc	2.40 ± 0.02ed
2.5 BAP	100.00 ± 0.00a	7.33 ± 1.37abc	2.95 ± 0.03a
5.0 BAP	100.00 ± 0.00a	8.08 ± 1.90ab	2.58 ± 0.04bc
7.5 BAP	100.00 ± 0.00a	5.42 ± 1.44abc	2.45 ± 0.03cd
10.0 BAP	83.33 ± 3.55b	8.92 ± 2.56a	0.98 ± 0.04i
1.0 KIN	100.00 ± 0.00a	2.58 ± 1.04bc	2.30 ± 0.02ef
2.5 KIN	66.67 ± 3.55c	4.33 ± 0.78abc	2.23 ± 0.04gf
5.0 KIN	100.00 ± 0.00a	2.83 ± 0.58abc	2.09 ± 0.06gh
7.5 KIN	83.33 ± 3.55b	3.33 ± 1.73abc	2.09 ± 0.10gh
10.0 KIN	100.00 ± 0.00a	2.00 ± 0.51c	2.07 ± 0.14h

Note: *Means followed by the same letter(s) within each column are not significantly different ($p \leq 0.05$) using DMRT. (Data for percentage of explants regenerating shoots has been transformed using arcsine and data for mean number of shoots per explants has been transformed using square root). Shoot proliferation measured in terms of the quantity of shoots (%) regenerated by explants, mean number of shoots per explant and mean length of shoots.

The quantity (%) of explants that regenerate shoots was significantly higher for control (0 mg/L of either BAP or KIN), 1.0 mg/L BAP, 2.5 mg/L BAP, 5.0 mg/L BAP, 7.5 mg/L BAP, 1.0 mg/L KIN, 5.0 mg/L KIN and 10.0 mg/L KIN compared to the rest of the treatments. The results showed that under these treatments, all (100%) of the explants managed to regenerate shoots (100.00% ± 0.00%). At 100% rate of explants able to regenerate shoots, these treatments of course show no significant differences with each other with respect to the quantity of explants able to regenerate shoots (Table 2). In other treatments, the quantity (%) of explants regenerating shoots vary from 66.67% (2.5 mg/L KIN) to 83.33% (10.0 mg/L BAP and 7.5 mg/L KIN). Statistically, there were no significant differences in % explants that regenerate shoots between 10.0 mg/L BAP and 7.5 mg/L KIN (both producing 83.33% explants regenerating shoots). However, 10.0 mg/L BAP and 7.5 mg/L KIN were significantly different from 2.5 mg/L KIN in producing higher quantity of explants (83.33%) that regenerate shoots compared with just 66.67% for 2.5 mg/L KIN. All treatments that produced less than 100% of explants that regenerate shoots were significantly different from those treatments that produce 100% of such explants (Table 2). For the BAP group, the results showed increasing BAP concentrations from 0 mg/L to 7.5 mg/L had no adverse effects on the quantity (%) of explants that regenerated shoots; all treatments within this range of BAP concentrations produced full quantity

(100%) of explants that regenerated shoots. At 10.0 mg/L BAP, however, the quantity of explants that regenerate shoots significantly dropped to 83.33% (Table 2). Further, there is a limit to increasing BAP concentration without negatively impacting the quantity of explants that can regenerate shoots: at 10.0 mg/L BAP, the shoot formation tends to be inhibited. As for the KIN group, the results showed that the quantity (%) of explants that can regenerate shoots fluctuates rhythmically with increasing concentrations of KIN. At 1.0 mg/L KIN, all the explants (100%) were able to regenerate shoots. But increasing the KIN concentration to 2.5 mg/L significantly reduced the quantity of explants able to regenerate shoots to 66.67% and yet, when KIN concentration was further increased to 5.0 mg/L, the quantity of explants able to regenerate shoots was restored to 100%. Astonishingly, this quantity fell significantly to 83.33% when the KIN concentration was further increased to 7.5 mg/L, yet achieving again a full 100% rate when the KIN concentration was increased to 10 mg/L, the final and the highest KIN concentration in this experiment. Results for the KIN group of treatments proved a rhythmic pattern of fluctuation in the quantity of explants able to regenerate shoots with increasing KIN concentrations. As for mean number of shoots per explant, results for the BAP group of treatments showed the mean number of shoots per explant varied from the lowest of 3.75 shoots per explant (in treatment consisting of 1.0 mg/L BAP) to the highest of 8.92 shoots per explant (in the treatment consisting of 10.0 mg/L BAP) (Table 2). Results for the BAP group of treatments showed a fluctuating pattern in the mean number of shoots per explant with increasing BAP concentration. At 0 mg/L BAP (control treatment), mean number of shoots per explant was 6.08 (Table 2). But at 1.0 mg/L BAP, the mean number of shoots per explant fell to 3.75 and then increased to 7.33 at 2.5 mg/L BAP. And yet, results for these three BAP concentrations (0 mg/L, 1.0 mg/L and 2.5 mg/L) showed no statistically significant difference from each other (Table 2). Further, increasing BAP concentration to 5.0 mg/L further increased mean number of shoots per explant to 8.08, and yet when BAP concentration was increased to 7.5 mg/L, mean number of shoots per explant significantly fell to 5.42 before rising again, to 8.92 (the highest in the experiment), when BAP concentration was increased to 10.0 mg/L. The results for the BAP group of treatments showed a fluctuating trend in mean number of shoots per explant with increasing BAP concentration, and yet, all results for these treatments were not statistically different from each other as they all shared at least one superscript (Table 2). As for KIN group of treatments, the results also showed a fluctuating trend in the mean number of shoots per explant with increasing KIN concentration (Table 2). Results for KIN treatments showed mean number of shoots per explant varied between 2.00 (in treatment consisting of 10.0 mg/L KIN) to 4.33 (in treatment consisting of 2.5 mg/L KIN). Yet, statistically, KIN results were not significantly different from each other as they all shared at least

one superscript. In general, mean number of shoots per explant for KIN treatments is much lower than those obtained in BAP treatment, signifying that BAP is better as a plant growth inducer in terms of number of shoots per explant compared with KIN.

Shoot Induction Using Shoot Tip and Different Node Positions

Shoot tip, node 1 and node 2 explants excised from shoots of seed-derived plants of *A. heterophyllus* were cultured on half-strength MS media supplemented with 2.5 mg/L BAP for shoot induction. Figures 2(a), 2(c) and 2(e), respectively show the shoot tip, node 1 and node 2 during week 1 of culture. It was observed that bud break occurred within 7 to 8 days of culture. Figures 2(b), 2(d) and 2(f) respectively, show multiple shoot formation from the shoot tip, node 1 and node 2 after four weeks of culture.

The quantity **(%)** of explants able to regenerate shoots from the shoot tip, node 1 and node 2, mean number of shoots per explant and mean length of shoots are shown in Table 3.

The mean number of shoots per explant ranged from 4.47 ± 0.96 (node 2) to 3.20 ± 0.40 (shoot tip). ANOVA results showed no significant difference among the treatments on mean number of shoots per explant. Nevertheless, numerically, the mean number of shoots per explant was the highest for node 2 at 4.47 ± 0.96. For the mean shoot length, the range was between 2.53 ± 0.04 cm (node 2) and 1.28 ± 0.05 cm (shoot tip) (Table 3). Significant differences were

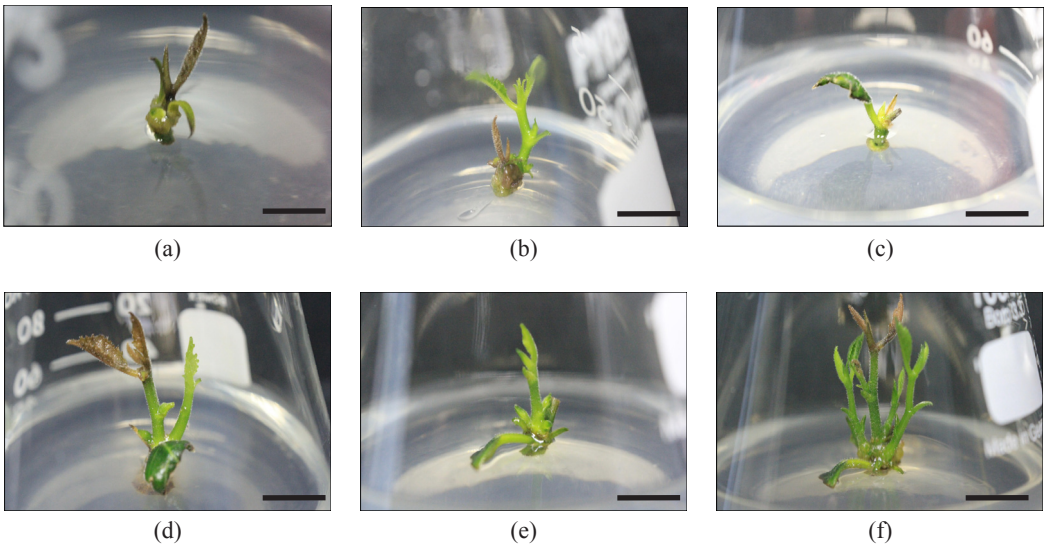


Figure 2. Shoot induction using shoot tip and different node positions of *A. heterophyllus*: (a) Week 1 shoot tip culture (Bar = 1.2 cm); (b) Week 4 shoot tip culture (Bar = 0.9 cm); (c) Week 1 node 1 culture (Bar = 0.7 cm); (d) Week 4 node 1 culture (Bar = 1.1 cm); (e) Week 1 node 2 culture (Bar = 0.8 cm); and (f) Week 4 node 2 culture (Bar = 1.1 cm)

Table 3
Effects of shoot tip and different node positions on shoot induction of A. heterophyllus *after 4 weeks of culture*

Explant	Quantity (%) of explants able to regenerate shoots	Mean no of shoots per explant*	Mean length of shoots (cm)*
Shoot Tip	100.00	3.20 ± 0.40a	1.28 ± 0.05c
Node 1	100.00	4.07 ± 0.42a	2.39 ± 0.05b
Node 2	100.00	4.47 ± 0.96a	2.53 ± 0.04a

Note: *Means followed by same letter within columns are not significantly different (p>0.05). Data for mean number of shoots per explant has been transformed using square root.
These effects are measured in terms of the quantity (%) explants able to regenerate shoots, mean number of shoots per explant and mean length of shoots.

observed between the treatments on mean shoot length based on the ANOVA results. Results from DMRT revealed that the mean shoot length for node 2 (2.53 ± 0.04 cm) was significantly higher than node 1 and shoot tip. Shoots derived from shoot tip only reached 1.28 ± 0.05 cm in length.

Effects of Decapitation on Shoot Proliferation

The effects of decapitation on *A. heterophyllus* shoots was observed after six weeks of culture on half-strength MS media supplemented with 2.5 mg/L BAP. Figures 3(a) and 3(c) shows respectively, decapitated and non-decapitated shoot on day 1. Bud break from the axillary buds occurred

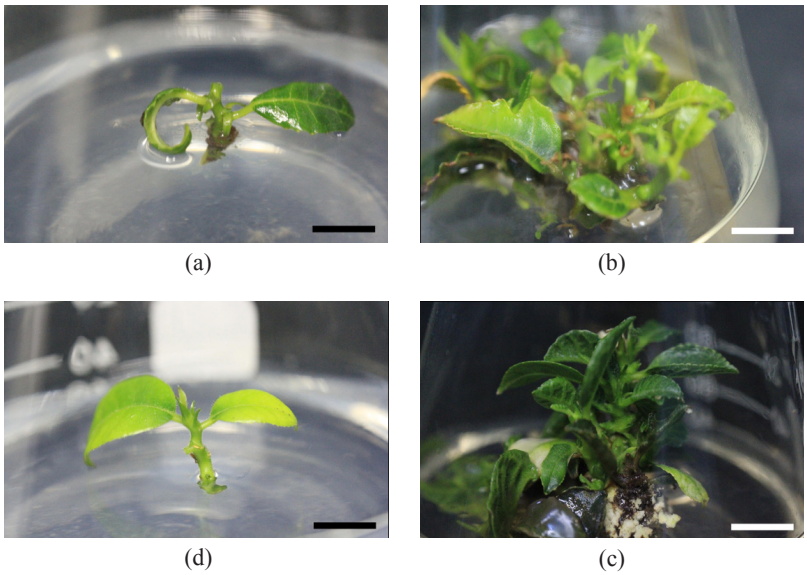


Figure 3. Effect of decapitation on shoot proliferation of *A. heterophyllus*: (a) Decapitated shoot at day 1 of culture (Bar = 1.1 cm); (b) Decapitated shoot after 6 weeks of culture (Bar = 0.9 cm); (c) Non-decapitated shoot at day 1 of culture (Bar = 1.2 cm); and (d) Non-decapitated shoot after 6 weeks of culture (Bar = 1.2 cm)

within 7 to 8 days of culture. Figures 3(b) and 3(d), respectively show the response of the decapitated and non-decapitated shoot after 6 weeks of culture. The quantity (%) of explants able to regenerate shoots from the shoot tip, node 1 and node 2, mean number of shoots per explant and mean length of shoots are shown in Table 4.

The result showed significant difference between the two treatments on mean number of shoots per explants. The mean number of shoots per explant for shoots without shoot tip (18.33 ± 2.12) was significantly higher than those attained by the non-decapitated shoots at 8.67 ± 2.31 (Table 4). As for the mean shoot length, there was no significant difference between both treatments. Nevertheless, shoots without shoot tip indicated the highest mean (2.31 ± 0.27 cm) compared with the shoots with shoot tip (1.89 ± 0.36 cm).

Table 4
Effects of decapitation on shoot proliferation from A. heterophyllus *shoot after 6 weeks of culture*

Explants	Quantity (%) of explants able to regenerate shoots	Mean no of shoots per explant*	Mean length of shoots (cm)*
With shoot tip	100.00	8.67 ± 2.31b	1.89 ± 0.36a
Without shoot tip	100.00	18.33 ± 2.12a	2.31 ± 0.27a

Shoot Multiplication

Nodal explants were cultured on half-strength MS media supplemented with different concentrations of BAP, KIN and TDZ for shoot multiplication. Bud break from the axillary buds began at day 7 to day 8 of culture. Shoot proliferation was observed after 2 weeks of culture. Table 5 shows the quantity (%) percentage of explants able to regenerate shoots, mean number of shoots produced per explant and mean shoot length attained after 12 weeks of culture.

The results show that quantity (%) of explants able to regenerate shoots varied from a low of 66.67% (in a treatment consisting 1.0 mg/L TDZ) to 100% (in treatments consisting of 0 mg/L (control treatment devoid of any cytokinin), 1.0 mg/L BAP, 2.5 mg/L BAP, 7.5 mg/L BAP, 10.0 mg/L BAP, 5.0 mg/ L KIN, 7.5 mg/L KIN, 10.0 mg/L KIN, 0.05 mg/L TDZ, 0.1 mg/L TDZ, 0.5 mg/L TDZ and 2.0 mg/L TDZ) (Table 5). All treatments, except for one that uses 1.0 mg/L TDZ, are not significantly different from one another in terms of the quantity (%) of explants able to regenerate shoots. For the treatment that uses 1.0 mg/L TDZ, the quantity (%) of explants able to regenerate shoots, at 66.67%, was significantly lower than values obtained in other treatments.

As for the mean number of shoots per explant, the results show that values ranged between 17.13 ± 3.54 (1.0 mg/L BAP) and 1.13 ± 0.09 (1.0 mg/L TDZ). Statistical analyses have bundled results into groups; within a group, results are not statistically

Table 5
Effects of different types of cytokinin (BAP, KIN and TDZ) at different concentrations on shoot multiplication of A. heterophyllus *after 12 weeks of culture*

Treatment (mg/L)	Quantity (%) of explants able to regenerate shoots	Mean no of shoots per explant*	Mean shoot length (cm)*
0 (control)	100.00 ± 0.00a	5.80 ± 1.27b	2.30 ± 0.33a
1.0 BAP	100.00 ± 0.00a	17.13 ± 3.54a	1.09 ± 0.08c
2.5 BAP	100.00 ± 0.00a	4.00 ± 1.18bcd	0.92 ± 0.30c
5.0 BAP	86.67 ± 2.52ab	4.13 ± 0.66bc	2.95 ± 0.77a
7.5 BAP	100.00 ± 0.00a	2.67 ± 0.57cdef	1.12 ± 0.10c
10.0 BAP	100.00 ± 0.00a	3.87 ± 0.38bc	0.76 ± 0.04c
1.0 KIN	93.33 ± 2.521a	3.40 ± 0.48bcd	2.18 ± 0.36ab
2.5 KIN	80.00 ± 7.56ab	1.13 ± 0.13f	1.40 ± 0.14bc
5.0 KIN	100.00 ± 0.00a	3.47 ± 0.95bcde	1.14 ± 0.09c
7.5 KIN	100.00 ± 0.00a	2.67 ± 0.50cdef	1.01 ± 0.02c
10.0 KIN	100.00 ± 0.00a	1.67 ± 0.13def	0.99 ± 0.08c
0.05 TDZ	100.00 ± 0.00a	3.40 ± 0.62bcd	1.30 ± 0.10bc
0.1 TDZ	100.00 ± 0.00a	4.93 ± 1.28bc	0.72 ± 0.05c
0.5 TDZ	100.00 ± 0.00a	4.07 ± 0.78bc	0.85 ± 0.06c
1.0 TDZ	66.67 ± 6.67b	1.13 ± 0.09ef	0.66 ± 0.03c
2.0 TDZ	100.00 ± 0.00a	2.27 ± 0.49cdef	0.86 ± 0.05c

Note: *Means followed by the same letter(s) within each column are not significantly different ($p \leq 0.05$) using DMRT. (Data for percentage of explants regenerating shoots has been transformed using arcsine; data for mean number of shoots per explants has been transformed using square root and data for mean length of shoots has been transformed using square root).
Effects were measured in terms of quantity (%) of explants able to regenerate shoots, mean number of shoots produced per explant and mean shoot length after 12 weeks of culture.

different from each other. There is also overlapping between groups: some results in a group are not statistically significant from some results in other groups. For example, the result from the treatment that uses 1.0 mg/L TDZ is significantly different from the one that uses 0.5 mg/L TDZ, yet both are not significantly different from the treatment that uses 7.5 mg/L BAP (Table 5). A single treatment, one that uses 1.0 mg/L BAP, is in a group of its own. It produces the highest mean number of shoots per explant, at 17.13, significantly different and higher than values from all the other treatments. The results thus show that at 1.0 mg/L, BAP induces the highest mean number of shoots per explant (17.13) compared with KIN (mean of 3.40 shoots per explant at 1.0 mg/L) and TDZ (mean of 1.13 shoots per explant at 1.0 mg/L). As concentration of each type of cytokine is increased from the initial 1.0 mg/L, the results showed that mean shoot number per explant for all types of cytokinin fluctuated and was at much lower value compared with 17.13 obtained from 1.0 mg/L BAP. Results showed that after

1.0 mg/L, mean shoot number per explant varied from a low of 1.13 (2.5 mg/L KIN and 1.0 mg/L TDZ) to 4.93 (0.1 mg/L TDZ). All values in this range are lower than 5.80 obtained for the control treatment (Table 5).

Results for mean shoot length meanwhile showed a range of values from a low of 0.72 cm (0.1 mg/L TDZ) to a high of 2.95 cm (5.0 mg/L BAP) (Table 5). The control treatment (0 mg/L and devoid of any growth regulator) yields a value of 2.30 cm, significantly high and not different from the numerically highest value of 2.95 cm obtained from concentration level of 5.0 mg/L BAP. Results from the control treatment and 5.0 mg/L BAP were significantly different and higher from those obtained in other treatments. Generally, shoot mean length values are statistically bundled in group based on results of statistical analyses. Within each group, mean shoot length values are not significantly different from each, but there is some overlapping between groups whereby values within a group may be statistically similar to values in other groups. Most values are in the lowest range with letter 'c' attached to them as shown in Table 5, with values ranging from just 0.66 cm to just 1.40 cm (Table 5).

Rooting of Shoots

Shoots of *A. heterophyllus* were cultured on half-strength MS media supplemented with two types of auxins which (IBA and NAA) at different concentrations. The root initiation began as early as by the second week of culture. The results for this experiment are presented in Table 6. The results showed the quantity (%) of explants able to regenerate regenerating roots ranged from 100.00% (in treatment consisting of 2.5 mg/L IBA) to 66.67% (in treatment consisting of 5.0 mg/L IBA), after 8 weeks of culture (Table 6).

Table 6
Effects of two different types of auxins (IBA and NAA) at different concentrations on root induction of A. heterophyllus *after 8 weeks of culture*

Treatment (mg/L)	Quantity (%) of explants able to regenerate roots*	Mean no of roots per explant*	Mean root length (cm)*
0 (control)	80.00 ± 4.36ab	6.80 ± 0.35b	3.37 ± 0.18a
1.0 IBA	86.67 ± 5.04ab	9.13 ± 0.87b	1.62 ± 0.19c
2.5 IBA	100.00 ± 0.00a	18.73 ± 2.71a	1.74 ± 0.15c
5.0 IBA	66.67 ± 2.52b	17.27 ± 2.10a	1.50 ± 0.21c
1.0 NAA	80.00 ± 4.36ab	4.20 ± 1.24c	3.07 ± 0.27ab
2.5 NAA	93.33 ± 2.52ab	6.20 ± 0.75bc	1.85 ± 0.17bc
5.0 NAA	73.33 ± 2.52b	7.53 ± 0.57b	2.00 ± 0.19bc

Note: *Means followed by the same letter(s) within columns are not significantly different ($p \leq 0.05$) using DMRT. (Data for percentage of explants regenerating roots has been transformed using arcsine; data for mean number of roots per explant has been transformed using square root and data for mean length of roots has been transformed using square root).
Effects are measured in terms of quantity (%) of explants able to regenerate shoots, mean number of roots per explant and mean root length.

Most treatments showed no significant differences from each other in terms quantity (%) of explants able to regenerate, except for treatments 2.5 mg/L IBA which produces result (100% explants able to regenerate roots) significantly different from those of treatments 5.0 mg/L IBA (66.67% explants able to regenerate roots) and 5.0 mg/L NAA (73.33% explants able to regenerate roots) (Table 6). The latter two treatments (5.0 mg/L IBA and 5.0 mg/L NAA) do not significantly differ from each other in the quantity (%) explants able to regenerate roots. In general, most treatments produced high quantity (%) of explants able to regenerate roots except for treatments 5.0 mg/L IBA and 5.0 mg/L NAA which respectively yield relatively lower values of 66.67% and 73.33% (Table 6).

It can be clearly seen that both auxins showed relatively the same trend. The results show that as concentration increases from 1.0 mg/L to higher levels of 2.5 mg/L and 5.0 mg/L for both auxins, the quantity (%) of explants able to regenerate root increases (at 2.5 mg/L) followed by a decrease (at 5.0 mg/L). This shows that for both IBA and NAA, increasing the concentration to more than 2.5 mg/L tend to inhibit root formation in *A. heterophyllus*. Results for the mean number of roots per explant show that the values for mean number of roots per explants ranged from a high of 18.73 roots per explant (at 2.5 mg/L IBA) to 4.20 roots per explants (at 1.0 mg/L NAA). Statistical analyses using ANOVA (using square roots transformed data) and DMRT results have bundled the treatments into groups. Within each group, mean number of roots per explant are not significantly different from one another (Table 6). Most groups are significantly different from each other in term of mean number of roots per explant, although there is a little overlapping between groups, whereby a mean within a group is statistically similar to a mean from another group. In general, IBA tends to produce significantly higher mean number of roots per explant (18.73 roots per explant to 9.13 roots per explant) compared with NAA (7.53 roots per explant to 4.20 roots per explant) (Table 6). The mean number of roots per explant for the control treatment which is devoid of any auxin (6.80 roots per explant) is generally significantly similar to values from NAA treatment but significantly lower than more values from IBA treatments.

As for the mean root length, overall results show a range of 3.07 cm (1.0 mg/L NAA) to 1.50 cm (5.0 mg/l IBA), not including the value from the control treatment (3.37 cm) (Table 6). The results for the control treatment (3.37 cm) is significantly higher than results from other treatment except 3.07 cm from treatment 1.0 mg/L NAA which is not significantly different. Most of the lower values are not significantly different from each other (Table 6). Results further indicate that mean root lengths for all NAA treatments are significantly higher than all values from IBA treatments, although IBA tend to result in significantly higher mean number of roots per explant (table 6). From this experiment it shows that IBA

is better than NAA for rooting of *A. heterophyllus* shoots as tap root (Figure 4(c)) was also obtained which is important to support the plant later on.

Acclimatisation

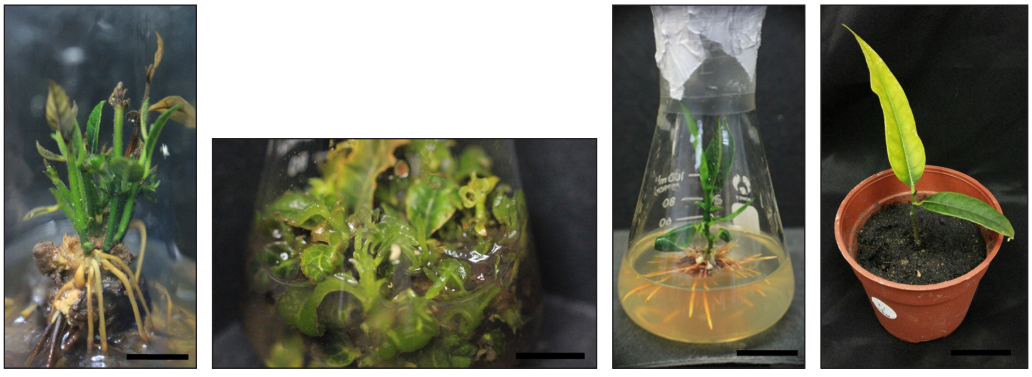
The survival rate (%) of *A. heterophyllus* plantlets was observed 8 weeks after transplanting them into different potting mixtures. The results for this experiment are show in Table 7.

The 0% survival rate for peat moss + sand at 1:1 (v/v) was due to the fact that all plantlets did not survive (Table 5). The results showed that survival rates range from 88.89% (organic soil + top soil at 1:1 ratio) to 22.22% (Organic matter + topsoil + sand at 1:1:1 ratio). The survival rate of 88.99% in organic soil+topsoil (1:1) was significantly higher than survival the 44.44% survival rate in perlite+sand (1:1) and the 22.22% survival rate in organic matter+topsoil+soil (1:1:1). The survival rates in the latter two potting mixtures (perlite+sand at 1:1 ratio and organic matter + topsoil + sand at 1:1:1 ration) were not significantly different from each other. The results thus showed that organic soil + topsoil (1:1) is the best potting mixture for growth for *A heterophyllus*. Figure 4(d) shows a plantlet surviving in the best potting mixture of (organic soil + topsoil (1:1)).

Table 7
Survival (%) of A. heterophyllus *plantlets after 8 weeks of acclimatisation in different potting mixtures*

Potting mixture	Survived plantlets (%)
Organic soil + topsoil (1:1)	88.89 ± 5.56a
Perlite + sand (1:1)	44.44 ± 5.56b
Peat moss + sand (1:1)	0.00 ± 0.00c
Organic matter + topsoil + sand (1:1:1)	22.22 ± 5.56b

Note: *Means followed by the same letter(s) within columns are not significantly different ($p \leq 0.05$) using DMRT. (Data for percentage of explants regenerating roots has been transformed using arcsine)



(a) (b) (c) (d)

Figure 4. Micropropagation stages of *A. heterophyllus*: (a) Shoot induction after 8 weeks of culture on 2.5 mg/L BAP; (b) Shoot multiplication on 1.0 mg/L BAP after 12 weeks of culture; (c) Rooting in 2.5 mg/L IBA after 8 weeks of culture; (d) Plantlet surviving in a potting mixture consisting of organic soil + topsoil (1:1) after 8 weeks of acclimatisation

DISCUSSION

Plants growing in the external environment are invariably contaminated with micro-organisms and pests (George, Hall, & De Klerk, 2008). Anabestani, Behjatnia, Izadpanah, Tabein and Accotto (2017) reported pathogenic seed infection had a negative effect on the development of seed and plant. Thus, it is crucial to eliminate pathogens at the seed level through surface sterilisation for tissue culture. In this study, the experiment on optimisation of culture sterilisation showed the sterilization regimes of 40% Clorox for 20 min + 20% Clorox for 15 min and 50% Clorox for 20 min + 20% Clorox for 15 min can eliminate contamination. Both sterilisation treatments showed low rate (%) of contamination and high rate (%) of survival.

In comparison to previous works, Choy and Chan (2005) had used 20% Clorox for 10 min followed by 10% Clorox for 10 min for sterilising the apical shoots taken from a 12-year-old *A. heterophyllus* tree. Their sterilisation procedure was evidently different from the one used in this experiment. According to Abd El-Zaher (2008), different sterilisation procedures required by different types of explant could be maybe due to the various anatomical structures that these explants had. According to Lizarraga, Tovar, Jayasinghe and Dodds (1986) ethanol was used because it has a wetting property that can easily penetrate between leaf hairs and the wet surface of explants. Additionally, ethanol can partially remove hydrophobic waxes and resins which protect the microorganisms from getting in contact with sterilant (Kunneman & Faaij-Groenen, 1988). On the other hand, sodium hypochlorite, also known commercially as Clorox, is mainly used as a surface sterilising agent in plant cell and tissue culture experiments (Çölgeçen, Koca, & Toker, 2011), and it is known to be an effective and powerful antimicrobial agent (Abd El-Zaher, 2008). The active components in Clorox bleach are water, sodium hypochlorite (it whitens and kills bacteria), sodium chloride, sodium carbonate (it removes alcohol and grease stains), sodium chlorate, sodium hydroxide (it removes soils that are fatty, oily, or acidic), and sodium polyacrylate (Anonymous, Wikipedia, 2018). Mng'omba, Sileshi, Toit and Akinnifesi (2012) reported that Clorox acts as an oxidising agent that kills a large range of pathogens. George (1993) reported that Clorox has the potential as a germicide which is related to its oxidation reaction properties.

In the experiment on shoot proliferation from seeds, results showed that the shoot-inducing regime of 10.0 mg/L BAP induced the highest number of shoots. Nonetheless, 2.5 mg/L BAP produced higher number of shoots of suitable length for multiplication. The discrepancy in 10.0 mg/L BAP regime is probably caused by the competition among the shoots in absorbing the hormones and nutrients from the agar which resulted in shorter shoots even though the number of shoots produced was higher. Ashraf, Aziz, Kemat and Ismail (2014) reported the same observation in *Chlorophytum borivilianum* when BAP concentration was increased.

Thomas (2003), in contrast**,** obtained the best result of shoot induction using 1.2 mg/L BAP from cotyledon explants of *Morus alba*. Verma, Choudhary, Ashish, Kumar and Lal (2015) used seeds of ripened fruits of *A. lakoocha* for preparation of nodal segments as explants for synthetic seed production. Hassan and Khatun (2010) used germinated seeds of *Ficus glomerata* to produce shoot tips and node explants for adventitious shoot regeneration. The BAP requirement could be different for different species even though they are from the same family. Buah et al. (2010) mentioned that the variation in performance of *in vitro* cultures could be due to the differences in the genomic constitution or phenolic contents of a cultivar or species. Arinaitwe, Rubaihayo and Magambo (2000) studied proliferation rate effects of cytokinins on banana (Musa spp.) cultivars and reported that shoot proliferation is cultivar-dependent.

Cytokinin promote the growth of axillary buds by reducing the apical dominance of buds during the micropropagation phase (Van Staden, Zazimalova, & George, 2008). In addition, cytokinin are known to stimulate plant cell division as well as their involvement in releasing lateral bud dormancy, inducing adventitious bud formation, inducing growth of lateral buds, and their role in the cell cycle control (Melara & Arias, 2009). However, based on observation in this experiment, BAP, as a cytokinin, appears superior to kinetin as BAP produced higher mean number of shoots per explant as well as higher mean shoot length. Bogaert, Van Cauter, Werbrouck and Dolezal (2006) stated that kinetin has a relatively low biological activity in certain bio-assays. Within the cytokinin group, BAP is more broadly used for in vitro shoot induction compared to other cytokinin as it exhibits higher shoot induction in many taxa (Isah & Mujib, 2013).

In the experiment on effects of shoot tip and different node positions on shoot regeneration of *A. heterophyllus,* the results obtained were similar to those of Rahman and Blake (1988). The researchers studied the effects of the same parameters on *A. heterophyllus* and found the number of shoots produced by nodal explants was not significantly different from the number of shoots produced from shoot tips, although numerically more shoots were produced by nodal explants than by shoot tips. However, the results on shoot length obtained in this study differed from those of Rahman and Blake (1988) who reported that shoot tip produced higher shoot length. The difference could be due to the fact that the cultivars used in this study are different from those used in the study by Rahman and Blake (1988). Apart from that, the varied response of different explant positions may be due to the variation in the endogenous auxin level of buds in different regions of the stem (Lane, 1978). Shirdel, Motallebi-Azar, Matloobi and Zaare-Nahandi (2013) also observed significant differences among node positions of Dog rose (*Rosa canina*), with the lower nodal position (node 2) having the longest shoot as well as having the highest number of shoots when cultured

in 6 mg/L BAP. Shekafandeh and Khosh-Khui (2008) also observed node 2 having the maximum number of shoots and shoot length in their study on effect of bud position in guava (*Psidium guajava* L.). Yadav, Lal and Jaiswal (1990) stated that shoot tips of *Syzigium cuminii* were less responsive than nodal explants. This is perhaps caused by the differences in the physiological state of the two explant types. Shoot induction from axillary or nodal bud is one of the most efficient methods of micropropagation in plants, considering the fact that buds emerging especially from meristematic tissues possess great potential for vigorous development due to their totipotency ability (Yadav, Malan, & Rajam, 1995). Taiz and Zeiger (2002) reported that carbohydrates played an important role in providing normal growth and development of shoots emerging from the nodes. Chern, Hosokawa, Cherubini, & Cline (1993) added that axillary bud growth was influenced by the node position in *Ipomoea nil*. They also observed that outgrowth of axillary buds at the lower part was reduced compared to that of the higher nodes. In addition, Punyarani and Sharma (2010) mentioned that cutting the stem of *Costus speciosus* into different node positions and then culturing them on suitable medium supplemented with suitable PGRs could break the dormancy of the bud, resulting in greater shoot proliferation. They also concluded that bud-break was affected by the position of the nodal buds on the stem.

Since there is no report on decapitation of *Artocarpus* species or any other species from the Moraceae family, the results obtained in this study were compared with those obtained from studies of plants of different families. For example, the findings from this study were similar to that reported by Singh and Tiwari (2012) who showed high frequency of shoot regeneration from decapitated embryonic axes of *Clitoria ternatea* in 2.0 mg/L BAP. Pumisutapon, Visser, & Klerk (2009) reported that decapitated rhizome of *Alstroemeria* showed the highest bud outgrowth whereby the axillary buds were released when rhizome tip and shoot tips were removed. Ngamau (2001) observed that seedling decapitation in *Zantedeschia aethiopica* 'Green Goddess' also resulted in an increased number of axillary shoot production. El Boullani, Elmoslih, El Finti, El Mousadik and Serghini (2012) reported that artichoke (*Cynara cardunculus* var. *scolymus* L.) seedlings produced greater shoot proliferation ratio (17 shoots per explant) when decapitated. Vieitez, Pintos, San-José and Ballester (1993) tested the effects of decapitation on a woody plant, *Quercus rubra* (Red Oak) and was able to attain vigorous axillary bud growth on woody plant medium with 0.2 mg/L BAP. Cline (1994) described apical dominance as the control exerted by the terminal bud (or shoot apex) over the outgrowth of lateral buds in order for plants to grow vertically. Apical dominance is said to significantly lessen shoot branching (Bressan, Kim, Hyndman, Hasegawa, & Bressan, 1982; Kucharska, Golis, Podwyszyńska, Wiśniewska-Grzeszkiewicz, & Orlikowska, 2000; Voyiatzi, Voyiatzis

and Tsiakmaki, 1995). Based on the auxin-inhibition hypothesis of apical dominance, Prasad *et al.* (1993) stated that auxin flowed down the stem and inhibits axillary bud outgrowth, either directly or indirectly. Dun *et al.* (2006) reported that decapitation had been widely used to study bud outgrowth which was best demonstrated via shoot tip removal. Tezuka *et al*. (2011) hypothesised that endogenous cytokinin synthesised after decapitation caused the promotive effect of CDM (complete decapitation method) on shoot regeneration. According to Punyarani, and Sharma (2010), it was possible to break bud dormancy in cultured nodal segments due to its separation from the shoot apex.

Punyarani, and Sharma (2010) in their experiment on shoot multiplication, observed that 1.0 mg/L BAP gave the highest mean number of shoots. The results were consistent with those of Amin and Jaiswal (1993) who also reported that 4.5 µM BAP (1.0 mg/L BAP) gave the highest multiplication of shoots of *A. heterophyllus*. However, Ashrafuzzaman, Kar and Prodhan (2012) and Khan, Rahman, Abbasi, Ibrahim and Abbas (2010) reported producing high shoot multiplication of *A. heterophyllus* on medium containing 2.0 mg/L BAP and 1.5 mg/L BAP respectively. Choy and Chan (2005) reported that 4.5 mg/L BAP was the best treatment in producing the most number of shoots per explant in *A. heterophyllus*. BAP is suitable for shoot regeneration and multiplication for most plants. Kumar, Krishna, Pradeepa, Kumar and Gnanesh (2012) reported that the effects of BAP perhaps lied in its capability to trigger plant tissues to metabolise the natural endogenous hormones for shoot organogenesis induction. Taiz and Zeiger (1998) opined the tremendous activity of this plant growth regulator was probably related to its chemical structure, but high concentrations could induce reduction in shoot height and increase in hyperhydricity rates.

The highest mean shoot length obtained in this study was in 5.0 mg/L BAP. Choy and Chan (2005) showed the best mean shoot length in 4.0 mg/L BAP. Amin and Jaiswal (1993) and Ashrafuzzaman *et al.* (2012) obtained the best shoot length in 2.0 mg/L BAP. Meanwhile, Amany, Ali and Boshra (2007) achieved the best shoot length in 3.0 mg/L BAP. The difference may be due to different varieties of *A. heterophyllus* used in these studies which gave different responses. Lima da Silva, Rogalski and Guerra (2003) found the mean height of shoots of Prunus 'Capdeboscq' was reduced with increased concentration of the different cytokinin used and similar results were observed in this study. This is probably caused by competition among the proliferated shoots in absorbing hormones and nutrients from the agar (Ali, Mulwa, Norton, & Skirvin, 2003). Ledbetter and Preece (2004) in their work on *Hydrangea quercifolia* reported that BAP was more effective than TDZ as shoots in TDZ failed to elongate. Similar results were also obtained in this study for *A. heterophyllus* using BAP and TDZ.

In the experiment on rooting, the results of this study showed that 2.5 mg/L IBA was the best treatment as it produced the highest number of roots per explant with 100% of explants regenerating roots. Abd El-Zaher (2008) recommended half-strength MS + 3 mg/L IBA as the optimal treatment for producing good rooting in *A. heterophyllus*. On the other hand, Amany *et al*. (2007) reported that half-strength MS supplemented with 1.5 mg/L IBA produced the highest number of roots per explant in jackfruit while full MS medium supplemented with 1.5 mg/L NAA gave the greatest root length. Ashrafuzzaman *et al*. (2012) reported that half-strength MS supplemented with 2.0 mg/L IBA was the best in producing the highest number of roots for jackfruit.

Apart from playing a role in root formation, auxins also exert a strong influence on initiation of cell division, meristem organisation giving rise to unorganised tissue (callus) or defined organs (shoots), cell expansion, cell wall acidification, apical dominance and promotion of vascular differentiation (Gaspar et al., 1996, 2003). In this study, the highest mean root length was obtained in half-strength MS medium without hormone (control treatment). Ashrafuzzaman *et al*. (2012), however, obtained the best mean root length for jackfruit in half-strength MS medium supplemented with 2.0 mg/L IBA while Amin and Jaiswal (1993) obtained it in half-strength MS medium supplemented with 2.25 mg/L IBA. Earlier, Epstein and Ludwig-Muller (1993) stated that the application of IBA to cuttings of many plant species resulted in the induction of adventitious roots, which in many cases was more effective than IAA.

According to Wareing and Phillips (1981), increasing the concentration of IBA from 10^{-7} to 10^{-5} M increased the number of roots, but the length was significantly decreased at the highest concentration. Such inhibition of root development by high concentrations of auxin may be due to the enhancement of ethylene biosynthesis in the root tissues. Sevik and Guney (2013) stated that auxins control the growth and development of roots in plants, including lateral root initiation and root gravity response. Generally, high level of auxin promoted the production of adventitious roots, despite the fact that it also inhibited root elongation (Blakesley, Weston, & Hall, 1991).

The results on acclimatisation of plantlets in this study were in accordance with that of Choy and Chan (2005) who also reported 70-80% survival of *A. heterophyllus* plantlets after 3 – 4 weeks maintenance in a mixture of organic soil + topsoil (1:1) in a chamber. On the other hand, Abd El-Zaher (2008) obtained the lowest survival percentage of *A. heterophyllus* plantlets in potting mixture consisting of peat moss + sand (1:1). In this study, the same potting mixture comprising peat moss + sand (1:1) resulted in no survival. Amin and Jaiswal (1993) reported that regenerated plantlets of jackfruit transferred to the soil resulted

in 50% survival rate. Roy, Rahmanand and Majumdar (1990) reported that they succeeded in the acclimatisation of jackfruit using potting mixture of sterile sand + soil + humus (1:2:1) where 75% of the plants survived.

CONCLUSION

This study showed the protocol for producing mass production of *A. heterophyllus* was successful in a short period of time. The technique can be used to produce clonal materials in place of the present method for propagating jackfruits using seeds. In this way, the seed recalcitrant problem and the lengthy grafting method often faced by farmers can be overcome. The established tissue culture protocol can be a platform for future transformation or future related studies on *A. heterophyllus*. As hormone combination was not tested during this study period, future study should consider that. Additionally, micrografting can be done using this protocol.

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Evaluation of Bouillon Cube Prepared with the Addition of Threadfin Bream *(Nemipterus japonicas)* Hydrolysate

Normah Ismail* and Nurfathin Saadah Sahibon

Food Science and Technology Program, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), Shah Alam, Selangor 40450, Malaysia

ABSTRACT

Threadfin bream was hydrolyzed by enzymatic hydrolysis for 120 min (60°C) at pH 8.5, enzyme:substrate ratio 1:3 using Flavourzyme 500 L to produce the threadfin bream hydrolysate (TBH). Bouillon cubes prepared by incorporating TBH or isolated soy protein (ISP) were then analyzed for physicochemical and functional properties, sensory properties and acceptability. Results showed that bouillon cubes added with TBH contain 23.05% protein and 18.53% fat. Solubility, hardness and fracturability of bouillon cubes containing TBH were better than those incorporated with ISP. SDS–PAGE results indicated the presence of short peptides of <20 kDa especially in bouillon cubes with the addition of TBH. The bouillon cubes had bitter and umami taste with intensities of respectively, 9.88 and 11.70 compared to the reference solutions; 12.10 and 12.01. Moreover, functional group analysis showed the existence of amines and carbonyl group peaks. Based on its binding capacity, TBH can be used as partial ingredient in the development of bouillon cube.

Keywords: Bouillon cube, hydrolysate, taste, threadfin bream

INTRODUCTION

Bouillon, commonly described as broth, is made by cooking meat, poultry, fish, or vegetables and other ingredients such as onion, tomatoes, salt and oil in water (Lukmanji et al., 2008). The broth can be further dehydrated and compressed into flavor-concentrated cubes known as bouillon cubes. The addition of bouillon cubes augments food flavor (Akpanyung, 2005). Apart from monosodium glutamate and salt as the main ingredients, bouillon cubes are also prepared with the addition of fat, starch, and spices (Caponio, Gomes, & Bilancia, 2003). Industrially, bouillon cube processing involves mixing of dry

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E-mail addresses:
norismel@salam.uitm.edu.my/normaisma@yahoo.com (Normah Ismail)
nurfathinsaadah@gmail.com (Nurfathin Saadah Sahibon)
* Corresponding author

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ingredients with molten fat before it is cooled, shaped, wrapped and packed (Gupta & Bongers, 2011). Bouillon cubes are used during cooking as it is one of the convenient ways to make variety of food such as condiment, gravies and soup. The commercial bouillon cubes available are chicken, mushroom, pork, and seafood broth cubes (Chiang, Yen, & Mau, 2007).

Protein hydrolysate is a product obtained from the hydrolysis of protein-rich sources (Jin et al., 2014; Klompong, Benjakul, Kantachote, & Shahidi, 2007; Mutilangi, Panyam, & Kilara, 1996). Hydrolysate has been produced from soybean, whey protein, fish, shellfish, chicken, and many others. The characteristics of fish and fish byproduct hydrolysates have been well documented (Chalamaiah, Hemalatha, & Jyothirmayi, 2012; Sathivel et al., 2003; Wiriyaphan, Chitsomboon, & Yongsawadigul, 2012). Isolated soy protein (ISP) is a highly refined and concentrated protein fraction produced from soybean (Crockett & Vodovotz, 2011). It is one of the most common ingredients available in commercial bouillon cubes. ISP has also been used as a binder in meat products such as sausages, beef patties, mortadell, meat ball, and corned beef while protein hydrolysate can be used as milk replacers, protein supplements, beverage stabilizers, and flavor enhancers (Li, Youravong, & Aran, 2010).

Incorporating fish protein hydrolysate may improve bouillon cube functionality in terms of solubility, texture and binding capacity. It could also enhance the flavor and nutritional aspect of the bouillon cube. The role of fish hydrolysate from threadfin bream (*Nemipterus japonicas*) as a binding agent in forming a good texture of bouillon cubes has never been explored. Besides, no study has been done on the incorporation of threadfin bream hydrolysate (TBH) in the form of bouillon cubes to serve as flavor enhancer. This study was carried out to evaluate the physicochemical, functional, and sensory properties of bouillon cubes added with TBH.

MATERIALS AND METHODS

Materials

The fish, threadfin bream *(Nemipterus japonicas)* and red snapper (*Lutjanus erythropterus)* were purchased from a wet market in Selangor, Malaysia. The fish were stored in an ice box filled with ice and immediately transported to the laboratory. Flavourzyme 500 L with a declared activity of 500 Leucine Amino Peptidase Units per gram (LAPU/g) was obtained from Novo Nordisk Industries (A/S, Bagsvaerd, Denmark). Bouillon cube ingredients such as salt, sugar and corn starch were bought from the nearest local hypermarket. Chemicals were purchased from Sigma Aldrich (M) Sdn Bhd. Malaysia.

Preparation of Threadfin Bream Hydrolysate (TBH)

TBH was prepared according to Normah, Jamilah, Saari and Che Man (2005). Minced threadfin bream was hydrolyzed using flavouzyme at the following hydrolysis conditions; 60°C, 120 min, pH 8.5, enzyme substrate ratio 1:3. After hydrolysis and

centrifugation, the supernatant was freeze dried in a freeze drier (SANYO- Biomedical freeze drier).

Preparation of Bouillon Cube

Bouillon cube was prepared according to Jin et al. (2014). The detail of the composition is presented in Table 1. Red snapper was degutted, washed thoroughly under running tap water and filleted to obtain the flesh. The fish was boiled in 2 L water (fish:water, 1:2) for 30 min at 80°C before it was cooked along with the basic ingredients. The fish broth was cooled for 10 minute and then filtered through muslin cloth. The filtrate was collected and cooled to room temperature. Subsequently, the filtrate was divided into three parts, which are control (as it is), addition of 8.05% ISP (F1) and addition of 8.05% TBH (F2). The mixture was freeze dried until consistent moisture content was obtained. The resulting powder was then moulded into cuboid shape with dimensions of 2.5 cm length, 2.5 cm width and, 2 cm height by using a customized mould tray.

Moisture, Fat and Protein Content

The moisture, fat, and crude protein was determined in triplicate and performed according to the AOAC methods (AOAC, 2005). Moisture content was determined using the oven dried method, fat content by soxhlet extraction and protein by Kjeldahl method.

Table 1
Ingredients used in the formulating of bouillon cube

Ingredients (%)	Control (C)	Formulation 1 (F1)	Formulation 2 (F2)
Red Snapper	71.09	71.09	71.09
Salt	9.48	9.48	9.48
Isolated Soy Protein Powder (ISP)	-	8.05	-
Threadfin Bream Hydrolysate Powder (TBH)	-	-	8.05
Granulated Sugar	7.11	7.11	7.11
Corn Starch	12.32	4.27	4.27
Total	100	100	100

Note: F1 Bouillon cube with the addition of isolated soy protein (ISP); F2 with the addition of threadfin bream hydrolysate (TBH)

Colour

Analysis of bouillon cube colour was determined by using Minolta chroma meter (CR400; Konica Minolta, Japan) based on the CIE system in terms of the L, a*, and b* to measure brightness, redness, and yellowness, respectively. The white calibration plate was used as reference. Measurement was performed in triplicate.

Water Activity

The water activity of the bouillon cube was measured by using dew point equipment (Aqualab 4TE, Decagon Devices Inc., USA). An amount of 5 g of the sample was placed into a small container with 3 cm diameter before the measurement at ambient temperature (25°C).

Protein Solubility

Protein solubility of the bouillon cube was determined according to the method by Caprita, Caprita and Cretescu (2010) using the potassium hydroxide (KOH) solubility test. Protein solubility was expressed in percentage and calculated as follow:

$$\text{Protein solubility (\%)} = \frac{\text{Protein content of the KOH extracted solution}}{\text{Protein content of the original bouillon cube sample}} \times 100$$

Determination of Cube Hardness

Fracturability and hardness test was performed by using TA.XT2 Texture Analyser (Stable Micro Systems, Surrey UK). The probe used was 0.5" Spherical Probe (P/0.5S) and the measurement was conducted on heavy duty platform (HDP/90) that was set with 10 mm distance from the probe.

Sensory Evaluation

Training of the Panelist and Sample Preparation. Sensory evaluation was carried out in the sensory laboratory. Nine panelists were trained for Quantitative Descriptive Analysis (QDA) to evaluate the bitterness, umaminess and fishy odor before testing the samples. Panelists were given caffeine solution (0.2-0.6 g/L), monosodium L-glutamate (MSG) solution (1-3 g/L) and fish aqueous solutions. Fish aqueous reference solutions were prepared by homogenizing red snapper flesh with water at the ratio of 1:3, 2:3, 3:3 (water: red snapper flesh). Panelists needed to determine the intensity of each taste and mark on the 15 cm QDA line anchored, with the word 'weak bitter/umami/fishy odor' on the left side of the scale and 'strongly bitter/umami/fishy odor' on the right side of the scale. The anchor in the center of the line represents the moderate taste intensity. Panelists were trained to identify each taste with the least intensity and memorize the intensity. The solution with the least intensity was used as a reference during the sensory evaluation session.

For sensory evaluation, each panelist was served with 15 mL fish soup. They were asked to mark the intensity of each taste on the QDA line by comparing with the reference solutions which had been identified during the training. Panelists were also given bouillon cubes that were kept in tightly closed bottles. They had to take deep sniffs of the samples and describe the aroma of the bouillon cubes.

Acceptance Test. The acceptability of bouillon cubes was evaluated by thirty panelists. The bouillon cube solution was prepared at the ratio of 1:50 (bouillon cube: water) followed by heating for 10 min. An amount of 15 mL bouillon cube solution prepared was served in transparent containers for the evaluation process. Samples were evaluated for their degree of acceptability for colour, taste, bitterness, aroma, and overall acceptability based on 9-point Hedonic scale (9 = like extremely and 1 = dislike extremely).

Functional Group Analysis of Bouillon Cube. The analysis of the functional group in bouillon cube was performed according to Normah, Siti Hafsah and Nurul Izzaira (2013) by using the Perkin Elmer Spectrum One FTIR spectrometer equipped with a deuterated triglycerine sulphate infrared detector. Perkin Elmer Spectrum Software was used to control the spectrometer and the data was collected over a wavenumber range of 4000-400 cm^{-1} with resolution of 4 cm^{-1} and 16 collections of spectra.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Molecular weight distribution was determined according to Normah and Nur Anati (2015). About 10 µl sample was loaded into the gel comprising of 12% resolving and 4% stacking gel. Benchmark™ protein ladder ranging from 3- 188 kDa was used as a marker. Electrophoresis was run for 50 min at 100-125 mA/gel using the XCell Surelock electrophoresis cell (Consort, Model EV231, Germany).

Statistical Analysis

Data was analyzed by using the Analysis of Variance (ANOVA) to determine significance at 5% level. Duncan Multiple Range Test (DMRT) was used to identify differences between means. The statistical software used was Statistical Analysis System (SAS Institute Inc., 2004).

RESULTS AND DISCUSSION

Moisture, Protein and Fat Content

Moisture, protein and fat content of bouillon cubes are shown in Table 2. Bouillon cube had a low moisture content ranging from 2.03-3.50% with no significant difference ($p > 0.05$) between formulations. Low moisture content will extend shelf life and limit deterioration in quality due to the microbial activity (Lillian, Prisca, Ozioma, Nkechi, & Ifeoma, 2013). It has been suggested that the ideal moisture content for bouillon cube is approximately between 2.25-2.92% (Akpanyung, 2005).

Protein content was significantly higher ($p<0.05$) in bouillon cube containing isolated soy protein (F1). However, it was only <2% higher than those containing TBH (F2). The high protein content could be due to the addition of isolated soy protein which has been reported to contain approximately 90% protein (Kolar, Richert, Decker, Steinke, & Van der Zanden, 1985). Fish protein hydrolysates with protein content varying between 69-87.69% has the potential to be used as protein supplements (Chalamaiah et al., 2012).

Table 2
Physicochemical properties of bouillon cube incorporated with isolated soy protein (F1) and threadfin bream hydrolysate (F2)

	Formulation		
Parameters	**Control**	**F1**	**F2**
Moisture content (%)	3.50 ± 1.30[a]	3.30 ± 0.97[a]	2.03 ± 0.47[a]
Protein content (%)	17.24 ± 0.16[c]	24.15 ± 0.43[a]	23.05 ± 0.27[b]
Fat content (%)	13.10 ± 0.01[b]	19.19 ± 0.21[a]	18.53 ± 1.42[a]
Colour			
L*	85.34 ± 0.11[b]	84.79 ± 0.50[b]	86.32 ± 0.44[a]
a*	-0.56 ± 0.03[c]	-0.25 ± 0.04[b]	-0.09 ± 0.03[a]
b*	11.95 ± 0.48[b]	13.65 ± 0.41[a]	10.65 ± 1.22[c]
Water activity	0.25 ± 0.01[a]	0.24 ± 0.01[ab]	0.21 ± 0.03[b]
Solubility (%)	32.08 ± 5.86[b]	16.07 ± 4.07[c]	46.98 ± 7.38[a]
Hardness (g)	1.73	1.73	2.46
Fracturability (mm)	4.41	3.04	2.41

[a,b,c] Values with different letters in the same row are significantly different ($p<0.05$)

Meanwhile, bouillon cubes prepared in the presence of ISP and TBH contained significantly higher fat content ($p < 0.05$) than the control bouillon cube. The increment in fat content could have been derived from ISP and hydrolysates itself, where protein concentrates and isolates contain fat of approximately 1-1.8 % (Mao and Hua, 2012). Hydrolysate also contributes to the increase in fat content as it contains 4-6.1% fat (Chalamaiah, Rao, Rao, & Jyothirmayi, 2010). It was reported that the fat content in 32 samples of bouillon cubes of the most commonly marketed brands ranged between 4.3 and 29.8% (Caponio et al., 2003).

Colour

Bouillon cubes containing TBH had significantly lighter colour ($p < 0.05$) than the others (Table 2). The addition of TBH affected the colour of the bouillon cubes since the hydrolysate colour was lighter than isolated soy protein. There was a significant difference ($p < 0.05$) in redness (a*) and yellowness (b*) between all the formulations. Most commercial cubes are pale yellow in colour; however it is affected by the material added which will reflect the colour of the cubes (Rodrigues, Pantoja, Soares, Nelson, & Santos, 2016).

Water Activity

Water activity is important in dried food as it will determine the quality of the bouillon cubes regarding spoilage and deterioration by microbes. Bouillon cube should have water activity lower than 0.65 (Smorenburg & Yamson, 2009). All bouillon cubes prepared in this study contain low water activity which varies from 0.21-0.25

(Table 2). Low water activity of bouillon cube indicates good flow-ability of powder (Gupta & Bongers, 2011).

Protein Solubility

Bouillon cubes prepared with the incorporation of TBH contain significantly ($p < 0.05$) higher soluble protein compared to control and F1. Higher protein solubility of F2 is associated with the presence of low molecular weight peptides in the hydrolysate which have been reported to be rich in hydrophilic amino acids (Taheri, Anvar, Ahari, & Fogliano, 2013). In contrary, low solubility of bouillon cube from F1 might be due to the addition of ISP. ISP contains bands with high molecular weight peptides mostly above 20 kDa compared to TBH with bands, most of them being below 20 kDa (Figure 4). Furthermore, ISP is a plant component which is rich in insoluble polysaccharides (Altschul & Wilcke, 2013).

Determination of Bouillon Cube Hardness

Incorporation with TBH resulted in harder bouillon cubes than others (Table 2). In contrary, it has the lowest fracturability reading, compared to others. Higher hardness and lower fracturability indicates good strength of bouillon cubes, as higher fracturability and lower hardness will cause the bouillon cubes to break easily (Paula & Conti-Silva, 2014). The differences in hardness and fracturability were affected by the addition of ISP and TBH. Moreover, hydrolysates have high potential to be used as binding agents (Sathivel et al., 2003).

Sensory Evaluation

Quantitative Descriptive Analysis (QDA). QDA was carried out by nine panelists and the results are presented in Figures 1 and 2. The umaminess, bitterness and fishy odor of bouillon cubes and fish soup, prepared from bouillon cubes, was evaluated and the score of reference solution by using 15 cm line scale from weak to strong was 12.01, 12.10, and 10.99 for umaminess, bitterness, and fishy odor, respectively.

No significant difference ($p > 0.05$) can be seen for intensity of umaminess between umami reference solution and F2. However, F2 showed significantly higher ($p < 0.05$) intensity than control and F1. According to Rodrigues et al. (2016), hydrolysates with low molecular weight peptides less than 500 Da could contribute to the umami taste.

As for bitterness, F2 was significantly less bitter ($p < 0.05$) than the reference solution but more bitter than F1 and control. F2 was bitter due to the addition of TBH which has bitter taste. According to Birhade, Bankar, Gaikwad and Pawar (2010), as a result of the hydrolysis process, hydrolysate consisted of amine group which could contribute to bitterness. For fishy odor, F2 showed no significant difference ($p > 0.05$) with the reference solution. However, the fishy odor was more significant ($p < 0.05$) than control and F1. The intensities for bitterness and fishy odor of the reference solution were identified by the trained panelists during the training sessions, whereby the set scales refer to the lowest possible intensity that the panelists can identify. With regards to acceptance, these

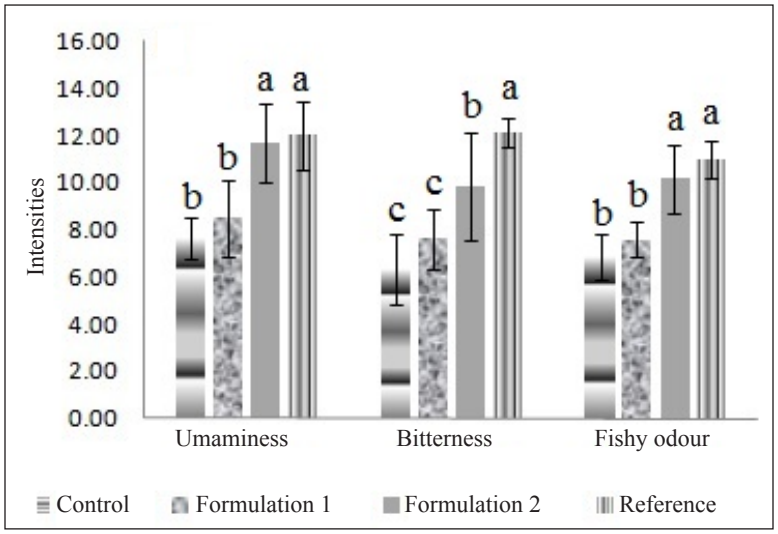

Figure 1. Quantitative descriptive analysis of bouillon cube with different formulations; control, Formulation 1 (incorporated with isolated soy protein) and Formulation 2 (incorporated with threadfin bream hydrolysate)
Note: [a-c] Values with different letters indicate significant difference at $p < 0.05$

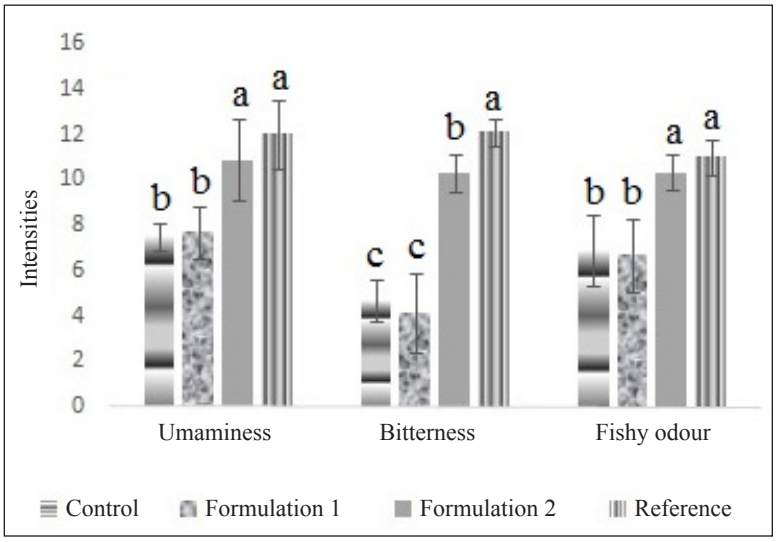

Figure 2. Quantitative descriptive analysis of fish soup with different formulations; control, Formulation 1 (incorporated with isolated soy protein) and Formulation 2 (incorporated with threadfin bream hydrolysate)
Note: [a,b,c] Values with different letters indicate significant difference at $p < 0.05$.

intensity scales are considered as the accepted values by the panelists. Thus, the result suggested that bitterness and fishy odor of F2 is acceptable.

For fish soup prepared from the bouillon cubes, there was no significant difference ($p > 0.05$) between the reference solution and F2 for umaminess and fishy odor (Figure 2). However, soup prepared from F2 has significantly higher intensity ($p < 0.05$) for both attributes than control and F1. In terms of bitterness, soup from F2 was significantly

less bitter ($p < 0.05$) than the reference solution but more bitter than control and F1. Hydrolysate having low molecular weight peptide contributes to the umaminess and bitterness (Geisenhof, 2009; Mouritsen, Styrbrik, Johansen, & Mouritsen, 2014). However, the umaminess in foodstuff is normally mild and of subtle taste, as it is not as intense as bitterness, sweetness and sourness (Hoehl, Schoenberger, & Busch-Stocks, 2014). On the other hand, the aroma, taste, and colour of food products usually depends on the processing method of the food (Meilgaard, Carr, & Civille, 2006).

Acceptance Test. Overall acceptability results showed that bouillon cubes from F1 were most acceptable (Table 3). However, no significant difference ($p > 0.05$) could be seen for colour and aroma for all samples. For taste, F2 was least accepted, probably due to its bitter taste.

Table 3
Acceptability of bouillon cube incorporated with isolated soy protein (F1) and threadfin bream hydrolysate (F2)

	Colour	Taste	Bitterness	Aroma	Overall acceptability
Control	7.10 ± 1.03^{a}	7.13 ± 1.04^{b}	6.63 ± 1.07^{b}	7.03 ± 1.13^{a}	7.00 ± 0.91^{b}
F1	7.53 ± 1.22^{a}	7.83 ± 1.12^{a}	7.30 ± 1.12^{a}	7.47 ± 1.04^{a}	7.83 ± 0.91^{a}
F2	7.13 ± 1.14^{a}	6.43 ± 1.31^{c}	6.37 ± 1.25^{b}	6.97 ± 1.59^{a}	6.87 ± 1.48^{b}

Values are expressed as means ± standard deviation. Different letters within columns indicate significant difference at $p < 0.05$

Functional Group Analysis in Bouillon Cube. Functional group analysis was conducted by using FTIR spectrum. FTIR peak ranges between 3250 to 3400 cm^{-1} represent the N-H of amine group while the peak ranges between 1760 to 1670 cm^{-1} represent the C=O of carbonyl group (Figure 3). The functional group of F2 showed peaks at 3400 cm^{-1} where it falls within the N-H region, suggesting the presence of bitter group. Amine group is usually present in bitter drugs (Agrawal & Chiddarwar, 2010). On the other hand, F2 also showed peaks at 1650 cm^{-1} where it lies within the carbonyl (C=O) stretch. These bands indicated the presence of strong amide I and II in protein amide group (-CONH2) which contribute to bitter taste (Charalambous & Inglett, 2012). Similar prominent peak that lay between N-H stretch and C=O stretch was also found in bitter gourd studies (Ekezie, Jessie-Suneetha, Uma-Maheswari, & Prasad, 2015).

Bouillon cube from control, F1 and F2 showed peaks at 1413.15 cm^{-1}, 1458.50 cm^{-1}, and 1407.45 cm^{-1}, respectively. These peaks have been shown to lie within C-N stretch of aliphatic amines where it is the similar functional group that contributes to the umaminess (McMurry, 1996). Umaminess is derived from monosodium glutamate (MSG) but it is only elevated when MSG

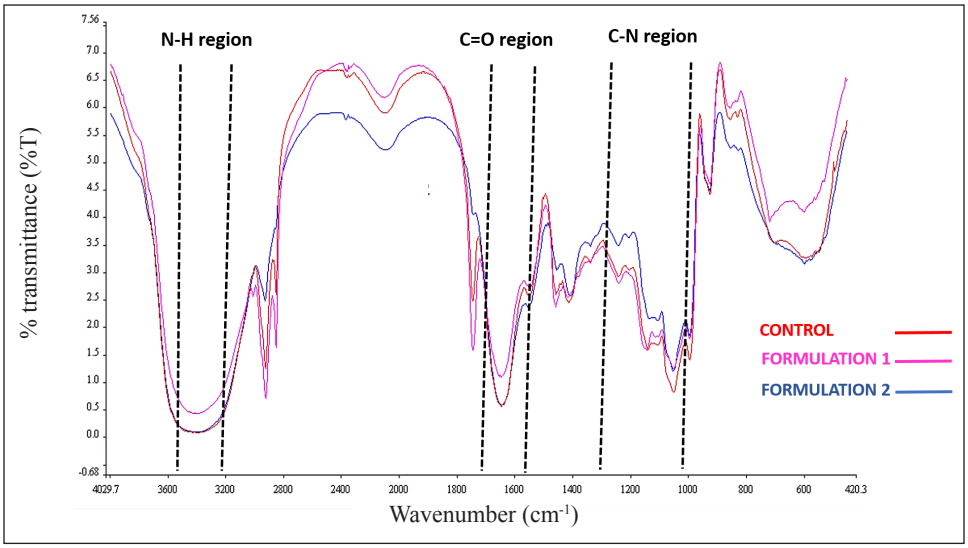

Figure 3. FTIR spectra for bouillon cube incorporated with isolated soy protein (F1) and threadfin bream hydrolysate (F2)

is incorporated with another component (Morini, Bassoli, & Borgonovo, 2011). Sodium chloride, 2,5-dimethylpyrazine, Maillard peptides and glutathionine are examples of components that can enhance and contribute to umaminess (Hayase, Takahagi, & Watanabe, 2013; Ogasawara, Yamada, & Egi, 2006; Ueda, Yonemitsu, Tsubuku, Sakaguchi, & Miyajima, 1997). Umami is a unique property as it has an apparent tactile component and has a weak, unpalatable taste by itself (Beauchamp, 2009).

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The molecular weight distribution patterns are shown in Figure 4. ISP shows an intense band with a wide molecular weight range from 10-70 kDa. Meanwhile, TBH had bands between 10-60 kDa in which bands at 10 and 20 kDa were very intense. The presence of low molecular weight peptide in TBH was due to the enzymatic hydrolysis (Normah & Nur Anati, 2015). Threadfin bream (TB) and red snapper (RS) flesh showed wide molecular weight peptides which can be visualized ranging from 10-220 kDA. The electrophoresis pattern for control, F1 and F2 showed almost similar pattern with red snapper (RS) flesh, since all the bouillon cubes were derived from red snapper flesh.

A similar band pattern of isolated soy protein (ISP) and F1 was discovered where intense band from 70 kDa and below was visualized as it indicates higher molecular weight compared to control and F2. Meanwhile, F2 showed intense band at lower range less than 13 kDa where these bands were not observed in control and F1. Similar band at less than 12 kDa were also exhibited by TBH. On the other hand, F1 showed very wide and intense

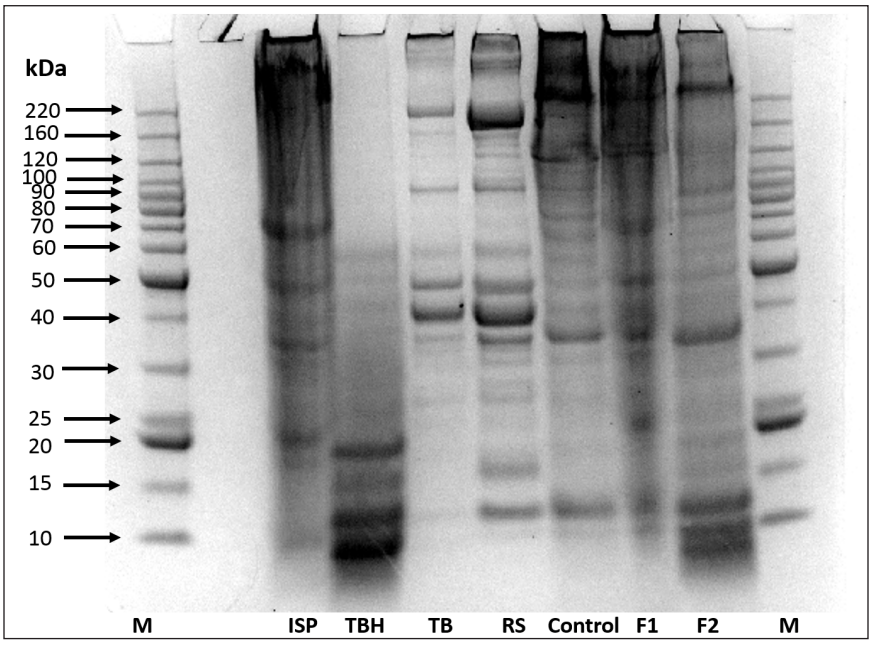

Figure 4. Electrophoresis pattern of bouillon cube incorporated with isolated soy protein and threadfin bream hydrolysate. From left: marker (M), isolated soy protein (ISP), threadfin bream hydrolysate (TBH), threadfin bream flesh (TB), red snapper flesh (RS), control, formulation 1 added with ISP (F1), and formulation 2 added with TBH (F2)

band compared to control and F2. Modified soy protein was reported to contain high molecular weight ranging from 38-80 kDa (Monagle, 2004). It has been stated that molecular weight of protein below 10 kDa will contribute to the bitter flavor (Kristinsson & Rasco, 2000). Thus, the presence of intense band at 13 kDa in F2 suggested that the sample was slightly bitter. Umami flavor has also been studied to have low molecular weight less than 500 Da, however, most umami peptides were weak or even undetectable (Lioe, Takara, & Yasuda, 2006). On the other hand, the presence of low molecular weight band at F2 indicates high solubility properties of bouillon cubes. In contrast, high molecular weight band of F1 shows low solubility properties of bouillon cubes. The studies indicate that low molecular peptides have potential application in functional food products (Roslan, Yunos, Abdullah, & Kamal, 2014).

CONCLUSION

TBH has the potential to be used as a binding agent as it performed well in texture analysis. Besides, incorporation of TBH into bouillon cubes was considered acceptable as it has good physicochemical and functional properties in terms of solubility and strength. TBH is also a good source of protein and has potential as a flavor enhancer due to the umaminess properties based on the sensory results.

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Morphometric Study of the Palm Weevils, *Rhynchophorus vulneratus* and *R. ferrugineus* (Coleoptera: Curculionidae) in View of Insular and Mainland Populations of Malaysia

Siti Nurlydia Sazali[1,2]*, Izfa Riza Hazmi[1], Fatimah Abang[2], Faszly Rahim[1] and Abdul Aziz Jemain[1]

[1]Faculty of Science & Technology, Universiti Kebangsaan Malaysia (UKM), 43600 Bangi, Selangor, Malaysia
[2]Faculty of Resource Science & Technology, Universiti Malaysia Sarawak (UNIMAS), 94300 Kota Samarahan, Sarawak, Malaysia

ABSTRACT

A morphometric analysis was conducted on *Rhynchophorus vulneratus* and *R. ferrugineus* (Coleoptera: Curculionidae) from insular and mainland populations. Twenty-three morphological characters were measured and the data were analysed using independent *t*-test, principal component analysis (PCA) and discrimination function analysis (DFA). Using independent *t*-test, all characters were found to be significant at $p < 0.05$, except distance between eyes (ED), mesotarsus length (F2Ta) and metatarsus length (F3Ta). In PCA, cumulative variations of 80.7% were recorded from the first two principal components, resulting from high loadings in elytra length (EL), elytra width (EW) and pronotum length (PL). For DFA, a single function explained a canonical correlation of 0.952 with 100.0% of variation and the Wilk's Lambda statistics (0.094) was strongly supported with $p < 0.0001$. The highest character loadings were the total length (TL), elytra width (EW) and pronotum length (PL), which were useful as diagnostic characters for separating both *Rhynchophorus* species. Therefore, this study suggests that *R. vulneratus* and *R. ferrugineus* are morphologically distinct species. This finding proves that the insular population of *R. vulneratus* fits the generality of the 'island rule' as being larger compared to the mainland counterparts of *R. ferrugineus*.

Keywords: Morphometrics, mainland, insular population, *Rhynchophorus*, weevils

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E-mail addresses:
ssnurlydia@unimas.my (Siti Nurlydia Sazali)
izfahazmi@ukm.edu.my/izfariza.hazmi@gmail.com (Izfa Riza Hazmi)
fatim@unimas.my (Fatimah Abang)
faszlyrahim@gmail.com (Faszly Rahim)
azizj@ukm.edu.my (Abdul Aziz Jemain)
* Corresponding author

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INTRODUCTION

The palm weevils of the genus *Rhynchophorus* (Herbst, 1795) are large insect pests that cause massive destruction to a broad range of palm species (family Aracaceae) including sago, coconut, oil palm and dates worldwide (Hoddle & Hoddle, 2011; Murphy & Briscoe, 1999; Wattanapongsiri, 1966). The red palm weevils, *R. ferrugineus* (Olivier, 1790) was reported to be indigenously distributed throughout the Southeast Asia and Melanesia; including Sri Lanka, Thailand, Cambodia, Vietnam, Peninsular Malaysia, Singapore, Sumatra, Java, Boneo, Philippines and Taiwan (Rugman-Jones, Hoddle, Hoddle, & Stouthamer, 2013; Wattanapongsiri, 1966).

A comprehensive taxonomic revision on the genus *Rhynchophorus* by Wattanapongsiri (1966) had separated the two species of the red stripe weevils, *R. vulneratus* and the red palm weevils, *R. ferrugineus*, primarily by the shape of pronotum and body coloration. However, the extreme colour polymorphism shared between the two species caught the attention of many taxonomists especially since 1990s (Rugman-Jones et al., 2013). Many taxonomical studies had been conducted including by Hallett, Crespi and Borden (2004), Hoddle and Hoddle (2011), Rugman-Jones et al. (2013), Abad et al. (2014), and Lannino, Sineo, Bianco, Arizza and Manachini (2016), but none could validate the specific status of these pests due to high polymorphism and phenotypic plasticity expressed by different populations.

Apart from that, the occurrence of the red stripe weevils, *R. vulneratus* singularly in Sarawak (Malaysian Borneo) had provided a good natural study site for examining the effect of island rule (van Valen, 1973) towards the body size of *Rhynchophorus* species. Due to the unique features of island environments, distinctive selection pressures led to less predators, relaxed competition and limited food supplies that can yield complex evolutionary trajectories in body size (McClain, Boyer, & Rosenberg, 2006; Palkovacs, 2003; van Valen, 1973).

Animal species inhabiting islands have a smaller body size (dwarfism) among large-sized species, while the opposite, a larger body size (being gigantism) among small-sized species are seen compared with mainland organisms (Lomolino, 2005; van Valen, 1973). However, it is not a conclusive evidence for every single organism, since the discrepancies of findings were reported from other vertebrates, such as in carnivores (Meiri, Dayan, & Simberloff, 2004, 2006) and elephant (Smith et al., 2003).

The objectives of this study were to identify unique characteristics of two *Rhynchophorus* species in Malaysia and to examine whether the size variation follows the generality of the 'island rule'. *R. vulneratus* represented the insular population, meanwhile *R. ferrugineus* represented the mainland population. In addition, this study is important to complement the lack of research on Malaysian specimens, especially in Borneo.

MATERIALS AND METHODS

A total of 98 adult specimens consisting of 53 individuals of the red stripe weevils, *R. vulneratus* from Samarahan, Sarawak and 45 individuals of the red palm weevils, *R. ferrugineus*, collected from Kuala Nerus, Terengganu was linearly measured. The specimens of *R. vulneratus* from Samarahan were collected from several field samplings while *R. ferrugineus* from Terengganu were obtained from the insects collection at Department of Agriculture, Semenggoh (Sarawak) and the Centre for Insect Systematics (CIS), Universiti Kebangsaan Malaysia (UKM).

Identification of field specimens follows the taxonomy suggested by Arnett, Thomas, Skelley and Frank (2002) and Wattanapongsiri (1966). Twenty-three characters (Figure 1) were linearly measured using a digital caliper (Mitutoyo TM) calibrated to 0.01 mm and recorded to two decimal points. Snout length (SL), snout width (SW), scape of antenna length (SAL), antenna length (AL), antenna width (AW), distance between eyes (ED), pronotum length (PL), pronotum width (PW), elytra length (EL), elytra width (EW), total length (without snout) (TL), profemur length (F1L), protibial length (F1Tb), protarsus length (F1Ta), mesofemur length (F2L), mesotibia length (F2Tb), mesotarsus length (F2Ta), metafemur length (F3L), metatibia length (F3Tb), metatarsus length (F3Ta), mesocoxal distance (MSD), metacoxal distance (MTD) and meso-metacoxal distance (MMD) were measured.

All 23 variables were subjected to independent *t*-test to define the significant characteristics among the examined species

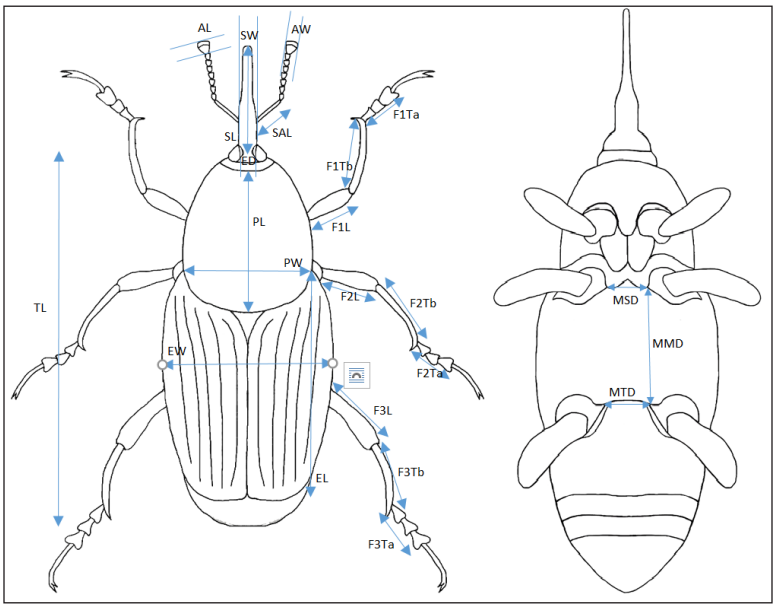

Figure 1. Twenty three morphological characters measured in this study

at *p*-value less than 0.05 (Hair, Rolph, Roland, & William, 1995). The variables were then subjected to Principal Component Analysis (PCA) to enable condensation of data on a multivariate phenomenon into its main, representative features by projection of the data into a two-dimensional presentation (Janžekovič & Novak, 2012) and analysed using Minitab program version 17.1 (Minitab Inc, 2013). The analysis was based on the correlation matrix and the first two components were visualised to facilitate the separation among the two species.

The discriminant function analysis (DFA) was also conducted to define the significant characters for distinguishing *R. vulneratus* and *R. ferrugineus*. The analysis was conducted following procedures described by Sazali and Aziz (2012), and Sazali and Juary (2012) using SPSS program version 22.0 for Windows (SPSS Inc., 2017). All character loadings were performed at once to investigate the integrity of pre-defined groups using the measure of distance of Wilk's Lambda, based on generalised squared Euclidean distance that adjusts for unequal variances (Hair et al., 1995). A cross-validation testing procedure was also performed to assess the ability of the predictive model built. Statistical differences are considered significant where *p*-value is less or equals 0.05 (Hair et al., 1995).

RESULTS

Descriptive data from the 23 morphological characters for *R. vulneratus* and *R. ferrugineus* is summarised in Table 1. Data was assumed to follow normal distribution as the sample size for each species is greater than 30, which satisfy the normality requirement for further statistical analysis. Using independent *t*-test, all tested variables were significant at $p < 0.05$ which were found useful for differentiating both *R. vulneratus* and *R. ferrugineus*. This was followed by principal component analysis (PCA) using all characters before the discriminant function analysis (DFA), except for three characters, namely, distance between eyes (ED), mesotarsus length (F2Ta) and metatarsus length (F3Ta).

In the PCA, the first two principal components showed the most variations among the two species with cumulative variations of 80.7%. Only those with eigenvalues greater than 1.0 were considered for data interpretation. In the first principal component (PC1), the characteristics that showed higher loadings were the elytra length (0.236), elytra width (0.236) and pronotum length (0.234), supported with eigenvalue of 17.815 and 74.7% variation. Meanwhile, for the second principal component (PC2), e higher loadings were due to metafemur length (F3L, -0.498) and antenna length (0.418) with eigenvalue of 1.368 and 5.9% variation. Although the species-grouping is not really obvious as shown in Figure 2 since few samples overlapped, the cumulative variations of more than 80% is proven and supported in the analysis.

Further test of discriminant function analysis (DFA) revealed a single significant function that explains a canonical correlation

Table 1
Summary of the 23 morphological characters measured (mm) in R. vulneratus *and* R. ferrugineus *for this study*

Species	*R. vulneratus* (n=53)				*R. ferrugineus* (n=45)				*p*
Character	Mean	SD	Min	Max	Mean	SD	Min	Max	
SL	10.7408	1.0917	8.88	13.28	9.1444	0.8513	7.8	11.05	<0.001
SW	1.6891	0.1263	1.41	2.06	1.6127	0.0769	1.40	1.78	0.001
SAL	4.0396	0.4065	3.24	5.04	3.4282	0.2720	2.38	3.98	< 0.001
AL	1.3779	0.0905	1.20	1.58	1.0831	0.0774	0.94	1.25	< 0.001
AW	1.9213	0.1729	1.57	2.33	1.7622	0.1088	1.50	1.97	<0.001
ED	0.9723	0.1255	0.80	1.30	0.9302	0.1017	0.73	1.20	0.075*
PL	12.8749	1.3656	10.10	16.53	11.5109	0.7034	9.11	13.00	< 0.001
PW	10.8866	1.2505	8.45	13.94	9.4089	0.6161	7.58	10.50	< 0.001
EL	16.6117	1.6107	13.15	20.05	14.1091	0.9092	11.50	15.86	< 0.001
EW	13.6032	1.4049	10.71	16.95	11.4389	0.7105	9.28	12.78	< 0.001
TL	31.3221	3.0401	25.84	38.75	28.1244	1.7203	22.77	32.12	< 0.001
F1L	6.6419	0.8911	4.91	9.07	6.2816	0.4960	4.53	7.25	0.018
F1Tb	7.1898	0.8183	5.22	9.26	5.9822	0.4372	4.70	6.93	< 0.001
F1Ta	3.2851	0.3513	2.42	4.08	3.0284	0.2667	2.31	3.62	< 0.001
F2L	7.3857	0.8059	5.66	9.30	6.1387	0.3763	4.93	7.00	< 0.001
F2Tb	5.8389	0.6402	4.44	7.47	4.9862	0.3760	4.14	5.77	< 0.001
F2Ta	3.0738	0.3795	2.25	4.05	2.9498	0.2601	2.27	3.70	0.067*
F3L	8.2860	0.7744	6.51	10.21	6.9000	0.3988	5.40	7.73	< 0.001
F3Tb	6.9877	0.7496	5.17	8.71	5.8822	0.3638	4.82	6.71	< 0.001
F3Ta	2.9155	0.3720	2.20	3.74	2.8600	0.2556	2.25	3.36	0.400*
MSD	2.6457	0.3314	2.02	3.44	2.3031	0.1920	1.78	2.86	< 0.001
MTD	3.2515	0.4146	2.53	4.42	2.9782	0.2555	2.46	3.76	< 0.001
MMD	7.8362	0.8222	5.86	9.68	6.8249	0.4485	5.57	7.76	< 0.001

Note: Snout length (SL), snout width (SW), scape of antenna length (SAL), antenna length (AL), antenna width (AW), distance between eyes (ED), pronotum length (PL), pronotum width (PW), elytra length (EL), elytra width (EW), total length (without snout) (TL), profemur length (F1L), protibia length (F1Tb), protarsus length (F1Ta), mesofemur length (F2L), mesotibia length (F2Tb), mesotarsus length (F2Ta), metafemur length (F3L), metatibia length (F3Tb), metatarsus length (F3Ta), mesocoxal distance (MSD), metacoxal distance (MTD) and meso-metacoxal distance (MMD). * Not significant

of 0.952 which accounted for 100.0% of the variation (Table 2). The Wilk's Lambda statistics of 0.094 was supported with a highly significant function ($p < 0.0001$) (Table 3). Meanwhile, the standardised canonical discriminant function coefficient (Table 4) shows the highest character loadings observed for the model, namely total length (TL), followed by elytra width (EW) and pronotum length (PL).

The discriminant function scores for each group are clearly separated for both *R. vulneratus* and *R. ferrugineus* in which all the examined parameters were useful in

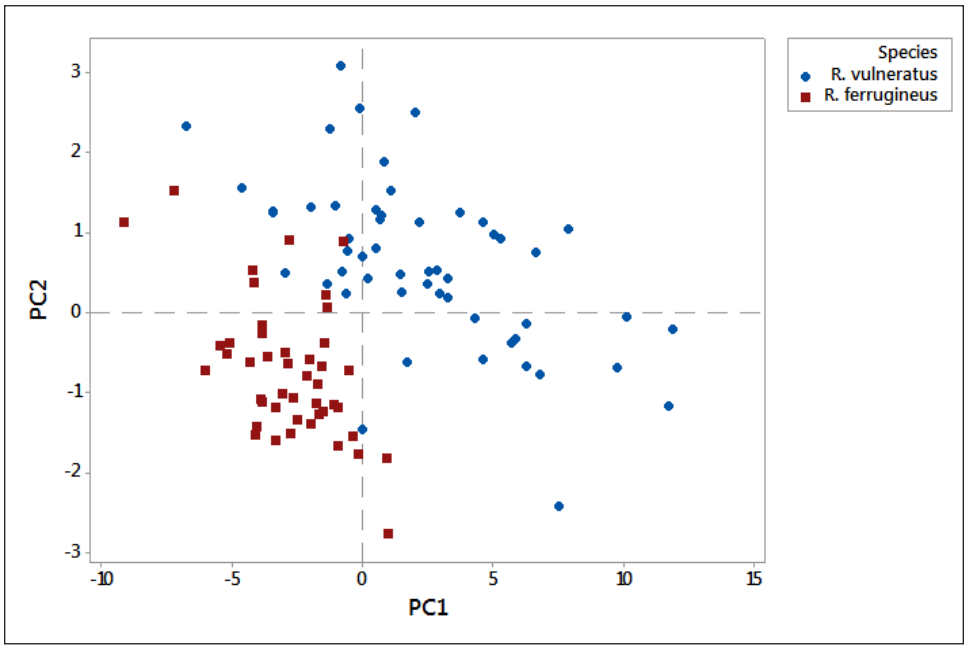

Figure 2. Principal component plot (PC1 vs PC2) of *R. vulneratus* and *R. ferrugineus*

Table 2
Eigenvalues for the DFA

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	9.588[a]	100.0	100.0	0.952

Note. [a] First 1 canonical discriminant functions were used in the analysis

Table 3
Wilks' Lambda values for the DFA

Test of Function(s)	Wilk's Lambda	Chi-Square	df	Sig.
1	0.094	202.939	20	0.000

separating both species as shown in Figure 3. Through a predictive group membership procedure, a perfect score of 100.0% of original group cases correctly classified into each respective species group, whereas in the cross-validating test, 98.0% of cases were correctly classified (data not shown). Hence, this outcome strongly suggests a valid species status of *R. vulneratus* and *R. ferrugineus*.

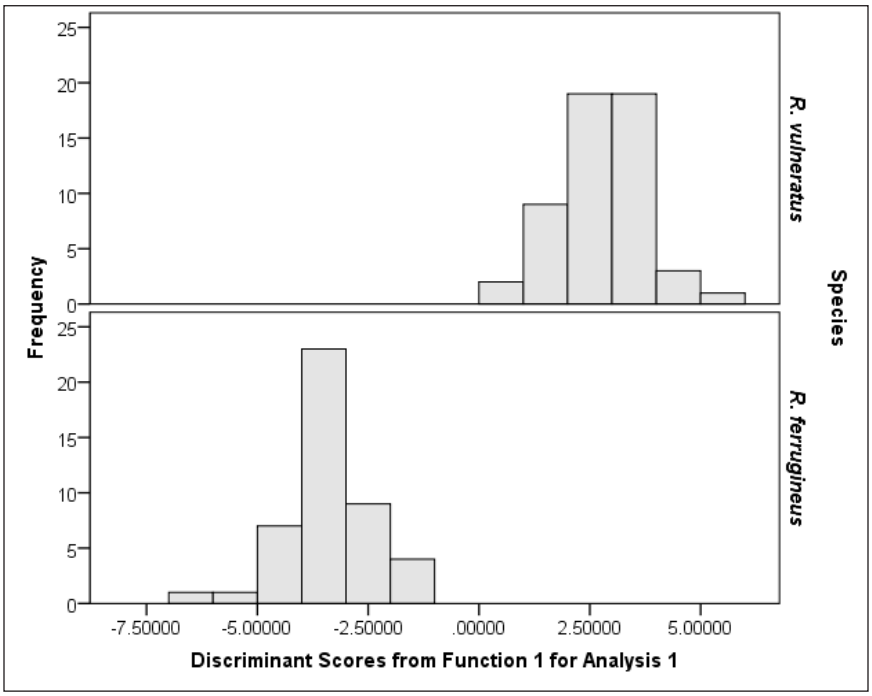

Figure 3. Separate-group histograms of discriminant function scores for *R. vulneratus* and *R. ferrugineus*

Table 4
Standardised canonical discriminant function coefficients for the 20 characters

	Function 1
SL	1.347
SW	-0.493
SAL	-0.070
AL	0.337
AW	0.021
PL	-1.583*
PW	0.163
EL	1.276
EW	1.769*
TL	-2.286*
F1L	-0.809
F1Tb	1.198
F1Ta	-0.107
F2L	0.473
F2Tb	-0.281
F3L	0.134
F3Tb	0.176
MSD	0.137
MTD	-0.856
MMD	-0.102

Table 4 *(continue)*

Note: *Diagnostic character(s) with high coefficient in Function 1

DISCUSSION

To date, the red palm weevils are taxonomically still considered as a single species of *R. ferrugineus* comprising other colour-morph type of R. *vulneratus* (Hallett et al., 2004; Rugman-Jones et al., 2013). It is important to understand the specific status of both colour morphs species because the red palm weevils have become a severe threat to date plantation and production, ornamental

landscape plants and native palm trees worldwide (Rugman-Jones et al., 2013).

In both principal component analysis (PCA) and discriminant function analysis (DFA), the differences between *R. vulneratus* and *R. ferrugineus* were found in the elytra width (EW) and pronotum length (PL). According to Wattanapongsiri (1966), the two species can be distinguished based on the shape of their pronotum and gular suture. In *R. vulneratus*, the pronotum is strongly and narrowly curved anteriorly, whereas in *R. ferrugineus*, its pronotum is curved at the sides and it has a more uniform anterior (Wattanapongsiri, 1966). Meanwhile for the gular suture, the shape is more concave at both sides before reaching the base of rostrum in *R. vulneratus*, whereas in *R. ferrugineus*, the shape is oval at base but less concaved than the former species (Wattanapongsiri, 1966).

However, the description by Wattanapongsiri (1996) was not informative enough as *Rhynchophorus* species expressed high polymorphism and phenotypic plasticity across a wide geographical distribution (Abad et al., 2014; Lannino et al., 2016; Rugman-Jones et al., 2013) leading to its taxonomic ambiguity. Phenotypic plasticity refers to changes in phenotypes and morphology induced by different surroundings and environment (DeWitt & Scheiner, 2004; West-Eberhard, 2003; Whitman & Ananthrakrishnan, 2009). Additionally, these changes are beneficial to insects for increasing survival, fecundity, fitness, population density and species range.

Moreover, the origin of specimens examined here has provided a platform to generally review the 'island rule' as proposed by van Valen (1973), who reported the body size variation in insular population of mammals. Many studies had been conducted to review the generality of the 'island rule' on vertebrates (Lomolino et al., 2013) but only one known study had been reviewed for invertebrate, particularly in tenebrionid beetle by Palmer (2002).

The size divergent of body and shape of wings in natural populations may represent adaptive modification due to island environments or habitat heterogeneity (Lee & Lin, 2012). Even when the geographical effect is considered, the evolutionary divergence of insects does not exactly follow the pattern of neutral evolution indicated by the genetic marker (Lee & Lin, 2012). This was the case study by Lee and Lin (2012) who proposed that morphological variations resulted from the morphology and wing shape of damselflies, *Euphaea formosa* and *E. yayeyamana* (Odonata: Euphaeidae) were most likely corresponded to natural and sexual selection for fitness optimization. Palmer (2002) in his study on tenebrionid beetle, *Asida planipennis* (Coleoptera: Tenebrionidae) also stated that size had evolved by selection related to site-specific environmental factors linked with island area.

The overall body size of *R. vulneratus* is relatively larger than *R. ferrugineus* from the two localities (Table 1). Wattanapongsiri (1966) noted the considerable variation in size for the two species was due to food

availability and limitation during the larval stage, which later affected the adult form. Being larger in size will give benefits to *R. vulneratus* as it provides greater fecundity in females, access to mates among males and advantages of resource sequestration (Blanckenhorn, 2000; Honik, 1993; Lighton, Quinlan, & Feener, 1994; Parker & Simmons, 1994; Rivero & West, 2002). In contrast, being smaller in size to *R. ferrugineus* may reduce viability costs of growth and development, enhance agility and reduced detectability, minimise maintenance costs of energy, lower heat stress and costs of reproduction and increase scramble competitive ability (Blanckenhorn, 2000, 2005; Moya-Lara*ño,* El-Sayyid, & Fox, 2007).

As a matter of geographical concern, the chosen localities for *R. vulneratus* and *R. ferrugineus* in the present study was believed to provide no beneficial impact towards the development of the weevils' larva, as the sampling sites for both species were not situated or located near any large plantation area that could give plenty of food supply. In fact, the sites were surrounded by human settlements and can be categorised as disturbed areas. Therefore, the effect of size variation in regards to possible consequence of food availability from the sites can be ignored or at least minimised.

The insular population of *R. vulneratus* was collected from the UNIMAS East Campus, Samarahan Division of Sarawak. The campus and its surroundings are developing areas, constructed from peat lands and surrounded by a secondary forest and are in close proximity to many human settlements. As a developing sub-urban, parts of Samarahan are transformed into agricultural lands for plantation of oil palm, coconut, pineapple, banana, paddy and vegetables farms, be it commercially cultivated or as a small-scale business managed by local people. However, the occurrence of *R. vulneratus* in these plantation areas are hardly detected and there is no established documentation that particularly report the damages caused by this red stripe weevil in the state, unless found abundantly in the sago plantations which is not commercially cultivated in this district.

Meanwhile, the mainland population of *R. ferrugineus* was collected from Seberang Takir, located in Kuala Nerus District of Terengganu. The sampling site is a non-commercial coconut plantation situated nearby fisherman villages. Being one of the most destructive insect pests of palms (Aracaceae) in the world (Rugman-Jones et al., 2013), this red palm weevil was first reported by the Department of Agriculture (Terengganu) when many of their coconut trees lose their leaf crowns at Rhu Tapai, Setiu (Wahizatul, Zazali, Abdul, & Nurul, 2013). Indeed, this weevil was never being reported as a lethal pest of coconut in the country (Murphy & Briscoe, 1999), hence the invasion of *R. ferrugineus* in Terengganu had urged the authority to take immediate actions to control the weevils before it could become aggressive enough to affect the

coconut and oil palm industry in Malaysia, which greatly contributes to the country's economic growth (Wahizatul et al., 2013).

As initially suggested by van Valen (1973), the insular population of the red stripe weevils, *R. vulneratus* fitted the generality of the 'island rule', as being larger compared with the mainland counterparts of the red palm weevils, *R. ferrugineus*. The larger size of *R. vulneratus* may be due to decreased competition or predators (Lomolino, 1985) in the Bornean island, since *R. ferrugineus* is a quarantine species in Sarawak (Department of Agriculture, personal communication, 2013). It survives due to abundance of food resources, especially sago trees. Meanwhile, the reduction of body size in *R. ferrugineus* might be related to resource limitation (Lomolino, 1985) in Peninsular Malaysia, as the competition with its sibling species, *R. vulneratus* and other insect pests, is also expected.

CONCLUSION

The findings of this study suggest that the red stripe weevils, *R. vulneratus* and the red palm weevils, *R. ferrugineus* are morphologically distinct species. The locality's selection of Kota Samarahan, Sarawak and Seberang Takir, Terengganu was believed to satisfactorily represent the nature of island and mainland populations. As an insect, the insular population of *R. vulneratus* in Sarawak is relatively larger in size compared with the mainland population of *R. ferrugineus* in Peninsular Malaysia, which adhered to the generality of 'island rule' proposed by van Valen (1973).

However, future studies should look at other study sites to examine the morphometric variation across the latitudinal or altitudinal gradients. Other possible factors including climatic condition, annual mean temperature, sexual size dimorphisms and island or mainland size should be included for detailed assessment.

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Phylogenetic and Expression of Atp-Binding Cassette Transporter Genes in *Rasbora sarawakensis*

Leonard Whye Kit Lim[1]*, Tan Hui Ying[1], Aimi Wahidah Aminan[1], Abdul Qawiem Jumaan[1], Mohd Zulfazli Moktar[1], Tan Say Yen[1], Clarissa Patrick Balinu[1], Arin Vynona Robert[1], Chung Hung Hui[1]* and Badiozaman Sulaiman[2]

[1]Resource Biotechnology Programme, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS), 94300 Kota Samarahan, Sarawak, Malaysia
[2]Animal Resource Science and Management Programme, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS) 94300 Kota Samarahan, Sarawak, Malaysia

ABSTRACT

The ATP-Binding Cassette transporters (ABC transporters) function in various physiological activity, allowing vertebrate to thrive even in polluted environment. The objective of this study is to discover ABC genes in *Rasbora sarawakensis*, a species endemic to Borneo and to understand the respective genes regulation. In this research, nine gene partial transcripts were isolated via RT-PCR and cloning approaches. Our study showed that most gene transcripts identified share high identities with conserved motif distributions across family. Further phylogenetic analysis revealed a clear divergence into three major functional clades (A2, E1, F1; D2; B4, B8, C2, G2). Expression profiles in six tissues (i.e., brain, eye, gill, intestine, muscle, and skin) revealed divergence that shed light on tissue-specific gene functional specialization, with highlight on B4, B8, and E1 which are expressed in all six organs. The brain and eye were also found to express all ABC genes selected in this study. In conclusion, nucleotide profiles of these genes are comparable to the phylogenetic analysis and expression patterns across family. This study implies that an alternative vertebrate model organism can possibly complement the current zebrafish researches.

Keywords: ABC transporters, expression profiles, isolation, phylogeny, *Rasbora sarawakensis*

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E-mail addresses:
lwkl1993@gmail.com (Leonard Whye Kit Lim)
hhchung@unimas.my (Chung Hung Hui)
hytan210@gmail.com (Tan Hui Ying)
aimiwahidah930415@gmail.com (Aimi Wahidah Aminan)
hitsugayaabdullah93@gmail.com (Abdul Qawiem Jumaan)
sanzfazli@yahoo.com (Mohd Zulfazli Moktar)
39046@siswa.unimas.my (Tan Say Yen)
40955@siswa.unimas.my (Clarissa Patrick Balinu)
40614@siswa.unimas.my (Arin Vynona Robert)
sbadiozaman@unimas.my (Badiozaman Sulaiman)
* Corresponding author

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INTRODUCTION

The ecosystem present in the aquatic environment greatly influences the overall health of the habitat. Various pollutants may be present naturally or introduced into the aquatic habitat by any means, causing significant impact on the aquatic biota (Ferreira, Costa, & Reis-Henriques, 2014; Liu, Li, & Liu, 2013). A handful of ATP-Binding Cassette (ABC) transporters can function to convert these polluting chemicals into their more execrable forms via biotransformation enzymatic reactions and detoxification pathways (Ferreira et al., 2014; Liu et al., 2013).

The ABC transporter superfamily is one of the most expanded family groups of the transmembrane proteins. Initially, this family was identified and characterized based on their functions in multidrug resistance (MDR) (Aryal, Laurent, & Geisler, 2015; Ferreira et al., 2014). The translocations of substances across membranes in biological systems of the host, especially the eukaryotes, are regulated by most members of the ABC proteins equipped with full transporters (Dermauw & Van Leeuwen, 2014; Ferreira et al., 2014).

To date, there are six subfamilies from ABCA to ABCH, that branch out from the ABC superfamily, each representing distinctive homology characteristics related to various functions and disorders (Andersen et al., 2015; Guo et al., 2015; Park et al., 2016). Of all members of the superfamily, only *ABCH1* gene is found to be absent in mammals (Popovic, Zaja, Loncar, & Smital, 2010). *ABCH1* gene had been discovered only in mold, insects and fish but not in other vertebrates. Subfamilies ABCA, ABCB, and ABCC are the groups containing full transporters and half transporters whereas other subfamilies (ABCD, ABCE/ABCF, and ABCG/ABCH) consist of only half transporters.

Thus, there is also a strong need to unravel the potential of using these gene biomarkers in selected endogenous organisms like the Sarawak rasbora for domestic environmental studies (Fedorenkova, Vonk, Breure, Hendriks, & Leuven, 2013; Luckenbach, Fischer, & Sturm, 2014), emulating *Rasbora caverii* as agrochemical model in Sri Lanka (Wijeyaratne & Pathiratne, 2006) and ABC gene discoveries in common carp (Liu et al., 2016). Rasbora genus represents one of the largest family members of the Cyprinidae with eighty-seven species recorded so far (Eschmeyer, 2015). Hence, this research focused on the isolation of nine selected ABC transcripts from *R. sarawakensis* and further followed by the transcript expression verification in various tissues and organs. By determining the presence and expression of these genes, this information can be fed into further studies involving functional characterization of these vital genes involved in ecotoxicology pathways in this fish species in the future.

MATERIALS AND METHODS

Total RNA Extraction

Sampling of *R. sarawakensis* was conducted at the Matang Wildlife Park under the permit NCCD.940 47(Jld1 3) -178 by the Sarawak Forestry Department. Live specimens

caught were then maintained at 26°C under 12-h light and 12-h darkness photoperiod under the Animal Ethics Committee of Universiti Malaysia Sarawak (UNIMAS/TNC(PI)-04.01/06-09 (17). The whole fish body was euthanized on ice and transferred to 1.5 mL microcentrifuge tube for RNA extraction using TRI reagents according to manufacturer's protocol (Sigma-Aldaich, USA). The aqueous phase was transferred to a new tube and total RNA was precipitated with isopropanol at room temperature. The pellet was subsequently dissolved in 20 µL nuclease free water and was stored at -80°C. A total of three biological replicates were used in this study.

Primer Design

In order to identify ABC superfamily genes sequence, FASTA sequences of each selected fish species were retrieved. The Clustal Omega interface website was used to conduct multiple sequence alignment for the ABC gene sequences of the six freshwater fish (Sievers et al., 2011). Based on the results obtained, suitable forward and reverse primers of 20-25 base pairs were selected on the regions of conserved domains. Primer3 software was used to assist in locating the right primer pair (Untergassar et al., 2012). The primers were screened for melting temperature, presence or absence of secondary structure, self-complementary as well as GC content using OligoCalc tool (Kibbe, 2007). The selected primer orders were sent to First BASE Laboratories Sdn. Bhd. for primer synthesis (Table 1).

First Strand cDNA Synthesis

EasyScript® Reverse Transcriptase (TransGen, China) was used to synthesize first-strand cDNA strand. Total RNA, primer and RNase-free water were mixed in a sterile PCR tube before incubating the tube at 65°C for 5 min, then on ice for 2 min. Then, other components such as dNTP mix, EasyScript® Reverse Transcriptase and

Table 1
The forward and reverse primers used in PCR amplification of selected ABC genes and β-actin

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
ABCA2	GATGT*D*GATGTGGCCTGTGA*R*AGA	CCAGC*W*GG*Y*TTGTC*K*GCATACTT
ABCB4	GTCCT*D*AATGG*Y*ATGAATCT	CCAGC*R*AT*K*ACATCAGCATT
ABCB8	TTCAGTTA*Y*CCAAC*R*AGACC	CCTCCTTTG*S*TCAG*Y*A*R*TTC
ABCC2	GG*K*AA*Y*CTGGT*R*GTGTTTTT	ACACA*R*CA*R*CTG*Y*CTCTGA
ABCD2	ATGGT*B*GC*Y*GTGCC*Y*ATCAT	CTGCTTCTCTCCAGA*H*A
ABCE1	GCCAA*Y*TCCTTCAA*R*CTGCA	ACAGCAGATGAAGTC*N*GACA
ABCF1	GCTGT*Y*ATCTGGCT*B*AACAACTA	TTGGA*R*ATCTG*R*AT*R*GTGTG
ABCG2	ACCGTCAGCTTCCACAACATC	GA*Y*GGAGAACA*R*GAAG*R*TGAAGA
ABCH1	CATCAGGCTTTTGAGGCTTT	TGATTGGCAGATCCATGTGT
β-actin	GGAGGAGATCTGGCATCACAC	GATCTCCTTCTGCATCCTGTCA

Reverse Transcriptase Buffer were added into the mixture and incubated consecutively according to the manufacturer's protocol.

Gradient RT-PCR

The optimum temperatures of all gene primers and *β-actin* were determined and applied for the following RT-PCR reactions in a T100™ Thermal Cycler (Bio-Rad, USA). RT-PCR was performed in a 20 µL reaction tube by preparing the 4X master mix containing 1X *EasyTaq* Buffer (with Mg^{2+}), 0.2 mM dNTPs, 0.2 µM forward and reverse primers respectively, 2.5 units *EasyTaq* DNA polymerase (TransGen, China), 2.5 ng/µL cDNA and nuclease-free water. The mixtures were subjected to one cycle of pre-denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55-60°C for 30 s and extension at 72°C for 40 s as well as one cycle of final extension at 72°C for 5 min. Three replicates were used for each gene in RT-PCR reactions. Gel extraction was conducted using Wizard® SV Gel and PCR Clean-Up System according to manufacturer's protocol (Promega, USA). Selected gene transcripts were cloned into pGEM-T® Easy vector (Promega, USA) according to manufacturer's protocol before they were subjected to sequencing.

Sequencing and Analysis

Purified PCR products and plasmids were sent to First BASE Laboratories Sdn. Bhd. for sequence determination using the same primers used for RT-PCR amplification (for PCR product sequencing) and T7 promoter primer (for plasmid sequencing). The electropherogram files were analyzed using the Mega7 (Kumar, Stecher, & Tamura, 2016) to trim and obtain consensus sequences through a quality check. Then, the results of the output obtained from direct sequencing were analyzed using basic local alignment search tool (BLAST) from NCBI (Altschul, Gish, Miller, Myers, & Lipman, 1990). Motif distribution was inspected by using MEME tool (Bailey & Elkan, 1994). ORFs were predicted from these gene transcripts using NCBI ORFfinder (Rombel, Sykes, Rayner, & Johnston, 2002), blasted using BLASTp and sequences of motifs were revealed via NCBI CDD (Marchler-Bauer et al., 2016). The amino acid sequences (predicted ORFs) were subjected to Best Protein Model test using Mega7 and Jones-Taylor-Thornton (JTT) model using maximum likelihood was revealed to be the best model. A maximum likelihood phylogenetic tree (Bootstrap method, replications of 500, Jones-Taylor-Thornton (JTT) model) was constructed to compare ABC genes across family members of Cyprinidae using Mega7 (Kumar et al., 2016).

Semi-Quantitative Expression Analysis

First, a total of ten *R. sarawakensis* fishes were euthanized by using Tricaine. Organs such as the brain, eyes, gills, intestine, muscle and skin were isolated. The six organs of *R. sarawakensis* were sliced on petri dish while on ice and then they were transferred into six sterile labeled 1.5 mL microcentrifuge tubes, respectively. The six organ samples were homogenized

with 500 µL of TRI® Reagent according to manufacturer's protocol (Sigma-Aldaich, USA) in respective sterile microcentrifuge tubes by using micropipette tips. These RNAs were extracted and used for cDNA synthesis according to the manufacturer's protocol. The cDNA of six organ samples were kept in -20°C for further expression study. These cDNAs of six organs were subjected to RT-PCR conditions as optimized above, using the ABC gene primers designed previously with three replicates, together with beta-actin gene as positive control to determine the semi-quantitative expression of these genes on agarose gel.

RESULTS AND DISCUSSION

Motif Distribution and Protein Analyses

To isolate ABC transporter genes, total RNAs were extracted from whole body of Sarawak rasbora. A total of nine ABC transporter gene transcripts have been isolated from *R. sarawakensis* with at least one representative member from a total of six subfamilies. Degenerate primers were designed based on conserved motifs that are shared within family members of Cyprinidae (i.e., zebrafish, common carp, and *Sinocyclocheilus* spp.). These transcripts isolated were found to have lengths ranging between 464-1383 bp (Table 2). Besides, they were also analyzed by BLASTn and most of them have identities ranging from 74-94% to that of zebrafish except for *ABCD2*, *ABCF1* and *ABCH1*. The BLASTp results (Table 2) were found to be consistent with that of BLASTn results, most of the gene transcripts isolated from the Sarawak rasbora hit the highest score to that of the zebrafish except for *ABCA2, ABCF1* and *ABCH1*. These ABC gene transcripts of Sarawak rasbora shared high identities in terms of protein to that of the zebrafish with percentage as high as above 95%. The BLASTp identities shared between close family members of Cyprinidae and the Sarawak rasbora are generally higher than that of nucleotides, indicating the strong functional conservation of these genes across the Cyprinidae family despite the presence of nucleotide variations due to speciation.

ORFs were predicted from these gene transcripts *in silico* using NCBI ORF finder, blasted using BLASTp and sequences of motifs were revealed via NCBI CDD (Table 3). These ORFs were found to be protein isoforms that are predicted from all 9 intronless transcripts. The motif distribution and location were found to be high conserved across the ABC family members of Sarawak rasbora except for *ABCH1* (Figure 1). The Walker A and Q-loop were found in all ABC gene transcripts with the exception for *ABCH1*. The other motifs such as ABC Signature, Walker B, D-loop, and Q-loop, were found to be conserved in terms of location and distribution in the gene transcripts they were found in, suggesting their concerted functional roles in detoxification that allows teleost to strive and adapt in polluted environments. The predicted ORFs used in this study was the product of *in silico* translation used to predict structural features of these genes.

Table 2
The summary of BLASTn and BLASTp results of the selected ABC genes

Gene isolated from Sarawak rasbora	Length of transcript isolated	BLASTn highest score potential candidate	Identities (%)	BLASTp highest score potential candidate	Identities (%)
ABCA2 (MG757499)	464 bp	*Danio rerio ABCA2* XM_005165231.4	92	Predicted *S. rhinocerous ABCA2-like* XP_016413565.1	100
*ABCB4** (MG757500)	534 bp	*Danio rerio ABCB4* NM_001114583.2	90	*Danio rerio ABCB4* isoform 2 NP_001108055.2	97
ABCB8 (MG757501)	668 bp	*Danio rerio ABCB8* NM_001017544.1	87	*Danio rerio ABCB8* NP_001017544.1	97
ABCC2 (MG757502)	696 bp	*Danio rerio ABCC2* NM_200589.2	85	*Danio rerio ABCC2* AAI55106.1	95
*ABCD2** (MG757503)	588 bp	Predicted *Sinocyclocheilus grahami ABCD2-like* XP_016107888.1	90	Predicted *Danio rerio ABCD2-like* XP_005174656.2	100
ABCE1 (MG757504)	600 bp	*Danio rerio ABCE1* AAH45882.1	94	*Danio rerio ABCE1* NP_998216.2	100
*ABCF1** (MG757505)	679 bp	Predicted *Sinocyclocheilus anshuiensis ABCF1-like* XP_016345625.1	91	*Oryzias latipes ABCF1* XP_004074386.3	100
*ABCG2** (MG757506)	1383 bp	*Danio rerio ABCG2a* NP_001036240.1	85	*Danio rerio ABCG2* NP_001036240.1	96
*ABCH1**	678 bp	*Cyprinus carpio* genome assembly common carp genome, scaffold: LG12, chromosome: 12 LN590717.1	74	-	-

Table 3
The ORFs of each partial gene fragment predicted by NCBI ORFfinder

Gene	Amino acid length*	Motifs available	Corresponding motif sequences
ABCA2	153 aa	Walker A/P-loop	GVNGAGKT
		Q-loop/lid	YCPQ
ABCB4	177 aa	Walker A/P-loop	GSSGCGKS
		Q-loop/lid	VVSQ
		ABC signature	MSGGQKQRIA
		Walker B	ILLLDE
		D-loop	SALD
		H-loop/switch region	IVVAHRL
ABCB8	221 aa	Walker A/P-loop	GESGGGKST
		Q-loop/lid	FISQ
		ABC signature	LSGGQKQRIA
		Walker B	ILILDE
		D-loop	SALD
		H-loop/switch region	LIIAHRL
ABCC2	231 aa	Walker A/P-loop	GRTGAGKS
		Q-loop/lid	IIPQ
		ABC signature	LSLGQRLLC
ABCD2	194 aa	Walker A/P-loop	GPNGCGKS
		Q-loop/lid	YIPQ
ABCE1	199 aa	Walker A/P-loop	GTNGIGKST
		Q-loop/lid	VKPQ
		ABC signature	LSGGELQRFA
		Walker B	IFMFDE
		D-loop	SYLD
		H-loop/switch region	IVVEHDL
ABCF1	212 aa	Walker A/P-loop	GPNGVGKS
		Q-loop/lid	FFNQ
ABCG2	231 aa	Walker A/P-loop	RATGSGKSS
		Q-loop/lid	YVVQ
		ABC signature	VSGGERKRTN
		Walker B	VLFLDE
		D-loop	TGLD
		H-loop/switch region	ILSIHQP
ABCH1	224 aa	No putative motifs detected	-

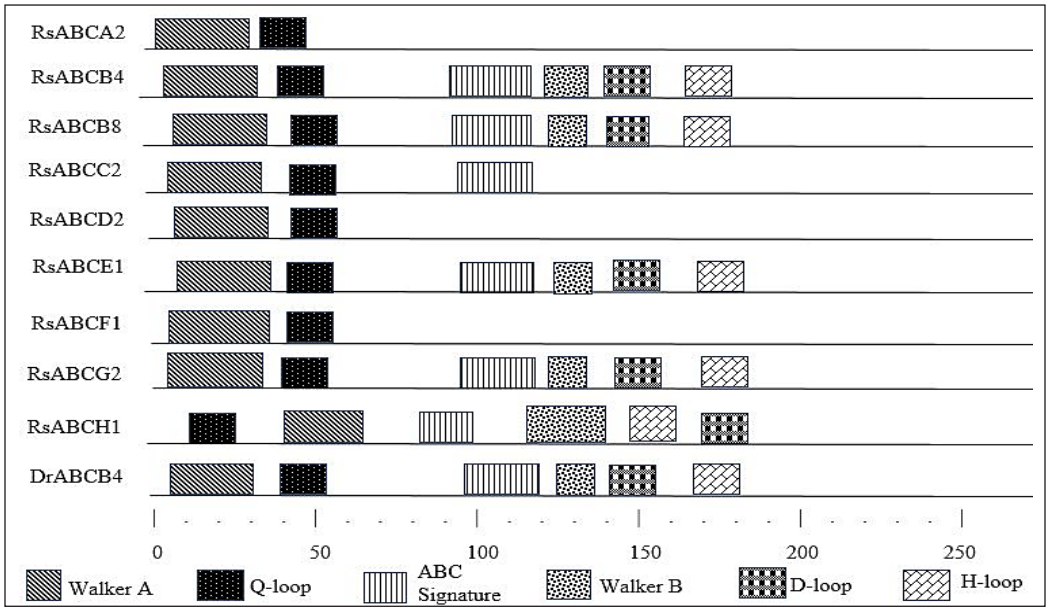

Figure 1. Motif distribution of selected ABC genes in Sarawak rasbora generated by MEME tool (Rs: *R. sarawakensis*; Dr: *Danio rerio*)

More advanced work has to be done in subsequent experiments to isolate the full ORFs using GeneRacer™ kit (Thermo Fisher Scientific, USA) in the future.

Phylogenetic Analysis

The phylogenetic tree constructed using maximum likelihood criterion gave a rather clearer point of clustering. Based on the ABC family tree constructed (Figure 2), it was observed that these ABC family members are clustered into three main clades where *ABCB4*, *ABCB8*, *ABCC2,* and *ABCG2* were grouped into Cluster A, *ABCD2* in its own Cluster B, while *ABCA2*, *ABCE1,* and *ABCF1* were grouped into Cluster C.

The phylogenetic clade where *ABCB4*, *ABCB8*, *ABCC2,* and *ABCG2* resided (Cluster A) has shown bootstrap values ranging up to 99% within clades. This cluster has been previously characterized as the canalicular ABC transporters (Cuperus, Claudel, Gautherot, Halilbasic, & Trauner, 2014; Paulusma et al., 1997; Smit et al., 1993). The canalicular phospholipid flippase (*ABCB4* protein) functions to regulate biliary excretion of phospholipids, synthesizing mixed micelles along with cholesterol as well as bile acids, thus provide protection for the bile duct epithelium against bile acids (Trauner, Fickert, Halilbasic, & Moustafa, 2008). Loss of function of *ABCB8* can result in interruption of iron homeostasis between cytosol and mitochondria in mouse. Besides, this gene, if found defective, can lead to severe cardiac dysfunction in mice (Ichikawa et al., 2012). *ABCC2*, *ABCG2* together with *ABCB1* are canalicular ABC transporters that are responsible for biliary

excretion of xenobiotics (Cuperus et al., 2014). These genes are major parts of the multidrug resistance mechanism which are greatly influenced by drug-drug interactions and these genes can contribute to cholestatic diseases, if found defective (Jäger, 2009).

Cluster B was only resided by *ABCD2* gene transcript of Sarawak rasbora. The *ABCD2* clade interestingly displayed the maximum branching within clade when compared to clade from other clusters. With bootstrap values within clade ranging between 72-99%, the *ABCD2* of Sarawak rasbora seem to branch off further than its counterparts from other fish from the same family.

Cluster C where *ABCA2*, *ABCE1* and *ABCF1* are clustered together has not been previously characterized in depth, unlike Cluster A. Cluster C depicted high bootstrap values up to 100%. This cluster shows a much closer clade relationship with less branching happening within clades in this cluster (Figure 2). The identities of clade members, especially clade for *ABCE1* and *ABCF1* within clades, were found to be higher and they were more evolutionarily conserved in terms of nucleotide and protein sequences compared to members from other clusters. More functional studies have to be done in future to further characterize them into sub-clusters based on their functional roles and tissue specificities.

Expression Analysis

Expression analysis was also done on these ABC gene transcripts (Figure 3) to study their expression patterns as well as levels of expression. Another internal reverse primer was designed to amplify a shorter fragment of *ABCG2* gene transcript for expression purposes. There were some transcripts of Sarawak rasbora that were found to express in all six organs selected (i.e., brain, eye, gill, intestine, muscle, and skin), namely *ABCB4*, *ABCB8,* and *ABCE1*. The *ABCA2*, *ABCC2*, *ABCF1,* and *ACBG2* however were discovered to have expression in five organs except the skin. It is also interesting to observe that the brain and eye organs were found to express all eight gene transcripts of Sarawak rasbora. The expression of *ABCD2* was rather specific, it was only being expressed in the brain and eye.

Currently, the expression profiles of ABC genes (although not all) are only available for zebrafish and common carp within the Cyprinidae family. From the comparisons of expression data between the two species and Sarawak rasbora, a few highlights had been focused on. The intestine organ was found to have high expression of *ABCA2* in Sarawak rasbora and common carp, *ABCG2* in zebrafish and Sarawak rasbora as well as *ABCB4*, *ABCC2* and *ABCE1* in all 3 species (Fischer et al., 2013; Liu et al., 2016; Long, Li, Zhong, Wang, & Cui, 2011; ZFIN, 2018). The expression of ABC genes in gill was seen for *ABCE1* in Sarawak rasbora and common carp, *ABCG2* in zebrafish and Sarawak rasbora as well as *ABCC2* for all 3 species (Kobayashi et al., 2008; Liu et al., 2016; Long et al., 2011). The brain expression was found common in terms of *ABCB4*, *ABCC2*, *ABCE1,* and *ABCF1*

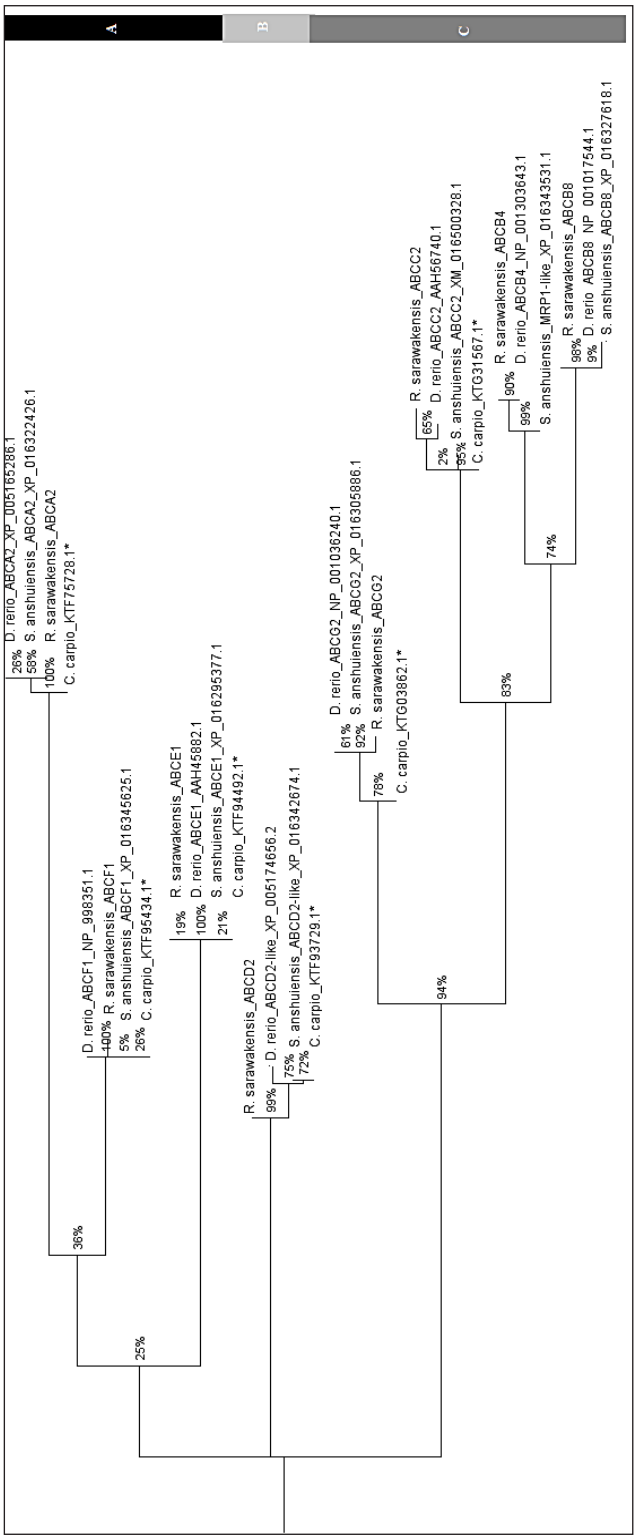

Figure 2. Phylogenetic maximum likelihood tree constructed using bootstrap with 500 replications, showing Cluster A, B, and C respectively. ("*" indicates hypothetical protein)

between Sarawak rasbora and common carp (Liu et al., 2016). The absence of *ABCD2* in both gill and intestine was found similar between Sarawak rasbora and common carp (Liu et al., 2016). These findings suggest that most of the conserved physiological roles of ABC genes take place in these three organs: intestine, gill and brain, across the Cyprinidae family.

From the expression patterns of the four gene transcripts of Sarawak rasbora (*ABCB4*, *ABCB8*, *ABCC2,* and *ABCG2*) from Cluster A, it was observed that most of them are expressed in almost all 6 organs except for *ABCC2,* and *ABCG2*. The *ABCG2* was not expressed in the skin of the Sarawak rasbora. This may be due to the functional compensation mechanism

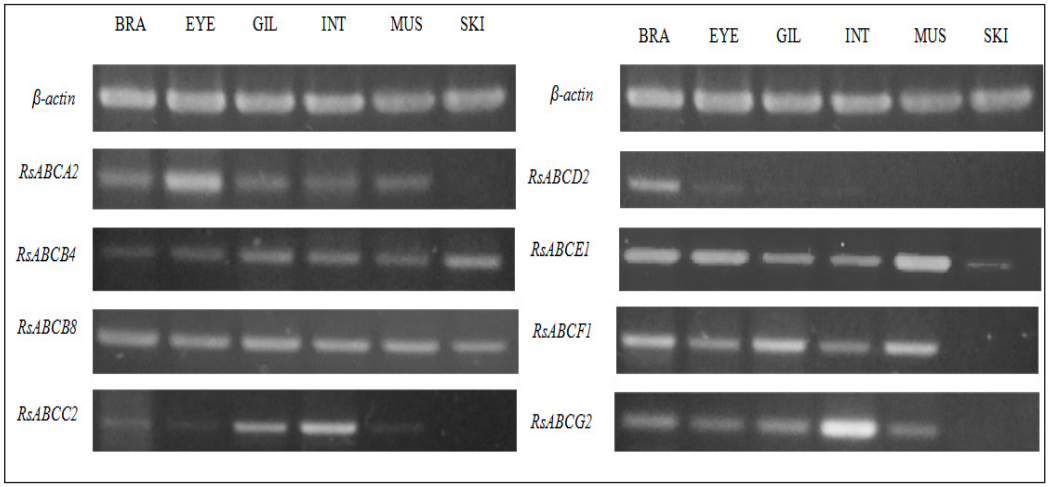

Figure 3. Expression patterns of ABC gene transcripts of Sarawak rasbora with *beta-actin* as positive control. BRA: brain, EYE: eye, GIL: gill, INT: intestine, MUS: muscle, SKI: skin.

where they share substrates as well as sites of expression and this is also why *ABCG2* knockdown mice did not suffer from severe phenotypes (Cuperus et al., 2014). The high expression pattern of *ABCC2* in the gill and intestine may explain its possible role in the detoxification mechanism as proven in the case of Nile tilapia, where expression of *ABCC2* was elevated up to 16-fold following benzo(a)pyrene exposure (Costa, Reis-Henriques, Castro, & Ferreira, 2012).

Considering the expression patterns of gene members of Sarawak rasbora from Cluster C, it can be observed that all gene members were expressed in the brain organ and the expression levels were relatively high. This may provide insights into the involvement of these ABC genes in the mechanisms of transport that are specifically localized in the brain.

CONCLUSION

In this study, a total of nine ABC transporter genes were identified in the Sarawak rasbora genome. The phylogenetic analysis had provided us more understanding towards the ABC gene family in the evolution of teleosts. The highly conserved motif distribution

across ABC gene family members suggests that they are the potential targets for the study of interactions involved in the mechanism of ecotoxicology especially in the teleosts, thus providing more detailed comprehension on how they can strive to live and adapt in polluted environment.

The expression profiles of most of the gene transcripts from the Sarawak rasbora correlate with the phylogenetic clustering where some similar characteristics were observed. This study serves as a preliminary exploration into evolution (i.e. divergence and convergence) of this gene family in the Sarawak rasbora. The functional significance and structural diversity of this gene family in teleost are yet to be explored in detail. In future, further characterization of the complete gene transcripts thoroughly will be performed via functional studies to verify their tissue-specific functions.

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First Report of *Rhizoctonia solani* Kuhn. Isolated from Parthenium Weed (*Parthenium hysterophorus* L.) in Malaysia

S. M. R. Karim[1]*, Laila Naher[2], Norhafizah M. Z.[1], Fatimah Kayat[1] and Nabilah Sarip[1]

Faculty of Agro-Based Industry, Universiti Malaysia Kelantan (UMK), Jeli Campus, 17600 Jeli, Kelantan, Malaysia
Institute of Food Secutiry and Sustainable Agriculture, Universiti Malaysia Kelantan (UMK), Jeli Campus, 17600 Jeli, Kelantan, Malaysia

ABSTRACT

Fungal pathogens are useful in biocontrol of parthenium weed (*Parthenium hysterophorus* L.) for efficient weed management. Although parthenium is a major problem in Malaysia, no initiative for developing biocontrol method has been taken yet. Therefore, a field study was conducted at Kg. Durian Tunggal, Melaka (Latitude: $2^0$20'55"N, Longitude: $102^0$17'33" E), Malaysia in August 2017 to isolate disease-causing pathogens of parthenium weed. Diseased parthenium leaves with symptoms of yellowish-brown leaf blight and the parthenium stem with reddish-brown cankers on the basal part were collected and examined after they were cultured in potato dextrose agar (PDA) medium. Isolated fungus was identified based on morphological and microscopy analyses. Two isolates, UMKRSPL1 and UMKRSPS1, were isolated qualitatively from the infected leaves and stem respectively, that yielded dark-brown colonies of sclerotia on PDA. Under the microscopic study, the mycelia with multi-nucleolus hyphal cells were noted, which were septate and hyaline. The hyphae branched at right and acute angles to the primary hypha. There was no conidium. Pathogenicity of the fungus proved to cause similar symptoms on new, fresh parthenium leaf. Based on cultural and morphological characteristics, the pathogen was identified as *Rhizoctonia solani* Kuhn. There is no published study on *R. solani* isolated from *P. hysterophorus* in Malaysia. This is the first research on the species found on parthenium weed in the country. The identification of pathogens from parthenium

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E-mail addresses:
rezaul@umk.edu.my (S. M. R. Karim)
lailanaher@umk.edu.my (Laila Naher)
norhafizah.mz@umk.edu.my (Norhafizah M. Z.)
fatimah@umk.edu.my (Fatimah Kayat)
nabilahsarip95@gmail.com (Nabilah Sarip)
* Corresponding author

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weed would help develop bioherbicide by extracting the toxins produced by the fungus in the media broth for effective control of *P. hysterophorus* in Malaysia.

Keywords: Biocontrol, mycoherbicide, parthenium weed, *Rhizoctonia solani*, soil-borne pathogen

INTRODUCTION

Parthenium weed (locally known as Rumpai Miang Mexico) is an invasive alien species (IAS) found in Malaysia. Crop yields suffer as a result by at least 40%. This species replaces native flora and lead to allergic reaction in o humans and compromises animal health through releasing toxins, which leads to serious socio-economic losses to the people (Adkins & Shabbir, 2014; Karim, Norhafizah, & Maszura, 2017a). Chemical analysis of parthenium plants confirms all plant parts, including trichrome and pollens, contain bitter glycoside parthenin, a significant sesquiterpene lactone which is responsible for causing allergic reactions in humans and animals (Patel, 2011).

The concerned authority of the country reported that the parthenium weed had affected more than 70 hectares of land in 10 states in Malaysia (DOA, 2015; Karim, 2015). Controlling this IAS is important for any country (Dilipkumar, 2016). Although the above-ground parthenium infestation has been reduced to some extent (Tashny, 2016) a vast amount of weed seedbank has remained in the soil (Karim, Nurzafirah, & Norhafizah, 2017b), indicating parthenium hazards have not been eradicated. Therefore, particular emphasis should be given by the concerned researchers to find ways to control the soil seedbank.

A few weed scientists at Universiti Malaysia Kelantan (UMK) identified this IAS in Malaysia in 2013 (Karim, 2013, 2014) and since then, the researchers of the Parthenium Weed Research Group (PWRG) of the university have been researching on the best method to control this invasive alien weed (IAW). Parthenium weed infestation can be prevented or managed by manual, chemical or biological methods. The manual method is suitable when the weed is still young. This is by removing the plants before they flower and subsequently burning them. However, it is an expensive method of control, and it not possible to completely eradicate them. The chemical control can suppress the IAS efficiently, but it leads to environmental pollution and can create herbicide resistance in the weed. Biological control using insect or pathogen, on the other hand, is the most eco-friendly and sustainable method of control (Singh & Srivastava, 2009). Biocontrol using mycotoxins from parthenium fungi is an eco-friendly and sustainable approach, and with current concerns related to biosafety and bioterrorism, the use of mycotoxins as weapons to control parthenium hazards cannot be ignored. It is well-documented that the fungal species produces

phytotoxic metabolites (mycotoxins) which induces symptoms similar to those of the pathogens themselves. Some of the metabolites of *Colletotrichum* sp. have been shown to play a significant role in pathogenesis creating large necrotic lesions on the leaves and stem of the host plant. Singh, Quereshi, Banerjee and Pandey (2010) observed the herbicidal potential of cell free culture filtrate of *Phoma herbarum* (FGCCPH#27) against parthenium weed.

In the integrated weed management approach, the use of mycoherbicide is beneficial (Kaur, Aggarwal, Yadav, & Gupta, 2016). No information regarding the control of parthenium weed using bioherbicide is available in Malaysia. Therefore, this research was carried out to isolate and identify the fungal pathogens that affect the growth of parthenium weed, so that it can be used to develop a bioherbicide for controlling the IAW, especially to control weed growth along the roadsides, residential areas and fallow lands in the country.

MATERIALS AND METHODS

Plant Materials

Five diseased leaves and one infected stem of parthenium weed were collected from a parthenium weed infested area in Melaka (Kg. Durian Tunggal; Latitude: 2°20'55" N, Longitude: 102°17'33" E), using zippered plastic bags and placing them in a cool box. The collected samples were carried back to the UMK laboratory for investigation.

Methods

Potato Dextrose Agar (PDA) was used for isolation of fungal pathogen. The PDA was prepared by mixing 19 g of commercial PDA premix with 500 ml of distilled water. The mixture was stirred on a hot plate for a few minutes to dissolve the powder completely (Kaur & Aggarwal, 2015a). The solution was then transferred into a sterilised media bottle for autoclaving at 121°C at a pressure of 15 psi for 15 minutes. 5 ml of streptomycin sulphate was added to the media after autoclaving to exclude the growth of any unwanted bacteria or other micro-organisms. The autoclaved PDA solution at the rate of 10 ml per plate was transferred to Petri dishes and placed in the laboratory for solidification. The PDA culture was ready after three hours (Aggarwal, Kaur, Kumar, & Saini, 2014).

The infected leaves and stems were cut into 6 mm sizes and were sterilised in 1% Sodium hypochlorite solution. They were washed in sterilised distilled water for four times. The sample plant parts were then placed on PDA medium of Petri dishes. The samples were incubated at 25°C in dark condition for seven days during which the fungi grew well on the PDA medium. In order to obtain a pure culture, the isolated fungi were aseptically transferred to new PDA plates and the cultures were incubated for seven days under the conditions mentioned earlier. The pure culture was maintained on PDA slants for further investigation (Kaur et al., 2016).

The identification of fungal isolate was done by preparing lactophenol cotton blue mounts from moist plate culture. Morphological characteristics of the fungal pathogens, such as the development of hyphae colour and septum of hyphae, conidia, conidiophores (if any), number of transverse and longitudinal septa and the size of the beak, etc. were recorded at different stages for identification of the pathogens. With the help of light stereomicroscope at 10×, 40× and 100× using micrometry, the size and shape of conidia (asexual spores) or conidiophores if present, type of hypha, number of the beak were observed.

The pathogenicity test was done following the technique of Koch's Postulate with slight modification in which the fresh susceptible parthenium leaves, one leaf per plate replicated three times, were placed on the newly prepared PDA culture of isolated fungus based on the procedure adapted from Kaur and Aggarwal (2015b) and kept for seven days under similar climatic conditions as described earlier. The microbial fungal mass, including hyphae, was the pathogenic unit since no conidia were found. The specimens were regularly observed for the appearance of symptoms after three days of incubation (Aggarwal et al., 2014; Aneja, Khan, & Kaushal, 2000).

The pathogen was identified based on basic morphological characteristics as discussed in the literature (Chen et al., 2014; Tredway & Burpee, 2006; Whitman et al., 2012). However, the molecular identification of the pathogen is in progress.

RESULTS AND DISCUSSION

The fungi were identified by their essential characteristics and the early symptoms that appeared on the parthenium leaf and stem. Symptoms of the disease, the colonial morphology that grew on PDA media, mycelia, and hyphal characteristics were observed under microscope are shown in Table 1.

Symptoms of Leaf and Stem of Parthenium Weed

The presence of the pathogen led to light-yellow coloured spots and blights on the leaf of parthenium weed. The fungus formed colonies of blackish sclerotia on the surface of PDA cultures after two weeks, which were irregularly shaped, >1 mm in width (Figures 1A and 1B). In the stem specimen, a concentric ring of sclerotia was also observed (Figure 2H).

Observation under the microscope showed the mycelia of the fungus was initially colourless, which turned brownish with maturity. No conidium or conidiophore was observed. It produced hyphae branches at right and acute angles to the main hypha. The branch hypha was slightly constricted at the branch origin, and there was a septum near the branch origin. The hyphae were observed with more than two nuclei.

Pathogenicity was proved on a detached fresh leaf of parthenium (Figure 3). The symptoms appeared on the fifth day after artificial inoculation which showed a water-soaked lesion. Later the light-yellowish lesions grew which later covered larger areas and the leaf tips became rotten.

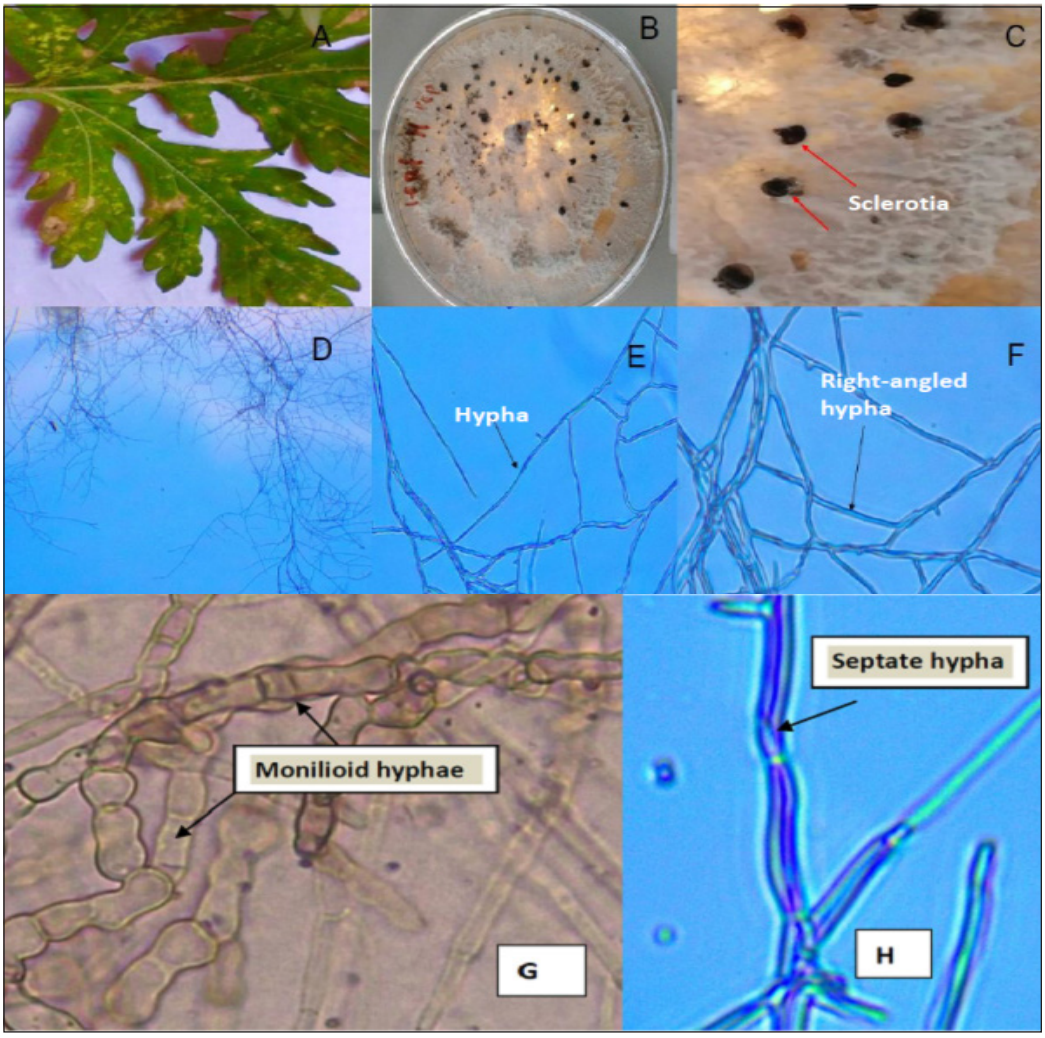

Figure 1. Isolated fungus from infected parthenium leaf. (A) Leaf blight disease on parthenium leaf; (B) Full colonial growth of the fungus with blackish sclerotia developed on the surface of PDA; (C) Magnified sclerotia (red arrow); (D) Mass of mycelia under microscope with 10× magnifying; (E) Fungal hyphae under microscope with 40× magnifying (black arrow); (F) Right angled hyphae of the fungus under microscope with 100× magnifying (black arrow); (G) Monilioid hyphae; and (H) Septate hyphae of the isolated fungus

Two weeks later, light brown mycelia and sclerotia of the pathogen were observed. The symptoms of the disease found under *in vitro* conditions were similar to that found in the field conditions. Kumar, Jayaraj and Muthukrishnan (1979) reported the wilting sign of parthenium weed caused by *R. solani* in India.

Ceresini (2011) reported the symptoms of the disease caused by *R. solani* depended on the host plant and the strain of the fungus. Usually, the symptoms are wilting, black necrotic collar rot of the seedling, and the blight on leave area. The symptoms appear at the lower and older leaves as a small brown spot with a circular ring. Dry

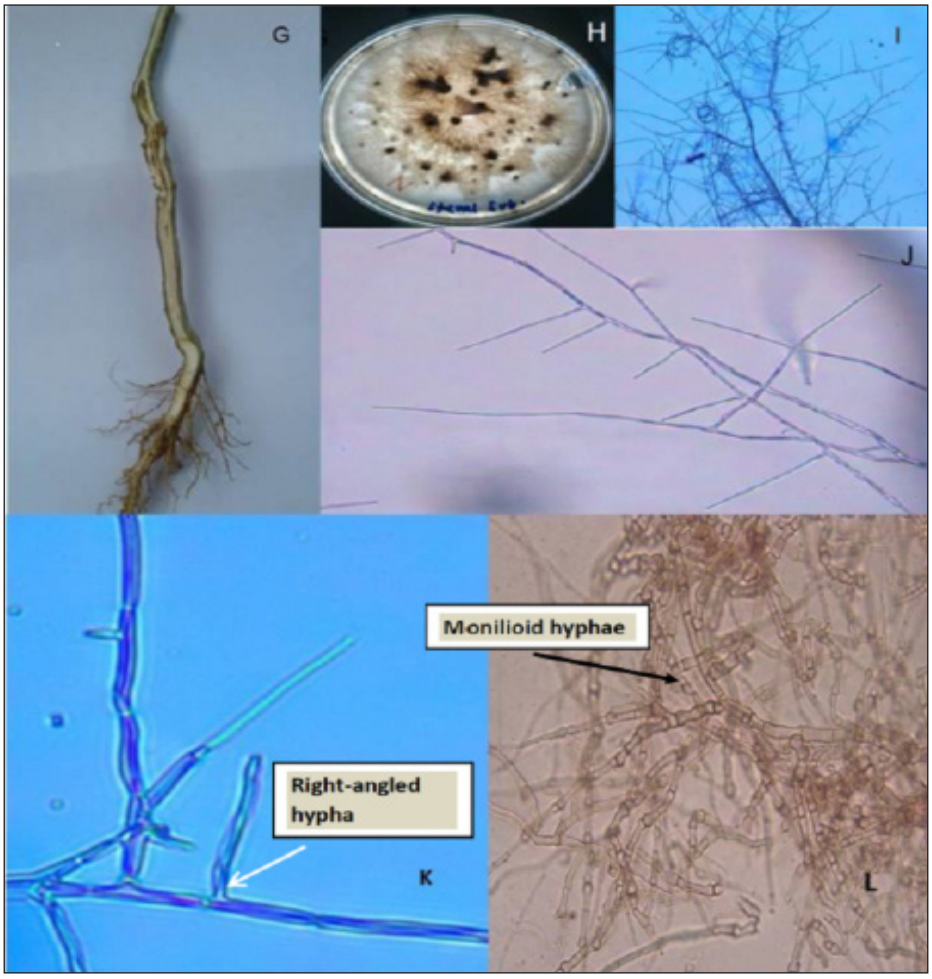

*Figure 2***.** Isolated fungus from infected parthenium stem. (G) Cankers on parthenium stem; (H) Fungal colony and sclerotia; (I) Fungal mycelia under the microscope with 10× magnifying; (J) Acute angle hyphae of under microscope with 40×; (K) Right angle hyphae (white arrow) under the microscope with 100×; (L) Monilioid hyphae

sunken, rusty-brown on stem and root that are near to soil line are also the symptoms of infection by *R. solani*. This disease leads to the stunting of the older plants and seedlings, wilting and yellowing. However, the illness sometimes does not appear as apparent damage to the hosts; it can girdle the stem causing the plant to become stunted and resulting its eventual death.

As per statement of Whitman et al. (2012), *R. solani* had septa that formed in the branch near constriction. As the hyphae mature, it becomes more rigid and uniform. The branches rise at 90° and also at acute angles of 45° from the primary hypha. Typically, most of the hyphal cells produce new arms near to the end of the principal hyphae. In the primary hyphae near the

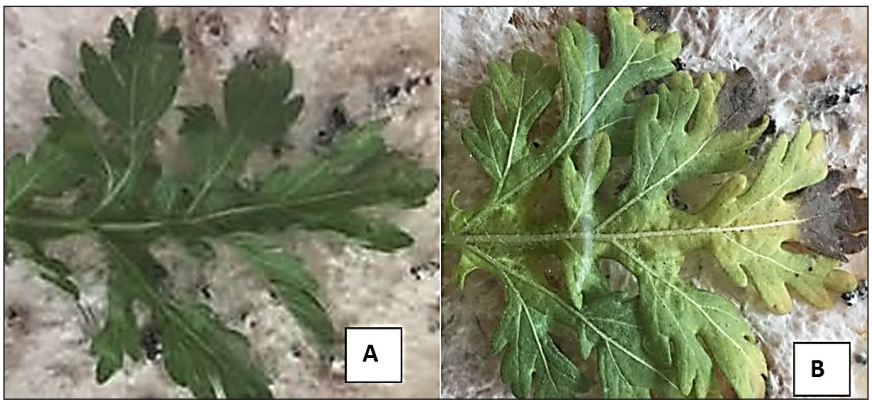

Figure 3. Pathogenicity test. (a) Fresh parthenium leaf after two days of inoculation; (b) Symptoms developed after five days of inoculation (after infection with the fungus the leaf turned to light yellowish color and tips are rotten)

branches, sometimes there is a single or no septum. Secondary septa are formed in older hyphae and with thinner or similar thickness as cell wall, while the primary septum is thicker at the junction with the cell wall. The young mycelia initially present as a white colour, but they turn to brown later.

Kaur et al. (2016) reported the pathogens with the production capability of lignin-degrading enzymes could play an essential role in the control of parthenium weed. Lignin is present in middle lamella, the secondary cell wall of xylem vessel and fibres that strengthen the plants. Only a small group of microorganisms are capable of degrading this lignin since the lignin is more resistant to enzymatic degradation than other plant substances. Wibberg et al. (2014) also recorded *R. solani* as an efficient producer of lignin-degrading enzymes. O'Briert and Zamani (2003) noted the production of pectic enzymes (polygalacturonase and pectin lyase) by the phytopathogenic *R. solani* AG-8 (ZG-1). These pectinases play a role in breaking the plant cell walls as well. The authors stated in their reports that plant cell walls are primarily polysaccharide in composition. In case of host-pathogen interactions, degradation of cell walls involves actions of polysaccharides secreted by the pathogens. Most of the degradative enzymes are glycoside hydrolases, which degrade the cellulose and pectate matrices by adding water to break down the glycoside bonds. The pectate network is also degraded by polysaccharide lyases, which cleave the glyosidic bonds via β-elimination mechanism (Herron, Benen, Scavetta, Visser, & Jurnak, 2000). This observation was also reported by Tredway and Burpee (2006) and Chen et al. (2014).

Based on the disease symptoms, characteristics of sclerotia on the PDA surface observed, attributes of fungal mycelia, hyphae, and hyphal branching habits (Table 1), reconfirmation through pathogenicity test, and the descriptions given by different authors, the present study confirmed the isolated fungal is *R. solani* Kuhn.

R. solani is the plant pathogenic fungus that is widespread and widely recognised species of Rhizoctonia. This fungus is commonly known as a soil-borne pathogen with a high diversity of host plant. The pathogen is a basidiomycete fungus, which does not produce any asexual spores like conidia. Sometimes it creates sexual spore (basidiospore), unlike other basidiomycete fungi. The basidiospores are not enclosed in the fruiting body. In nature, *R. solani* reproduces asexually through vegetative mycelia and sclerotia (Uchida, 2011).

Table 1
Identified characteristic of R. solani from infested parthenium plant

Fungal Species	Parthenium Sample	Colony colour on PDA	Sclerotia	Spore and hyphal structure	Isolated code
R. solani	Leaf	Produce dense mycelia with whitish-brown colour on PDA media	Produced sclerotia with irregular shapes and dark brown colour. Sclerotia grew in loose group.	Did not produce any spore. Septa formed in the branches. Hyphae become more rigid and uniform when it matures. Main hyphae branched at 90° and acute angle of 45°. Hypha produced specialised form called monilioid cells.	UMKRSPL1* (UMK *R. solani* Parthenium leaf)
R. solani	Stem	Produce dense mycelia with light brownish colour on PDA.	Produced sclerotia with irregular shapes and dark brown colour. Sclerotia grew in loose groups.	Did not produce spore. Septa formed in the branches. Hyphae become more rigid and uniform when matured. Main hyphae branched at 90° and acute angle 45°. Hypha produced specialised monilioid cells.	UMKRSPS1 (UMK *R. solani* Parthenium stem)

*The number indicates that time of investigation

These soil-borne fungi can be used to make bioherbicide to control the parthenium weed, especially those growing along the roadsides, fallow lands and residential areas where there are no susceptible crops. The mycoherbicide can be applied to the soil of parthenium infested area as the mycelia may reside in the soil, which can attack the host plants. The fungi are attracted to the host by the chemical stimuli released by the growing plants nearby and decomposing plant residue. The pathogen attaches itself to the host by direct penetration of the plant cuticle or using natural openings in the

hosts. Hyphae come in contact with the plant and attach themselves to the host producing an appressorium, which penetrates the plant cells. After making a connection, the pathogen obtains nutrients from the plant cell. The pathogens also release ligninolytic enzymes that break down the cell walls and continue to colonise and grow inside the dead tissue. In severe infections, the plants die.

CONCLUSION

The study identified *Rhizoctonia solani* as a soil-borne pathogen which has strong pathogenicity with parthenium weed. Therefore, these pathogens may be the right candidates for producing bioherbicide for controlling parthenium weed in Malaysia for application on non-cropped areas. More research is needed to increase the virulence of the pathogen so that biocontrol efficacy of the identified pathogens can be increased. In addition, more studies on molecular characterisation and identification of anastomosis groups of the collected isolates of *R. solani* will be carried out. There is no published information on the species *R. solani* isolated from *P. hysterophorus* in Malaysia and this is the first report of the species found on parthenium weed in the country.

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Chemical Profile, Total Phenolic Content, DPPH Free Radical Scavenging and α-Glucosidase Inhibitory Activities of *Cosmos Caudatus* Kunth Leaves

Wan Nadilah Wan Ahmad[1], Khozirah Shaari[1]*, Alfi Khatib[2], Azizah Abdul Hamid[3] and Muhajir Hamid[4]

[1]Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[2]Kuliyyah of Pharmacy, International Islamic University Malaysia (IIUM), 25200, Kuantan, Pahang, Malaysia
[3]Faculty of Food Science and Technology, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[4]Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

ABSTRACT

Herbs and medicinal plants are major sources of traditional or folk medicines for many countries of the world, including Malaysia. This study evaluated the bioactive potential of the leaf ethanolic extract and solvent fractions of *Cosmos caudatus* Kunth, in scavenging free radicals and inhibiting the enzyme α-glucosidase. In addition, their metabolite profiles were also characterized using liquid chromatography–mass spectrometry. The bioactivity was found to be concentrated in the EtOAc and BuOH fractions which largely contained rutin, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinofuranoside, quercetin 3-*O*-rhamnoside, and quercetin 3-*O*-galactoside, as profiled by LC-MS/MS. It was further shown that the flavonoids glycosides contributed to the free radical scavenging and glucose lowering effects of *C. caudatus* leaves. The results indicated that the leaves of *C. caudatus* are a rich source of bioactive compounds and could be prospective materials for development of new anti-diabetic agents.

Keywords: α-glucosidase inhibition, *Cosmos caudatus*, free radical scavenging activity, LC-MS/MS

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E-mail addresses:
wnadilahahmad@gmail.com (Wan Nadilah Wan Ahmad)
khozirah@upm.edu.my (Khozirah Shaari)
alfikhatib@iium.edu.my (Alfi Khatib)
azizahah@upm.edu.my (Azizah Abdul Hamid)
muhajir@upm.edu.my (Muhajir Hamid)
* Corresponding author

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INTRODUCTION

Cosmos caudatus Kunth, locally known as "ulam raja", is a small branched perennial aromatic herb that is 30-250 cm tall with finely dissected leaves (Bunawan, Baharum, Bunawan, Amin, & Noor, 2014). The plant belongs to the family *Asteracecae* and is commonly found in Southeast Asian countries, particularly Indonesia and Malaysia. A decoction of the leaves was recommended for use against diabetes, high blood pressure, arthritis and fever (Abas, Shaari, Lajis, Israf, & Kalsom, 2003; Burkill, 1966; Rasdi, Samah, Sule, & Ahmed, 2010) as well as for several other health benefits such as for longevity and aiding digestion (Bunawan et al., 2014; Ong & Norzalina, 1999). Some major chemical constituents of *C. caudatus* have been investigated and reported in scientific literature. Among these, the phenolic constituents were implicated in the antioxidant and antidiabetic properties of the plant (Kerem, Bilkis, Flaishman, & Sivan, 2006; Kunyanga, Imungi, Okoth, Biesalski, & Vadivel, 2012; Mai, Thu, Tien, & Chuyen, 2007; Ranilla, Kwon, Apostolidis, & Shetty, 2010). An increased production and ineffective scavenging of reactive oxygen species are also known to play a critical role in diabetes mellitus. *C. caudatus* has been reported to have an exceptionally high antioxidant capacity, mainly due to its polyphenolic content (Shui, Leong, & Wong, 2005). Polyphenol-rich foods are known to be potent antioxidants (Dai & Mumper, 2010), protecting cells against oxidative stress and reducing the risk of chronic disease (Art & Hollman, 2005). Thus the antioxidant property of *C. caudatus* has the potential to reduce the harmful effects of oxidative stress.

Hyperglycemia, or raised blood sugar, is a common effect of uncontrolled diabetes. If left unmanaged, it will lead to serious damages to various systems of the body, especially the heart, eyes, nerves, blood vessels, and kidneys. Postprandial glucose is an indicator for glycemic control. Postprandial hyperglycaemia is strongly associated with an increased risk of cardiovascular disease in diabetic patients (Hanefeld et al., 1996). One way to assess this is through postprandial glucose. Postprandial hyperglycaemia can be decreased by delaying carbohydrate absorption in the gastrointestinal tract (Dehghan-Kooshkghazi & Mathers, 2004). This can be achieved by inhibiting carbohydrate-hydrolysing enzymes and slowing glucose uptake by intestinal α-glucosidase. Apart from being costly to produce and associated with a decline in efficiency with long term use, modern synthetic hypoglycemic agents have been reported to cause side effects (De Melo Junior, Raposo, Neto, & Diniz, 2002; Satyanarayana, Katyayani, & Latha, 2006). To a certain extent, these reasons have fuelled continued chemical and biological exploration of medicinal and edible plants for the discovery of new and alternative agents with antidiabetic properties. Natural product based α-glucosidase inhibitors are the key targets to identify new compounds for the therapeutic management of diabetes

(Franco, Rigden, Melo, & Grosside, 2002; Yin, Zhang, Feng, Zhang, & Kang, 2014). *C. caudatus* has been shown to have hypoglycemic property in both *in vitro* (Loh & Hadira, 2011) and in *in vivo* study (Perumal, Hamid, & Ismail, 2014). Thus, it is interesting to ascertain if this property could be further exploited for the management of diabetes and to identify the bioactive constituents of the plant. The free radical scavenging activity combined with α-glucosidase inhibitory activity of the ethanol extract and its different polar, non-polar fractions from *C. caudatus* have not been fully studied. Therefore, this study investigated the *in vitro* antioxidant and α-glucosidase inhibitory activities of the ethanolic extract of *C. caudatus* and profiled the probable bioactive chemical constituents in the ethanolic extract by LC-MS/MS technique.

MATERIALS AND METHODS

Materials

C. caudatus Kunth was obtained from UPM Agriculture Park. A voucher specimen SK 2511/14 was deposited in the mini herbarium of the Institute Bioscience, University Putra Malaysia. Taxonomic verification of the species was carried out by Dr. Shamsul Khamis, Biodiversity Unit, Institute Bioscience, UPM).

Plant Extraction and Solvent Fractionation

The leaves of plant material were washed under running tap water and dried under shade. The sample dried leaves were ground to a fine powder in a Waring blender (model 32 BL 80, New Hartford, CT, USA). The powder material was macerated in EtOH:water, 80:20, plant powder (g) to solvent (mL) ratio 1:3, at room temperature. To facilitate extraction, the mixture was sonicated for 1 h, at two intervals of 30 min with a break of 15 min to avoid solvent overheating. The extraction procedure was repeated three times, the pooled filtrates filtered through Whatman Filter No 1, and evaporated to dryness using a rotary evaporator to yield 41.2 g of total crude extract. About 40.0 g of the crude extract was resuspended in 2 L of water and partitioned sequentially with 2 L of hexane, 2 L of DCM, 2 L of EtOAc, 2 L of BuOH to yield the respective hexane (2.69 g), DCM (15.56 g), EtOAc (0.86 g), BuOH (0.73 g) and aqueous (6.09 g) fractions. The extract and solvent fractions were stored at -4°C before subjecting them to bioassay and LC-MS/MS analysis.

Measurement of Total Phenolic Content

The Folin-Ciocalteu assay was used to determine the total phenolic content (TPC) of the extracts based on the procedure described by Zhang et al. (2006), with minor modifications. In triplicate, 20 µL of each serial dilution (6.25, 12.5, 25, 50, 75, 100, 125, 250, 500 ppm) was introduced into test tube, alongside the same series of serial dilution for quercetin as the positive standard. Folin-Ciocalteu reagent (100 µL) was added to each well, mixed thoroughly using a vortex mixer, and allowed to rest for 5 minutes at room temperature. This

was followed by the addition of 80 µL of 7.5% (w/v) sodium carbonate solution and made up to a final volume of 200 µL with distilled water. The solutions in the test tubes were allowed to stand for 30 min prior to the measurement of the absorbance at 765 nm. TPC was expressed as the gallic acid equivalent (GAE) in milligrams per gram of extract.

In Vitro Biological Assays

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Free Radical Scavenging Assay. The DPPH free radical scavenging assay was conducted according to methods described by Li and Seeram (2011), and Wan, Yuan, Cirello and Seeram (2012) with slight modification. The assay was performed in 96-well microplate and performed in triplicates. Aliquots of 50 µL of each serial dilutions (6.25, 12.5, 25, 50, 100, 200, 400 and 500 ppm), of the test samples and quercetin (positive control), made from the stock solutions 0.5 mg/mL and 0.2 mg/mL, respectively, were placed in each well. This was followed by the addition of 150 µL 1,1-diphenyl-2-picryl-hydrazyl (DPPH) which was prepared beforehand at a concentration of 59 mg/L to each well. The microplate was then incubated in the dark at room temperature for 30 min. The analysis was performed in triplicates. The absorbance of the reaction mixtures was measured at 517 nm using a microplate reader. The percentage of inhibition of each test sample was calculated from the following formula, % Inhibition = $[(A_o - A_s)/A_o] \times 100$ where, A_o = absorbance of the reagent blank and A_s = absorbance of the test samples.

The IC_{50} value was then determined from a plot of % inhibition versus concentration of the test samples. The IC_{50} value (µg/mL), for the free radical scavenging activity refers to the concentration at which the scavenging activity was inhibited by 50%.

Alpha-glucosidase Inhibition Assay. The α-glucosidase inhibitory activity was tested following the method described by Collins, Ng, Fong, Wan and Yeung (1997) with slight modifications. The property was determined by measuring the release of *p*-nitrophenyl from the substrate *p*-nitrophenyl-α-D-glucopyranose (PNPG) (Sigma-Aldrich, N1377-1G). Release of *p*-nitrophenyl was observed as a formation of a yellow color upon addition of the reaction stopping reagent glycine (pH 10).

First, stock solutions of the test samples were prepared by dissolving 0.2 mg of the extract in 1 mL ethanol. Meanwhile, 0.4 mg of quercetin, the positive control, was dissolved in 1 mL ethanol (Subramaniam, Asmawi, & Sadikun, 2008). Aliquot of 10 µL of each serial dilutions (3.125, 6.25, 12.5, 25, 50, 100, 200 ppm) made from the stock solutions (0.2 mg/mL) and for quercetin (positive control), made from stock solution (0.4 mg/mL) were placed in each well. The substrate and enzyme were dissolved in 50 mM buffer. Briefly, 10 µL of the sample extract were added to 100 µL of α-glucosidase type 1 from *Saccharomyces*

cerevisiae (Sigma G5003) solution (0.02 U/well) in 30 mM phosphate buffer (pH 6.5). The sample mixture was then incubated for 5 min at room temperature (Deutschländer, Van de Venter, Roux, Louw, & Lall, 2009). In the meantime, 60 mg of 4-nitrophenyl-α-D-glucopyranoside (PNPG) which is the substrate, was dissolved in 20 mL of 50 mM phosphate buffer (pH 6.5). This solution has been reported to be comparable to that of intestinal fluid (Lee, Mediani, Nur Ashikin, Azliana, & Abas, 2014). The PNPG solution (75 µL) was added to each well and the reaction mixtures were incubated for 15 min at room temperature. The reaction was stopped by adding 50 µL of 2M glycine (pH 10) to each well. The optical densities (ODs) were then immediately read off at 405 nm using a microplate reader (Deutschländer et al., 2009). The analysis was performed in triplicates.

The α-glucosidase inhibition activity of the test sample was expressed as percentage (%) inhibition and calculated using the following formula, % inhibition = $[(A_c-A_e/A_c]$ X 100%, where A_c = difference in absorbance between the control (with enzyme) and the blank control (without enzyme) and A_e = difference in absorbance between a sample (with enzyme) and the blank sample (without enzyme). The percentage inhibition was plotted against the concentrations of each sample to determine the concentration required to inhibit 50% of the α-glucosidase enzyme (IC_{50} value) in µg/mL.

Analysis of Extract and Solvent Fractions by LC-MS/MS

Test samples were prepared by dissolving 1 mg of the ethanolic extract and each solvent fraction in 1 mL methanol, followed by ultrasonication for 30 min at room temperature. The test samples were then filtered and kept at 4°C prior to the LC-MS/MS analysis.

The LC column used for the LC-MS/MS analysis was a Dionex C18 Reversed-Phase column (Dionex, Sunnyvale, USA) with column dimensions 250 (l) × 2.0 (i.d) mm and 2.5 µm particle size. Analysis was performed on a Dionex Ultimate 3000 HPLC, at 26.9°C (thermostated column compartment). Mobile phase used was double distilled water containing 0.1% acetic acid (solvent A) and HPLC grade acetonitrile containing 0.1% acetic acid (solvent B). The addition of acetic acid to the mobile phase was to enhance compound peak sharpness by inducing ionization of metabolites (De Moraes, Gregório, Tomaz, & Lopes , 2009). Sample elution was performed in a gradient manner with 10 to 100 mL for solvent A, and 90 to 0 mL solvent B, respectively. The injection volume was 15 µL with constant flow rate of 1.00 mL/min. The flow was split to allow 200 µL/min of eluent into the mass spectrometer. The total LC run time was 35 min.

API-ESI (Atmospheric Pressure Ionization – Electrospray Ionization) mass spectral measurement of the sample was performed on a MicroTOF mass spectrometer (Bruker Daltonic GmbH,

Bremen, Germany). The source conditions were: nebulizer gas nitrogen (N_2) at 0.2 bar, dry gas (N_2) at 3.0 L/min, dry temperature at 180°C, capillary voltage at 4500 V and end plate offset at -500 V. Data acquisition was performed by HyStar Application version 3.2 while data processing was carried out with DataAnalysis Version 3.4 by Bruker Daltonik GmbH. The MS acquisitions were performed in the negative electrospray ionization mode, for the mass range of 50-1000 *m/z*. Additionally, MS/MS experiments were carried out in the automatic and multiple reaction monitoring (MRM) mode. Automatic MS/MS low-energy collision dissociation (CID) was performed at 5- 8 eV collision energy. For the MRM mode, MS/MS spectra were acquired manually by selecting the mass of the parent ion to be fragmented and by setting the amount of collision energy. Neutral loss spectra were extracted from the MS/MS fragment spectra. The neutral loss spectrum was calculated by subtracting the *m/z* value of each fragment ion present from the *m/z* of the precursor ion. The resulting difference values were exhibited with the intensity of the corresponding fragment ion. To add a neutral loss chromatogram, a neutral mass value was entered in the 'Edit' menu after a chromatogram of interest was chosen (DataAnalysis Help, Bruker Daltonik GmbH). The conventional nomenclature used by Domon and Costello (1988), and Wu, Yan, Li, Liu and Liu (2004), were used in representing the fragment ions of glycoconjugates.

Statistical Analysis

The results were presented as the mean ± standard deviation (SD). The statistical significance of the results was evaluated using one-way ANOVA with Duncan's post hoc test. Significant differences were based on p values where $p < 0.05$ are considered significantly different and vice-versa. All the bioassay analysis was performed in triplicates.

RESULTS AND DISCUSSION

Total Phenolic Contents

Table 1 shows the TPC of the *C. caudatus* leaf ethanolic extract and its various solvent fractions, expressed in terms of g gallic acid/g dry weight (GAE/g DW) extract. The TPCs of the solvent fractions ranged from 0.21-0.72 g GAE/g DW extract. The content was highest, and not significantly different from each other, in the EtOAc and BuOH fractions with 0.72 and 0.60 g GAE/g DW, respectively. Although the ethanolic extract consisted of more DCM solubles (37.7%), the nature of these metabolites were mainly non-phenolics since the TPC of the DCM fraction was only 0.22 g GAE/g DW and were not significantly different from the TPCs of the hexane (0.21 g GAE/g DW) or the aqueous (0.36 g GAE/g DW) fractions. Thus, it was concluded that the phenolic constituents of the *C. caudatus* leaf sample were present mainly in the EtOAc (2.1%) and BuOH (1.8%) soluble fractions.

The observed differences in the TPCs of the solvent fractions can be attributed to the difference in polarity of the solvents

used for the extraction. The more polar solvents have a higher ability of extracting phenolic constituents as compared to the less polar and moderate polarity solvents, such as hexane and DCM (Dehghan, Sarrafi, & Salehi, 2016; Fatin et al., 2012; Hatipoĝlu et al., 2013; Robya, Sarhan, Selim, & Khalel, 2013). Other studies have also demonstrated the efficiency of EtOAc in extracting phenolic constituents, for example from outenga (Abdille, Singh, Jayaprakasa, & Jens, 2005), pomegranate (Li et al., 2006), onion (Peschel et al., 2006) and citrus peel (Rehman, 2006). Meanwhile, Bonoli, Verardo, Marconi and Caboni (2004) reported that the maximum phenolic compounds were obtained from barley flour with mixtures of ethanol and acetone. Similarly, aqueous methanol was found to be more effective in recovering higher amounts of phenolic compounds from rice bran (Chatha, Anwar, Manzoor, & Bajwa, 2006) and *Moringa oleifera* leaves (Siddhuraju & Becker, 2003). Meanwhile, Anwar, Jamil, Iqbal and Sheikh (2006) extracted antioxidant compounds from various plant materials including rice bran, wheat bran, oat groats and hull, coffee beans, citrus peel and guava leaves, using aqueous 80% methanol (methanol:water, 80:20 v/v).

DPPH Free Radical Scavenging Activity

The DPPH free radical scavenging activity of the solvent fractions was determined in order to gauge their antioxidant potential. As seen in Table 1, the EtOAc and BuOH fractions exhibited very good DPPH free radical scavenging activity, with 84.5% and 71.5% inhibition, respectively, at the test concentration of 0.5 mg/mL. The inhibition percentages of the EtOAc and BuOH fractions were not significantly different ($p > 0.05$) from each other, with quercetin (91.1%) at a test concentration of 0.2 mg/mL. The DCM and hexane fractions showed lower percentage inhibitions (24.5-33.4%) and were not significantly different from each other. Similarly, the aqueous factions showed lower DPPH free radical scavenging activity of 44.4%. Thus, it was clearly seen that solvent fractions with higher TPCs (EtOAc and BuOH fractions) also exhibited higher free radical scavenging activity, and vice versa.

With respect to the IC_{50} values, the EtOAc (255.20 µg/mL) and BuOH (257.61 µg/mL) fractions exhibited lower values than the crude EtOH:water (80:20) extract (197.87 µg/mL). The IC_{50} values of the EtOAc and BuOH fractions were not significantly different ($p>0.05$) from each other but were both significantly different ($p < 0.05$) from the crude EtOH:water (80:20) extract. The bioactivity of the crude extract and solvent fractions were significantly lower ($p < 0.05$) than that of the positive standard, quercetin with 69.56 µg/mL. Therefore, it was concluded that, although *C. caudatus* was as efficient as quercetin in inhibiting DPPH free radical, it was, however, less potent, based on the higher IC_{50} value in comparison to the positive standard.

Alpha-glucosidase Inhibitory Activity

The α-glucosidase inhibitory activity of the solvent fractions was determined in order to gauge their glucose lowering potential. As shown in Table 1, the solvent fractions of the crude extract showed variable α-glucosidase inhibitory activities. The highest α-glucosidase inhibition percentage was exhibited by the EtOAc fraction (79.3%) which was as high as and not significantly different ($p > 0.05$) with that shown by the crude extract (81.6%) at a test concentration of 0.2 mg/mL, and by the positive standard, quercetin (95.1%) at a test concentration of 0.4 mg/mL. Meanwhile, both the BuOH and DCM showed significantly lower inhibition values than the EtOAc fraction. Their inhibition percentages of 57.2% and 55.6%, respectively, were moderate and not significantly different from each other. The hexane and aqueous fractions showed much lower percentage inhibitions among all the solvent fractions.

The crude extract and solvent fractions exhibited significantly more potent α-glucosidase inhibition (27.56-85.73 µg/mL) than quercetin (109.30 µg/mL). In particular, the EtOAc fraction, with an IC_{50} value of 40.90 µg/mL, seemed to be the solvent fraction that contained most

Table 1
Yield of extracts, TPC, DPPH free radical scavenging and α-glucosidase inhibitory activities of C. caudatus *Leaf EtOH:water 80:20 Extract, and its various solvent fractions*

Sample	Yield of extract (g)	Yield of extract (%)	Total phenolic content (g GAE/g DW)	DPPH free radical scavenging activity		α-glucosidase inhibitory activity	
				Inhibition (%) [Test conc: 0.5 mg/mL]	IC_{50} (µg/mL)	Inhibition (%) [Test conc: 0.2 mg/mL]	IC_{50} (µg/mL)
Crude extract	41.2	25.9	0.73 ± 0.13[a]	85.6 ± 4.41[a]	197.87 ± 13.59[a]	81.6 ± 6.89[a]	27.56 ± 6.31[a]
Hexane fraction	2.69	6.5	0.21 ± 0.04[b]	33.4 ± 3.86[b]	nd	29.2 ± 6.58[b]	nd
DCM fraction	15.56	37.7	0.22 ± 0.14[b]	24.5 ± 4.95[b]	nd	55.6 ± 5.53[c]	85.73 ± 5.94[b]
EtOAc fraction	0.86	2.1	0.72 ± 0.19[a]	84.5 ± 5.37[a]	255.20 ± 19.41[b]	79.3 ± 1.27[a]	40.90 ± 6.55[a]
BuOH fraction	0.73	1.8	0.60 ± 0.18[a]	71.5 ± 5.93[a]	257.61 ± 11.20[b]	57.2 ± 3.31[c]	74.84 ± 5.83[b]
Aqueous fraction	6.1	14.8	0.36 ± 0.03[b]	44.4 ± 4.05[c]	nd	31.4 ± 7.46[b]	nd
Quercetin	nd	nd	nd	91.1 ± 3.52[a]	69.56 ± 2.39[c]	95.1 ± 6.41[a]	109.30 ± 4.30[c]

Note: Values are expressed as mean ± standard deviation (n=3). The concentration of quercetin for DPPH radical scavenging activity and α-glucosidase inhibitory activity were 0.2 mg/mL and 0.4 mg/mL, respectively. Values with different superscript letters (a, b, c) are significantly different ($p < 0.05$).

of the bioactive metabolites. Its IC_{50} value was close and not significantly different ($p > 0.05$) from that of the crude extract. Previous studies on other plant extracts have also found that EtOAc is a suitable solvent for extracting α-glucosidase inhibitory compounds from a plant (Ablat et al., 2014; Ado, Abas, Ismail, Ghazali, & Shaari, 2014; Hyun, Hyoun-Chol, & Ju-Sung, 2014; Hyun, Hyoun-Chol, Yeong-Jong, & Ju-Sung, 2016; Moein, Moein, & Ahmadizadeh, 2008; You, Chen, Wang, Jiang, & Lin, 2012). Overall, results from this work have provided additional support to the conclusion that *C. caudatus* can potentially reduce postprandial hyperglycaemia by delaying carbohydrate digestion.

Metabolite Profiling of *C. caudatus* using LC-MS/MS

Since the EtOAc and BuOH fractions exhibited significant bioactivities, these solvent fractions were chemically profiled using API-ESI LC-MS/MS in order to gain better insight into the phenolic constituents that may be contributing to the observed biological activities. The negative ion mode was used in the analysis since most of the constituents in the solvent fractions exhibited a higher response in the negative mode rather than in the positive mode. Six and five compound peaks were observed in the total ion chromatograms (TIC) of the EtOAc (Figure 1) and the BuOH (Figure 3) fractions, respectively. The compounds were tentatively identified, based on their MS/MS fragmentation and subsequently supported by comparisons with literature data and/or MassBank Software (High Quality Mass Spectral Database). The compounds in these bioactive fractions were found to consist mainly of quercetin derivatives.

The MS/MS fragmentation of the individual peaks from the TIC of the EtOAc fraction are shown in Figure 2 and listed in Table 2. Peak **1** exhibited an $[M-H]^-$ ion at *m*/*z* 609, which was further fragmented to daughter ions at *m*/*z* 463 and 300, consistent with rutin (Mediani, Abas, Ping, Khatib, & Lajis, 2012; Shui et al., 2005; Stobiecki, Malosse, Kerhoas, Wojlaszele, & Einhorn 1999; Stobiecki, 2000). Peaks 2 and 3, both showing $[M-H]^-$ ions at *m*/*z* 463, were identified as quercetin 3-*O*-galactoside and

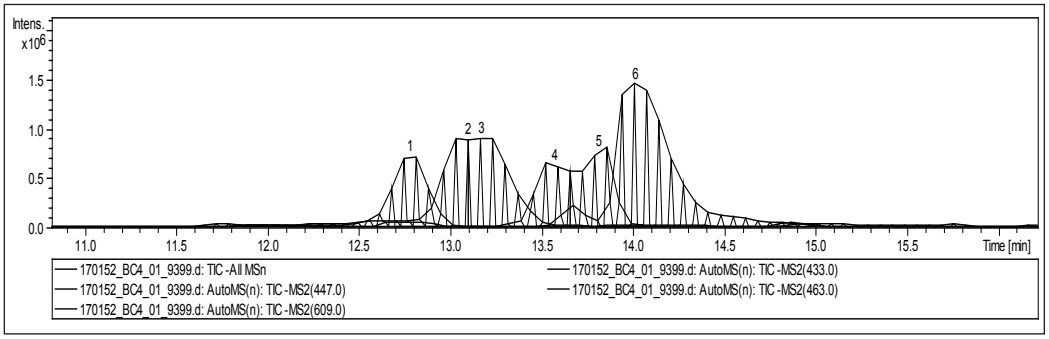

Figure 1. Total ion chromatogram (TIC) of the EtOAc fraction of leaves extract of *C. caudatus*. **1**, rutin; **2**, quercetin 3-*O*-galactoside; **3**, quercetin 3-*O*-glucoside; **4**, quercetin 3-*O*-xyloside; **5**, quercetin 3-*O*-arabinofuranoside; **6**, quercetin 3-*O*-rhamnoside.

quercetin 3-*O*-glucoside, respectively, due to the characteristic MS^2 ions at *m*/*z* 300, 179 and 151 (Qu, Liang, Luo, & Wang, 2004; Tahir et al., 2013). Peak 4 and 5 with their $[M-H]^-$ ions at *m*/*z* 433, which were also fragmented further to the characteristic ions at *m*/*z* 300, 179 and 151, were identified as quercetin 3-*O*-xyloside (Shui et al., 2005) and quercetin 3-*O*-arabinofuranoside (Mediani et al., 2012), respectively. Similarly, peak **6** with an $[M-H]^-$ ion at *m*/*z* 447 was identified as quercetin 3-*O*-rhamnoside, as previously reported by Mediani, Abas, Khatib and Tan (2013).

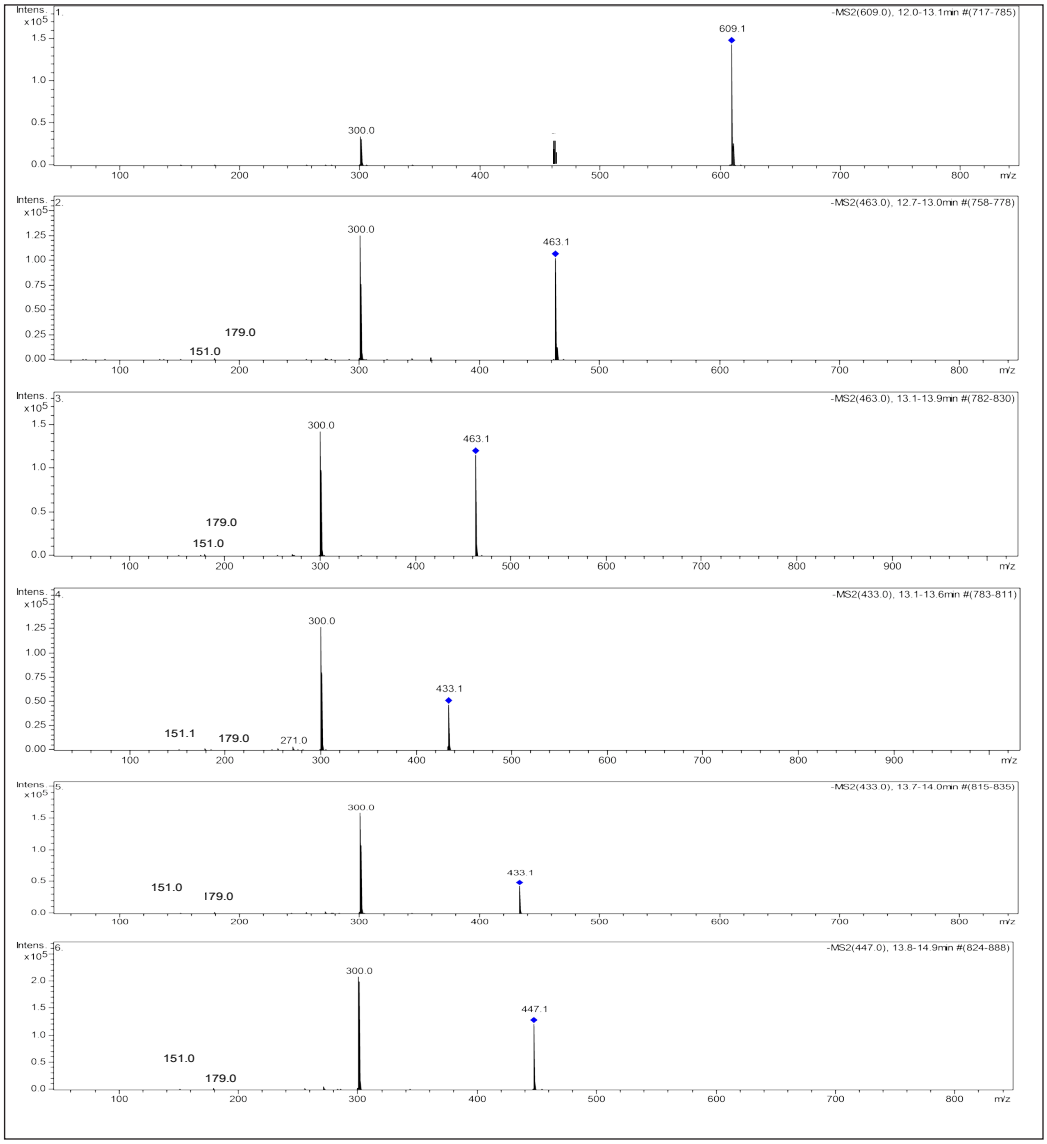

Figure 2. Negative mode API-ESI MS spectra of compounds 1-6. **1**, rutin; **2**, quercetin 3-*O*-galactoside; **3**, quercetin 3-*O*-glucoside; **4**, quercetin 3-*O*-xyloside; **5**, quercetin 3-*O*-arabinofuranoside; **6**, quercetin 3-*O*-rhamnoside.

Table 2
Tentative identification of phenolic compounds in the EtOAc Fraction of leaves extract of C. caudatus

Peak no	Retention time (min)	$[M-H]^-$ ion	MS/MS fragment ions	Tentative identification
1	12.8	609	463, 300	Rutin
2	13.1	463	300, 179, 151	Quercetin 3-*O*-galactoside
3	13.2	463	300, 179, 151	Quercetin 3-*O*-glucoside
4	13.6	433	300, 179, 151	Quercetin 3-*O*-xyloside
5	13.8	433	300, 179, 151	Quercetin 3-*O*-arabino-furanoside
6	14.1	447	300, 179, 151	Quercetin 3-*O*-rhamnoside

The TIC (Figure 3) and MS/MS data of the BuOH fraction was similarly analyzed and found to contain the same quercetin derivatives, tentatively identified as compounds 1- 5.

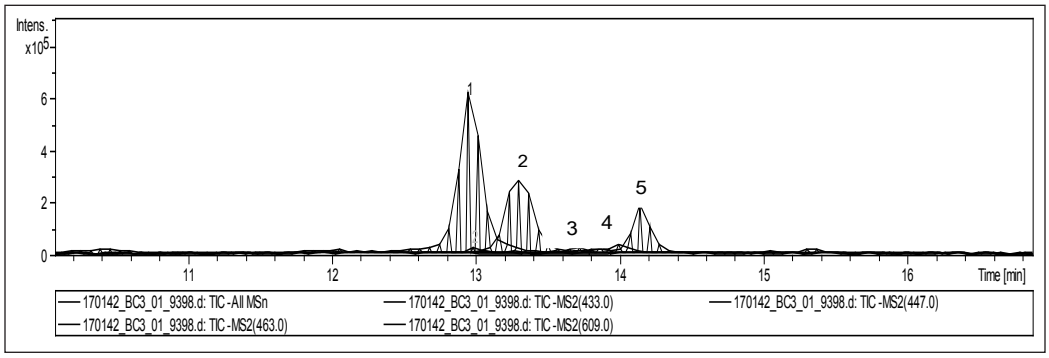

Figure 3. Total ion chromatogram (TIC) of the BuOH fraction of *C. caudatus*. **1**, rutin; **2**, quercetin 3-*O*-glucoside; **3**, quercetin 3-*O*-xyloside; **4**, quercetin 3-*O*-arabinofuranoside; **5**, quercetin 3-*O*-rhamnoside.

Table 3
Tentative identification of phenolic compounds in the BuOH Fraction of leaves extract of C. caudatus

Peak	Retention time (min)	$[M-H]^-$ ion	MS/MS fragment ions	Tentative identification
1	13.0	609	463, 300	Rutin
2	13.3	463	300, 179, 151	Quercetin 3-*O*-glucoside
3	13.7	433	300, 179, 151	Quercetin 3-*O*-xyloside
4	14.0	433	300, 179, 151	Quercetin 3-*O*-arabinofuranoside
5	14.1	447	300, 179, 151	Quercetin 3-*O*-rhamnoside

CONCLUSION

This study demonstrated that the EtOAc and BuOH fractions, derived from the ethanolic extract of *C. caudatus* leaves, have good antioxidant and antidiabetic activities based on the significant DPPH radical scavenging and α-glucosidase inhibition activities, respectively. The phytoconstituents that could be responsible for the antioxidant and α-glucosidase inhibitory activities, presumably acting in synergy, included rutin, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinofuranoside, and quercetin 3-*O*-rhamnoside. The chemical information and biological activity obtained are relevant as a starting point for studies on plant phytochemicals, which can contribute to further development of the medicinal plant into functional food, nutraceutical, and medicinal preparation.

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Interlinkage between Agri-Production System and Livelihood in Songkhla Province, Thailand

Ornaong Luanrak*, Buncha Somboonsuke and Prawat Wettayaprasit

Department of Agricultural Development, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

ABSTRACT

Due to rubber price fluctuations in Thailand, the rubber smallholders there have been forced to adjust their farming strategies to cope with the new economic, social conditions. The objective of this study is to analyse the livelihood system and factors influencing the sensitivity of livelihood system and show the relationship between the production management, and livelihood system in order to get the highest productivity and sustainable livelihoods of farmer households. The study area is Songkhla Province in Ratthaphum (Tha Cha Moung), Na Thawi (Klongsai) and Khuan Niang (Ratthaphum) districts selected using a purposive sampling method. Using a structured questionnaire interview, in-depth interview the key performance and focus group were used to collect data from 228 rubber farmer households. Sixty representative farms from three communities were selected evaluated based on their net farm income, comparing the farm type, again using a semi-structured interview and in-depth interviews. Key performance and focus group discussions were used to collect data, then analysed using content analysis, frequency, percentage, mean and multiple regression. The study found that the total income of monocrop system had the least. The highest was fruits (durian, mangosteen, rambutan, wollongong and banana). The livelihood of rubber smallholders practicing monocrop system reflected high economic capital but moderate social capital when compared to other rubber farming systems. Comparing all four rubber farming systems, integrated fruit estate offered the best interest for rubber smallholders.

Keywords: Agri-production system, rubber farming system, smallholders, sustainable rubber production management, livelihood

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E-mail address:
ornna_srg@hotmail.com (Ornaong Luanrak)
buncha.s@psu.ac.th (Buncha Somboonsuke)
dwettayaprasit@gmail (Prawat Wettayaprasit)
* Corresponding author

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INTRODUCTION

Rubber is an important export crop in Southern Thailand not only as a crop but for livelihood of smallholders (Bahuguna, 2006). In Thailand 93.1% of total rubber plantation areas are owned by smallholders. In 1995, Thailand emerged as top world rubber producing country. The rubber production was around 87% (3.77 million tons) of total output (4.32 million tons), and it was for both domestic use and export whereby the former accounted for 13% of total output (541,003 tons). An annual income from the rubber export is almost 200,000 million THB per year (Office of Agricultural Economics, 2015). Following the economic crisis of 2011 in Thailand, the Rubber small holding farming system was forced to alter their farming strategies and ensure sustainability of farming (Manivong and Cramb, 2008).

Now, rubber production is vital for the welfare of many of smallholder households in southern Thailand, who earlier were depending on shifting rubber plantation for their livelihood (Reang et al., 2014). Two factors affect rubber productivity: controllable and uncontrollable factors. The controllable factors have direct effect on rubber smallholder farming plan and strategies, such as decision-making process, soil and farm management practice, fertiliser, labour and farm capital investment. On the other hand, the uncontrollable factors that have on indirect impact on rubber farm and strategy plans, marketing system, trend, climate and group dynamics (Somboonsuke et al., 2002). Thus, the rubber smallholder have to identify factors involved in their farming system, for decision making and analyse how to control and manage. Thus, the government agency has been conducting training to the rubber small holders on latest cultivation technology and practice (Feder et al., 2014).

The rubber smallholders Thailand are facing income issues which have affected their quality of life. Additionally, they are challenged to adopt modern farming practices and use appropriate agricultural cultivation technologies (Nanda, 2011).

The rubber smallholding farm-household system consists of three basic sub-systems, which are inter-linked (Thungwa, 1998) and have an impact on farm productivity: the farm and its crop (rubber) and livestock activity providing employment cash, and food for farm family, the household decision-making unit and the off-farm component that is important to the well-being of rubber smallholder farming households (Renang, 2014). Thus, this study examines the economic performance of rubber smallholders, and other socio-economic factors that impact on their income (Ruthenberg, 2014). Then livelihood system and factors influencing the sensitivity of livelihood system are also analysed to determine the main constraints to rubber smallholders farm improvement and a solution to improve their farming methods. The results were synthesised to create a model that connected production management, and livelihood of small holders in Songkhla, for better farm productivity and sustainable livelihoods.

METHODS

The study area is Songkhla Province in Ratthaphum (Tha ChaMoung), Na Thawi (Klongsai) and Khuan Niang (Ratthaphum) districts divided into 3 agro-ecozones (Conway, 1985, p.92). Using a structured questionnaire and in-depth interview methods with small focus groups, data was collected from 228 rubber smallholdeholds, in addition to 60 representative farms from three communities. They were selected and evaluated based on their net farm income and farm type using a semi-structured interview and in-depth interview. Their key performance indicators were measured and focus group data was analysed using content analysis, frequency, percentage, mean and multiple regression.

RESULTS AND DISCUSSION

The Survival Livelihoods of Integrated Rubber Plantation Farming with Different Farming Systems in Songkhla, Thailand

Table 1 shows the livelihood of farmer which shows that S2 system had highest achievement, whereas the sensitivity and vulnerability of this system was also highest, due to high risk from natural disaster. The human resources and natural asset of the S2 system had the highest average score, while the capital asset and social relationship had lower and lowest score respectively because rubber farmers did not have official representation and hence, did not have the power to negotiate the price. The S2 survival strategies were focused on productivity increasing, innovation and capacity increasing, and also government support. For all 4 systems, the livelihood achievement under monetary, both capital and natural assets were very high, whereas food security and clothing, health and hygiene, and social relationship scores were all in the range of medium to high. These are similar to the findings reported by Thongyou (2014) in Rubber Cash Crop and Changes in Livelihoods Strategies in a Village in Northeastern Thailand. He reported that smallholders have to adjust themselves to the capitalist driven standardised farming practices and suffer due to unequal access to capital assets, particularly financial capital, changing socio-economic conditions and constrains related capital assets and access to them. The rural households have little choice but adopt integrated livelihood strategies.

The Analysis of Influential Factors towards Survival livelihoods of Integrated Rubber Plantation Farming with Different Farming Systems

Table 2 shows S1 has the highest sensitivity and vulnerability factor at 98.10%, whereas S2, S3, and S4 has 32.50%, 38.50%, and 39.50% of sensitivity and vulnerability respectively. From overall influential factors towards survival livelihoods of integrated Rubber Plantation Farming with different farming systems, the analyses show sensitivity and vulnerability factor were the highest in S1 and the lowest in S2. Consequently, the Monoculture Rubber System (S1) had the highest risk for survival livelihoods. On the other hand, the Rubber-Fruit trees Integrated Farming System (S2) had the lowest risk

Table 1
The survival livelihoods of Integrated Rubber Plantation Farming with different farming systems

Factors	S1	S2	S3	S4
Sensitivity and Vulnerability (Time/Year)				
Natural disaster	0.88	2.63	2.19	1.90
Flood	1.98	1.98	1.98	1.98
Draught	0.15	0.12	0.13	0.12
Wind/ Depression	0.57	0.57	0.57	0.57
Trend of changing of:	65.54%	67.18%	67.41%	70.67%
Price	87.23%	81.08%	84.21%	84.19%
Labour	79.06%	76.43%	84.10%	79.86%
Natural resources	85.18%	85.09%	70.35%	77.87%
Technology	39.21%	40.45%	39.91%	52.86%
Career	59.87%	61.84%	64.66%	67.45%
Market	50.00%	62.37%	53.33%	65.94%
Social	65.20%	63.01%	75.29%	66.50%
Human asset	2.93	3.39	3.24	3.24
Social assets	2.77	2.35	3.83	2.96
Physical assets	3.26	3.59	3.48	3.44
Natural assets	2.91	3.00	2.90	2.97
Capital assets	3.08	3.29	3.43	3.16
Structural and procedure	80.25%	78.10%	69.55%	75.97%
Supporting agencies farmer and Co-Op				
Bank:	54.39%,	60.96%	25.88%	40.56%,
Co-Op:	27.10%,	26.10%		29.10%
Saving group:	23.23%	23.23%		23.23%
Positive impact and benefits for better livelihood	70.98%	81.58%	94.30%	88.45%
Survival strategies	Productivity increasing (54.82%) Production diversification (52.86%) - Consumption Adaptation (58.07%)	- Silviculture improvement (75.09%) - Effectivity of Productivity increasing (82.81%) - Productivity increasing (67.55%)	- Production diversification (60.53%) - Consumption Adaptation (61.05%) -Social Adaptation (55.12%)	- Productivity increasing (54.05%) - Production diversification (54.17%) - Consumption Adaptation (59.49%)
Livelihood achievement	Medium	Medium	Medium	Medium

Table 1 *(continue)*

Factors	S1	S2	S3	S4
Monetary				
Food security and clothing	High	Medium	High	High
Capital assets	Medium	Medium	Medium	Medium
Health and hygiene	High	Medium	High	High
Natural resources	Medium	Medium	Medium	Medium
Social relationship	Medium	High	Medium	Medium

Note: S1- Rubber monoculture; S2- Rubber-fruit tree; S3- Rubber-rice; S4- Rubber-livestock

Table 2
The analysis of influential factors towards survival livelihoods of Integrated Rubber Plantation Farming with different farming systems

Factor	S1	S2	S3	S4
Sensitivity And Vulnerability	0.981	0.325	0.385	0.395
Capital And Assets	0.358	0.534	0.235	0.282
Structural And Procedure	0.234	0.303	0.262	0.289
Survival Strategies	0.343	0.303	0.405	0.351

for survival of the farmers, due to having highest capital and natural assets with high structural and procedure applied, resulting in low requirement to adapt their survival strategies. This finding is in line with Somboonsuke *(*2003) in The Sustainable Livelihood of Rubber Small holder: A case study of Rubber-Fruit Tree Farming System in Kao Phra Community, The Southern Thailand who found social capital at middle level but financial capital at low level.

The Characteristics of Small Rubber Plantations Management in Different Farming Systems

This study examined the economic performance of the rubber smallholders farming system and identify what socio-economic factors impact on their household income. Table 3 shows that all 4 integrated rubber plantation farming systems are aimed at income generation with general characteristic of sandy-loam soil on flat plain, low hill, and highland area. The size of plantation was 1-2 ha. The age of the population under study ranged from 45-55 years old, with an annual income of 104,000 – 271,000 THB per household. Most of the farmers were male having elementary to vocational level of education. The average number of family members was 4, and between 2 and 4 of them were rubber farm. Most of the farmers (82.36%) received Replanting Fund from the Rubber Authority of Thailand (RAOT). Buddhism is the main religion. Their average debt ranged from 50,000 – 81,000 THB. The Agricultural Production System showed the proposed recommendations were to reduce production cost, price control, economic

multi-crops with rubber plantation, alternative income from other activities in integrated rubber farming systems, with supporting information about market access, and lastly to minimise exploitation of the middleman. The challenges faced by the farmers were drop in the price of rubber leading to decreased income, inability to adapt silviculture technique in different topography, lack of tapping skills, and labour shortage. The advantages the farmers had were land tenure (farmer own their lands) and long experiences in rubber farming. The annual productivity of the rubber farming was 1,500 - 2,000 kg with 90,500 – 271,000 THB annual income from rubber farming, and average saving was 5,800 – 14,200 THB which correspondsed to Reang et al., (2014) in determinants of small rubber growers' adaptation behavior in Dhalai district of Tripura, which showed the social capital was at middle level, and human capital was at low level.

Table 3
Characteristics of small rubber plantations management in different farming systems

Factors	S1	S2	S3	S4
Socio-Economic Factors				
1. Annual income/household (THB/Year)	120,240.50	270,320.50	104566.00	180,050.25
2. Income from rubber farming (THB/Rai)	90,500.00	104,016.50	50,700.50	216,020
3. Average Rubber Productivity (Kg/Year)	2,040.15	2,217.77	1,410	1,380
4. Average Household Saving (THB/Year)	9,200.50	12,400.50	5,800.00	14,200.50
5. Average Household Debt (THB/Year)	50,000	68,500	62,500	80,500
6. Labour in Agricultural activities (No.)	2.70	3.30	3.10	2.10
7. Education	College	Primary	Primary	College
Physical and Biological Factors				
1. Average Rubber Plantation (Rai)	12.15	11.45	11.33	6.18
2. Rubber Clone				
- RRIM600	94.20%	100%	87.72%	95.32%
- RRIT251	5.80%	-	12.28%	4.68%
3. Number of Rubber Tree (Tree/Rai)	76	72	74	71
4. Spacing				
- 3×7 m^2	75.50%	100%	4.68%	24.68%
- 3×8 m^2	10.00%	-	-	4.68%
- 6×4 m^2	14.50%	-	-	4.68%
- Others	-	-	95.32%	65.96%
5. Fertiliser (Kg/Rai)	240.50	252.10	244.5	269.23
6. Weeding				
- Machine	70.14%	80.00%	86.08%	73.68%
- Herbicide	4.68%	4.68%	4.68%	26.32%

Table 3 (*continue*)

Factors	S1	S2	S3	S4
7. Taping System				
- 1/3s 3d/4	90.75%	80.75%	94.60%	95.60%
- Others	9.25%	19.25%	5.40%	4.40%
8. Products				
- Fresh Latex	92.32%	100.00%	95.40%	91.80%
- Others	7.68%	-	4.60%	8.20%
9. Market				
- Local buyers	83.25%	92.50%	90.10%	90.10%
- Group/Co-Op	16.75%	7.50%	9.90%	9.90%
10. Human Resources	2.93	3.39	3.24	3.24
11. Social Assets	2.77	2.35	3.83	2.96
12. Physical Assets	3.26	3.59	3.48	3.44
13. Natural Assets	2.91	3.00	2.90	2.97
14. Capital Assets	3.08	3.29	3.43	3.16
15. Livelihood achievement				
- Monetary	Medium	Medium	Medium	Medium
- food security and clothing	High	Medium	High	High
- Capital assets	Medium	Medium	Medium	Medium
- health and hygiene	High	Medium	High	High
- Natural resources	Medium	Medium	Medium	Medium
- Social relationship	Medium	High	Medium	Medium
The influential factors of survival livelihoods				
1. Sensitivity and vulnerability	0.981	0.325	0.385	0.395
2. Capital and assets	0.358	0.534	0.235	0.282
3. Structural and procedure	0.234	0.303	0.262	0.289
4. Survival strategies	0.343	0.303	0.405	0.351

Proposed Recommendations

(1) Regarding rubber and fruit market price fluctuation, the lowest price guarantee shall be applied.

(2) Set up the central market system for good governance for farmers. Integrated rubber farm for alternative income with food securities shall be promoted for additional income and healthy livelihoods.

(3) Group management training shall be provided for farmers to reduce market barriers imposed by local middlemen.

(4) Value-added of fruit production and innovation shall be promoted to farmers.

(5) Cost of living among farmers is very high. Therefore, they should be encouraged to use organic fertiliser, forming groups with other farmers for negotiation to buy/sell products, and access to fertilisers.

(6) The study showed natural and capital asset was low. Thus, it is important for the government to build capacity for farmers in rubber/marketing process and ensure the diversity of their income especially during periods where rubber and fruit price are low.

Suggestions for Further Research

1. A detailed study of financial issues related to rubber plantations should be conducted to thoroughly analyse the livelihood of rubber smallholders.

2. A study on rubber plantation career should be conducted.

3. A comparative study on livelihood among rubber farmers across many areas should be conducted so that the derived data can be used for future farmer's livelihood planning.

CONCLUSION

The study found that Songkhla province had an integrated rubber farming system (four vital farming activities). Most of the farmers in rubber plantation were adversely impacted by low price of rubber aggravated by increased cost of living which ultimately affected their livelihood. Additionally, the arrival of migrant workers for solving labour shortage in rubber plantations affected the employment of people in the area. It is recommended farmers shift from monoculture to hybrid rubber in response to changing economic conditions in the future. The current rubber farming systems in Thailand are monocrop, integrated fruit-estate, integrated rice-farming and integrated-animal-raising. The rubber monoculture system has the lowest efficiency and diversity. The total income of each system shows that farmers practicing monocrop system had the least total income and the highest was the integrated fruit estate. The study also indicate moderate social capital among rubber smallholders but their economic capital is high. Comparing the livelihood of farmers in four systems, the study shows that the net income and the strategy of integrated fruit-estate has the best rating. Thus, the public sector or related agencies should focus on the increasing awareness on the importance of agriculture, to improve the conditions of rubber plantations to suit the type of land they have. Group farming and management training are important for rubber smallholder farmers to reduce market barrier due to the presence of local middlemen. Additionally, the government should provide lowest price guarantee and farmers should practise value-added in terms of fruit production and innovation. This new type of management can improve the farmers' quality of life, reduce poverty, and increase their standard of living.

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Immunomodulatory Potential of *Eucheuma serra* as Haemocyte Cell Production Enhancer on *Litopenaeus vannamei*

Kartiko Arif Purnomo[1]*, Merdeka Agus Saputra[2], Shobrina Silmi Qori Tartila[2], Fariz Kukuh Harwinda[2], Sri Umida Setyaningsih[2] and Woro Hastuti Satyantini[2]

[1]Faculty of Pharmacy, Universitas Airlangga, Jl. Dharmawangsa Dalam, 60286 Surabaya, Indonesia
[2]Faculty of Fisheries and Marine, Universitas Airlangga, Mulyorejo, 60295 Surabaya, Indonesia

ABSTRACT

Litopenaeus vannamei is one of the most well-known fishery products and has high market value. In contrast, production of vannamei shrimp is facing threats from several diseases caused by bacteria, virus, and even parasites that attack the shrimp immune system. The purpose of this study is to identify the immunomodulatory effect of *Eucheuma serra* extract to enhance haemocyte cells production in *Litopenaeus vannamei* as the non-specific internal immune substance. The research method used was a complete randomised design with 5 concentrations of injected *Eucheuma serra* extract treatments and one placebo as control. *Eucheuma serra* extract administered on shrimp abdomen and the haemocyte cell samples collected from shrimp on day 0 and day 6. Treatment using 8 ppm injected *Eucheuma serra* extract showed the highest haemocyte cell amount increase about 15.90 million cells/ml in 6 days after injection. Statistical calculation using ANOVA test showed a significant difference in the amount of haemocyte cell at before and after treatment.

Keywords: Eucheuma serra, haemocyte cell, immunomodulator, *Litopenaeus vannamei*

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E-mail addresses:
kartiko.purnomo@gmail.com (Kartiko Arif Purnomo)
merdeka.merdekaagussaputra@wur.nl (Merdeka Agus Saputra)
shobrina.silmi@gmail.com (Shobrina Silmi Qori Tartila)
farizkukuh@hotmail.com (Fariz Kukuh Harwinda)
umidas918@gmail.com (Sri Umida Setyaningsih)
worohastuti79@gmail.com (Woro Hastuti Satyantini)
* Corresponding author

INTRODUCTION

Crustacean aquaculture represents a major industry in tropical developing countries (Vazquez et al., 2009). *Litopennaeus vannamei* (white leg shrimp) is an important aquaculture species (Sirirustananun et al., 2011). In Indonesia, *L. vannamei* has a major market. Its production volume increased 99.27% from 206.6 tonnes in 2010 to 411.7 tonnes in 2014.

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Shrimp farming has also led to prevalence of disease. Crustaceans are affected by opportunistic pathogens causing huge economic losses (Vazquez et al., 2009). Shrimp farming has suffered from problems linked to deteriorating pond environments, subsequently resulting in stress-induced disease incidences mainly of viral and bacterial aetiologies (Sirirustananun et al., 2011). Commercial shrimp farming has faced disease outbreaks, especially white shrimp *L. vannamei*, such as *Vibrio alginolyticus*, *Vibrio damsela*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*, infectious Myonecrosis Virus (IMNV), Monodon Baculovirus (MBV), White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Yellow Head Virus (YHV) and Taura Syndrome Virus (TSV). In Taiwan, the total farmed production of *L. vannamei* shrimp declined almost 90%, from 82,598 tonnes to 8,878 tonne, in the space of 15 years (Cheng, Liu, Yeh, & Chen, 2004).

There is also evidence of *L. vannamei* infected with WSSV, TSV, IHHNV and IMNV in several regions in Indonesia. Lampung, West Java, East Java and South Sulawesi are the regions which are exposed to various types of infection. The IMNV has led to death of 60% of the shrimps (Sugama, Novita, & Koesharyani, 2006). *V. alginolyticus* bacterium isolated from infected *L. vannamei* is known to cause high mortality rate among shrimp in stressful environments (Wang & Chen, 2005). The WSSV is considered to be an extremely virulent pathogen and may cause death within a few days post infection (Chou, Huang, Wang, Chiang, & Lo, 1995).

Physical barriers are the biggest challenge to prevent pathogenic microorganisms in most invertebrates (Vazquez et al., 2009). Invertebrates do not possess a specific, adaptive immune system such as found in vertebrates. In the absence of lymphocytes and functional antibody, they have been traditionally thought to rely instead entirely on innate or non-specific immunity for internal defence against parasites and pathogens (Rowley & Powell, 2007; Wei et al., 2012). Haemocyte cell in shrimp has similar function with leukocyte in vertebrate as non-self matter recognition and elimination (Sritunyalucksana & Soderhall, 2000). Circulating haemocytes are generally classified into three types: hyalinocytes, semi-granulocytes and haemocytes with refractile granules. Haemocyte common defence mechanisms include phagocytosis and encapsulation of extrinsic fungal spore, yeast and organisms (Cheng et al., 2004; Vazquez et al., 2009). Other defence mechanisms of haemocytes are activated depending on the pathogen characteristics, such as prophenoloxidase and lectin as sugar-binding properties of crustacean proteins (Vazquez et al., 2009).

Extensive use of human antibiotic drugs in shrimp farming as preventive purpose is potentially damaging and the residues can still enter contaminate the environment and enter the human food chain (Cabello, 2006). Moreover, the European countries have strict regulations on residues of antibiotics

in shrimp product (Food and Agricultural Organization of the United Nations, 2006). Therefore, proper immune modulators for shrimp are important (Pope et al., 2011).

MATERIALS AND METHODS

Materials

Materials used were acetone (Merck, Darmstadt), ammonium sulphate (Merck. Darmstadt), ethanol (Merck, Darmstadt), phosphate buffer saline pH 7.4 / PBS (Brataco Chemica, Jakarta), sea water (Surabaya, Indonesia) and sodium ethylenediaminetetraacetic (Merck, Darmstadt).

Animal and Plant Materials

Litopenaeus vannamei shrimp was obtained from a shrimp farmer in Gresik, Indonesia. *Eucheuma serra* was collected in Pandawa Beach, Bali, Indonesia.

Eucheuma serra Extraction

E. serra was dried inside a closed and clean room. Dry *E. serra* was later powdered and weighed at 30 g. Phosphate buffer saline pH 7.4 solution was dissolved into the dried *E. serra* powder with a ratio 2:1. The solution was stirred using homogeniser for 2 hours at 4°C and then centrifuged at 3200×g for 15 minutes. Supernatant was collected then added with acetone 1:1. The solution was centrifuged at 3200×g in 15 minutes. The pellet was collected as crude extract of *E. serra.* The yield of extract is 10 g.

Lectin concentration in *E. serra* crude extract is determined using spectrophotometry method for protein analysis at wavelength λ 540 nm. Protein standard solution was used for quantitative analysis as external standard. The lectin concentration in *E. serra* crude extract is 5.60% or 2.58 ppm.

Litopenaeus vannamei Treatment

L. vannamei shrimp was acclimatised in aquarium with controlled salinity water at 15 ppt and temperature at 29°C for 48 hours. During acclimatisation period and treatment process, the subject was fed using commercial shrimp food three times a day. Probiotic was given only once in three days. The treatment aquarium was cleaned every day to reduce stress impact on shrimp and avoid contamination of ammonia from shrimp waste.

The treatment was conducted using completely randomised design consisting of five treatment groups and a control group (Table 1). Each group was replicated four times. Each replication contained four *L. vannamei*.

Haemolymph Collection and Total Haemocyte Count

Haemolymph was collected (0.50 ml) at fourth segment of the shrimp abdomen using disposable syringe and later stored inside an Eppendorf tube which already contained Na-EDTA in cold temperature storage. Haemocytometer was used for Total

Table 1
Prerequisite of dosage injection on treatment subject

Group	Treatment
Control (G0)	0.50 ml injection of PBS
Treatment 1 (G1)	0.50 ml injection of 2 µg/ml extract solution
Treatment 2 (G2)	0.50 ml injection of 4 µg/ml extract solution
Treatment 3 (G3)	0.50 ml injection of 6 µg/ml extract solution
Treatment 4 (G4)	0.50 ml injection of 8 µg/ml extract solution
Treatment 5 (G5)	0.50 ml injection of 10 µg/ml extract solution

Haemocyte Count (THC) of haemolymph of each group. Blank haemocyte (H0) was collected from haemolymph before lectin was injected. Final haemocyte result for THC was collected from haemolymph after six days (H6).

Survival Rate Count

Survival rate was counted as percentage of shrimps still available until the end of treatment compared with the amount before the treatment. Survival rate count was conducted in every treatment and control group.

Statistical Analysis

Analysis of Variance (ANOVA) statistical test was conducted to test the differences between the results.

RESULTS AND DISCUSSION

Total haemocyte count (THC) measures innate immunity and non-specific immune activity of invertebrates (Cheng et al., 2004). THC before treatment was calculated as average from all group. While THC after treatment was calculated as average haemocyte on a shrimp in each treatment group. According to THC result (Figure 1), shrimp in Group 4 (G4) via treatment with 8 µg/ml extract injection, had the highest haemocyte cell increase compared with another treatment group, including control group (G0). Haemocyte cell amount in G4 increased 15.29 million cells/ml from 3.16×10^6 cells/ml at H0 to 18.45×10^6 cells/ml at H6. Other treatment groups also had various increase in haemocyte cell. The lowest increase was group 3 (G3), which saw an increase of 9.88 million cells/ml from H0.

Control group also showed a slight increase in haemocyte after six days, even though the subject did not get any lectin treatment injection. The haemocyte number increase in control group was caused by the influence of substances and nutrition from feed and probiotic which was given to all of subject (Olmos, Ochoa, Michael, & Contreras, 2011). ANOVA test give the result of F calculation (F_{calc}) as 19.04 and F table 0,01 ($F_{tab\ 0.01}$) as 4.25. The higher value of F_{calc} compared with $F_{tab\ 0.01}$ shows the significant difference of *L. vannamei* haemocyte amount at before and after treatment using lectin extract solution via

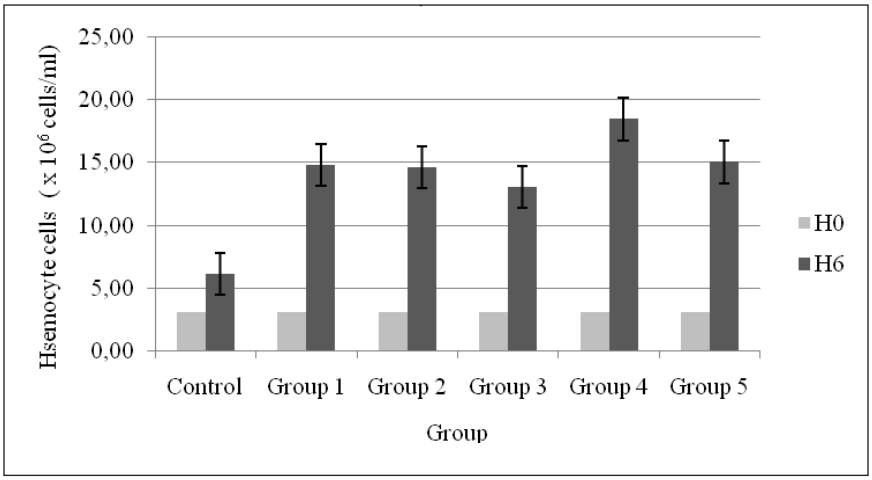

Figure 1. THC comparison of each treatment at H0 and H6

injection method. The group with the highest survival rate is G0 (87.50%) in control group and the lowest is G5 with 56.25%.

Based on the THC result of treatment groups, *E. serra* extract could be considered as the novel treatment as immunomodulator agent for *L. vannamei*. The increase in haemocyte cell means the innate immunity system has become stronger to encounter infectious pathogens, such as virus and bacteria. *E. serra* extract is more acceptable than using antibiotic to deter microorganism growth in pond.

E. serra gives high yields of isolectins - ESA–1 and ESA–2. The yield could be up to 1% from powdered algae with 0.1% as ESA–1 and 0.9% as ESA–2. *E. serra* could be a valuable source of lectin molecules that work even at relatively high temperatures and over a wide pH range that have preferential affinity for glycoproteins bearing high mannose–type N–glycans (Hori et al., 2007; Kawakubo, Makino, Ohnishi, Hirohara, & Hori, 1997). Lectin is defined as carbohydrate-binding proteins of non-immune origin that agglutinate cells or as carbohydrate-binding proteins other than antibodies or enzymes. It is a group of non-immunogenic proteins possessing at least one noncatalytic domain that binds reversibly to specific carbohydrates (Janeway & Medzhitov, 2002; Teixeira et al., 2012). Lectin has been regarded as primary candidates for pattern recognition receptors in animal innate immunity due to its ability to bind to specific carbohydrates on the surfaces of microorganisms (Zhao et al., 2009). Lectin can specifically recognise the carbohydrates from the membrane or surface of cell. Moreover, it is able to induce agglutination of these cell or can lead to diverse cellular events, such as phagocytosis (Marques & Barracco, 2000).

The carbohydrate-binding profile of ESA–2 was examined as 45 different complexes. N-acetyl glucosamine (GlcNAc) is related in 35 different complexes and N-acetyl galactosamine (GalNAc) takes

part in 4 different complexes (Hori et al., 2007). Lectin with structural characteristics and specificity has been identified in *L. vannamei*, known as C-type lectin (Ma, Tin, He, & Chan, 2007; Vazquez et al., 2009). C–type lectins has ligand binding specificities for carbohydrates, such as GlcNAc, GalNAc, sialic acid and lipopolysaccharide, indicate antimicrobial activity against several bacteria and fungi, also in response to WSSV (Vazquez et al., 2009; Zhao et al., 2009). The similarity of ligand binding carbohydrates of ESA–2 and C–type lectin is necessary for increase the immunity on *L. vannamei.*

Furthermore, immune memory in invertebrates that is referred as immune priming or specific immune priming, appears to be passed from brood stock to offspring (Pope et al., 2011). *L. vannamei* subjected to treatment and has an increase of haemocyte cell can be maintained as particular brood stock called Specific Pathogen Free (SPF). *L. vannamei* SPF can produce progeny that have can resist pathogens (Sugama et al., 2006).

CONCLUSION

L. vannamei is vulnerable to infection by pathogen microorganisms, such as virus and bacteria. *E. serra* extract contains lectin which serves as immunomodulator for *L. vannamei* to provide an innate immunity. *E. serra* extract as injection treatment for *L. vannamei* leads to a significant increase in haemocyte. Treatment of 8 µg/ml extract injection enhances haemocyte production as much as 15.29 million cells/ml in six days. Lectin from *E. serra* is a novel solution to enhance immunity of *L. vannamei* against pathogens.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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Influence of *Lactobacillus plantarum* Fermentation on Functional Properties of Flour from Jackfruit (*Artocarpus heterophyllus* Lamk.) Seeds

Jay Jayus[1,2]*, Dani Setiawan[1] and Cipto Giyarto[1]

[1]Agricultural Product Technology Department, Faculty of Agricultural Technology, University of Jember, Indonesia.
[2]Centre for Development of Advanced Science and Technology, University of Jember, 37 Kalimantan St., Jember 68121 Indonesia

ABSTRACT

Effort to improve the functional properties of jackfruit seed flour has been made by introducing *Lactobacillus plantarum* fermentation on the jackfruit chips, which are latter processed into flour. The jackfruit chips were fermented using *L. plantarum* for up to 32 hours, and air dried before they were grounded to pass through an 80 mesh sifter. The results of the experiment showed water holding capacity (WHC) of fermented flour was higher compared with that of the unfermented. The longer fermentation time, the higher the WHC of the flour. In contrast, the flour oil holding capacity (OHC) decreased during longer incubation time, indicating that some shorter amyloses were released during fermentation. Earlier studies on pasting characteristics of jackfruit seed showed that the peak, setback and breakdown viscosity of fermented jackfruit seed flour were higher compared with that of unfermented flour, indicating that fermented flour will have a higher thickening power and is more susceptible to heat and mechanical shear. This finding indicates *L. plantarum* fermentation will increase the potency of jackfruit seed flour for food industry application either as a thickener and or biodegradable film. Furthermore, HPLC analysis of the soluble saccharide of fermented flour showed the differences of oligosaccharides content such as raffinose, stachiose and verbaschose, indicating the capability of the enzymes released by *L. plantarum* during fermentation to hydrolyse the starch to become shorter oligosaccharide but without monosaccharide.

Keywords: Oil holding capacity, pasting properties, raffinose, stachiose, water holding capacity

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E-mail addresses:
jayus.ftp@unej.ac.id (Jay Jayus)
danisetiawansp@gmail.com (Dani Setiawan)
giyarto.ftp@unej.ac.id (Cipto Giyarto)
* Corresponding author

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INTRODUCTION

The demand for healthy foods has increased in tandem with health awareness. Consumers are also becoming concerned about how their diet contributes to obesity, cardiovascular disease, and hypertension (Bruhn, Cotter, & Diaz-Knauf, 1992). There have been attempts to lower the fat content of fat-rich food by promoting several types of flour as a source of fibre without damaging the physicochemical and sensory attributes of food, such as low fat high fibre meat *kofta* formulated using oat flour and carrageenan (Modi, Yashoda, & Naveen, 2009). Cassava has been used in the food industry to supplement wheat flour in the production of confectionaries (Gyedu-Akoto & Laryea, 2013; Kulchan, Boonsupthip, & Suppakul, 2010; Shittu, Dixon, Awonorin, Sanni, & Maziya-Dixon, 2008). Based on this evidence, several types of potential flour from different sources have been investigated to be developed as functional foods. However, many flours are reported to be deficient in some functional properties, which limits their industrial use. Therefore, several techniques have been proposed to resolve this problem including fermentation technique using microorganism, such as lactic acid bacteria, to find a simpler and cheaper method to improve their sensory characteristics, nutritional value, and technical-functional qualities (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003). Fermentation-based flour process could be a potential technique not only to improve physicochemical characteristic of the flour (Chowdhury, Bhattacharyya, & Chattopadhyay, 2012), but also to increase the functional nutritional compounds, which include dietary fibre, minerals, antioxidants, prebiotics and vitamins (Coda, Rizzello, Trani, & Gobbetti, 2011). The application of fermentation method for flour production has been established to produce various food forms which include bread, beverages, and porridge from the raw materials of several cereals origin including rice, wheat, corn and sorghum (Helland, Wicklund, & Narvhus, 2004); tubers and legumes (Noorfarahzilah, Lee, Sharifudin, & Hasmadi, 2014), where these fermentation techniques were mainly used to produce a physico-chemically and functionally better flour. Noorfarahzilah et al. (2014) reviewed studies that looked at formulation of composite flour from different local source, such as tubers, legumes, cereals, and even from underutilised fruit and vegetables to substitute wheat flour to produce variety of a higher quality of many local food products. It was found that other than cereals, tubers and legumes, flour from fruit by-product such as mango kernel (Menon, Majumdar, & Ravi, 2014) and seed (Kittiphoom, 2012), rambutan seed (Eiamwat, Wanlapa, & Kampruengdet, 2016), pumpkin seed (Białek, Rutkowska, Adamska, & Bajdalow, 2016) and jackfruit seed (Retnowati, Ratnawati, & Purbasari, 2015) have also been explored as composite flour. However, the functional properties of those native seed-based flour need to be improved since many did not fulfil the technical-functional properties for food uses. Fermentation method to produce, especially jackfruit seed as the composite

flour, has been understudied. Therefore, this research was undertaken to study the effect of *L. plantarum* fermentation on jackfruit seed characteristic changes and the functional properties of its flour.

MATERIALS AND METHODS

Preparation of Jackfruit Seed for *L. plantarum* Fermentation Processes

Jackfruit seed was used for this experimentation, and the inoculums used for chips fermentation were prepared using the method used by Jayus, Setiawan and Giyarto (2016). The *L. plantarum* were sub-cultured using medium containing 0.03% (w/v) refined sugar, 0.05% (w/v) raw jackfruit seed flour, and 0.02% (w/v) commercial skim milk to produce a stock culture. These sub-culturing processes were maintained to provide a starter culture with a minimum population of *L. plantarum* at 10^8 cfu/ml.

Fermentation of Jackfruit Seed Chips

The jackfruit chips were fermented in a submerged culture using the starter culture as previously reported by Jayus et al. (2016) for 8, 16, 24, and 32-hour incubation time. Fifty grammes of approximately 2cm thickness of jackfruit chips were UV radiated for 15 minutes before fermentation process. The fermented chips were rinsed 3 times to eliminate any salt residue. The chips were then sun dried, and dry-milled to achieve 80-mesh size of flour particles.

Measurement of Jackfruit Seed Flour Water Holding Capacity (WHC)

Water Holding Capacity (WHC) of jackfruit seed flour was calculated using the method described previously by Traynham, Myers, Carriquiry and Johnson (2007) with some modification. The sample of 0.5 g flour and 3.5 mL distilled water suspension was stirred for three minutes. The 10-min centrifugation of the samples were carried out at 6000×g using a refrigerated centrifuge (Himac compact RXII series, Hitachi). Decantation was used to separate the water. The WHC values were calculated using the following equation:

$$WHC = \frac{[(\text{weight of bottle and flour after decanting} - \text{dry bottle weight}) - \text{weight of flour}]}{\text{weight of flour}} \times 100\%$$

Measurement of Oil Holding Capacity (OHC)

The OHC values of jackfruit seed flour were determined using the modification of method described previously by Mirhosseini and Amid (2012). The sample of 0.5 g flour and 10 mL refined palm oil suspension were mixed for three minutes. The centrifugation of the samples was carried out at 6000×g for 10 minutes using a refrigerated centrifuge (Himac compact RXII series, Hitachi). The OHC values were calculated using the following equation:

$$OHC = \frac{[(\text{weight of bottle and flour after decanting} - \text{dry bottle weight}) - \text{weight of flour}]}{\text{weight of flour}} \times 100\%$$

Studies on Pasting Characteristics of Jackfruit Seed Flour

The pasting properties or characteristics of the flour samples were determined using RVA (Tec Master, Australia). Jackfruit seed flour samples (3 g, 10% moisture) were suspended into 25g distilled water homogenised under constant shear rate at 160×g for 13 min, controlled using heating and cooling cycle system, heated from 50 to 95°C. Initially, the slurry was heated to 50°C and agitated at 960×g for 10 seconds and then the mixing speed was lowered to 160×g. After 4 minutes and 42 second mixing time, the slurry was heated up to 95°C for 11 minutes. The flour temperature was then lowered to 50°C for 2 min. The parameters obtained were the *peak viscosity* (PV), breakdown (B), *minimum viscosity* (MV), *final viscosity* (FV), set back (SB), *pasting temperature* (PT), and *time to peak* (Ptime).

Identification of Dissolved Oligosaccharide in the Flour of Jackfruit Seed. HPLC analyses were undertaken to identify the dissolved oligosaccharide content of the flour, preparing 1 g of flour sample to be dissolved in mili-Q water (25 ml) and heated at 70°C for 60 minutes. The mixture was then filtered using 0.45 µm filters, before injected to Metacharb 87C column eluted using water at 0.6 mL/min flow rate using RID detector.

Reproducibility of the Data

Data obtained here are the means from at least triplicates. The variations of the means represent standard deviation.

RESULTS AND DISCUSSION

Influence of Fermentation on the WHC and OHC of Jackfruit Seed Flour

The WHC of fermented jackfruit seed flour was slightly higher compared with that of the unfermented one (0 h incubation time, see Figure 1), indicating that only a small amount of macromolecule was degraded during fermentation. *L. plantarum* enzyme produced during fermentation may only be able to degrade a few macromolecules to a shorter oligosaccharide (Jayus et al., 2016). This finding is in contrast to what had been observed by Kee and Saw (2010) on the jackfruit flour produced by fermentation of its raw flour using *Lactobacillus sp*., where the WHC of this fermented flour decreased. This could be due to differences of the *Lactobacillus* strain used. The enzymes released by the various strains may differ in terms of their mode of action, which could be either exo- or endo-action, releasing different oligosaccharides as the hydrolysis product. Different mode of action of enzyme secreted by microorganism had been reported by Talamond, Noirot and de Kochko (2006), and Jayus, McDougall and Seviour (2001; 2004). Afoakwa, Aidoo and Adjonu (2010) also pointed out fermentation

can lower the capacity of cowpea-fortified nixtamalised maize to absorb water because of the reductions in the availability and action of the hydrophilic groups which bind water. Even though the fermentation of the cowpea was spontaneous, but some amylase-rich flour added to the composite will increase the activity of the micro-flora during fermentation and the amylolytic enzymes will degrade the starch.

Meanwhile, the OHC of fermented jackfruit seed flour is lower compared with that of the unfermented one (Figure 2). The reduction in OHC occurs when the degradation of some poly- and oligosaccharide by enzyme of *L. plantarum* may reduce the hydrophilic side of the molecules and increases the presence of non-polar side in the flour (Adebowale, Afolabi, & Lawal, 2002). Many of OHC of the flour are affected by the method of process, even just a peeling process has been reported to lower the OHC of pumpkin (Aziah & Komathi, 2009).

Pasting Properties Studies

The pasting temperature of unfermented flour is similar (88.90°C) to that of the fermented one (88.00°C). This finding is

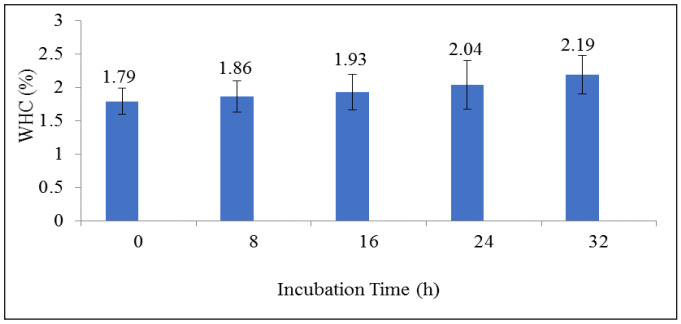


Figure 1. The WHC value of unfermented (0 h incubation time) and *L. plantarum* fermented jackfruit seed flour

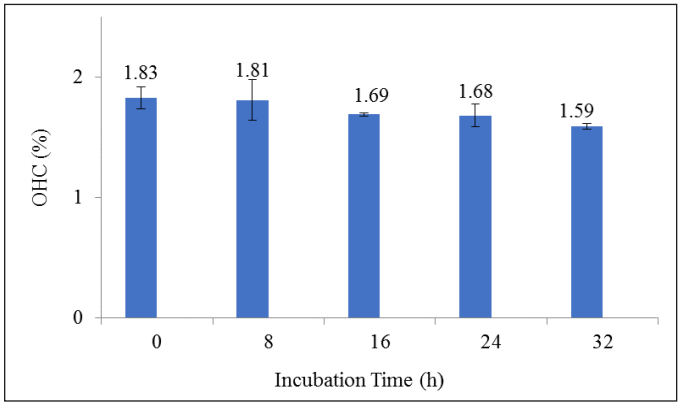


Figure 2. The OHC value of unfermented (0 h incubation time) and *L. plantarum* fermented jackfruit seed flour

consistent with the milled prepared jackfruit seed flour measured by Mukprasirt and Sajjaanantakul (2004), though jackfruit seed starch had higher pasting temperature (81.58 °C) as reported by Rengsutthi and Charoenrein (2011). Jackfruit seed flour pasting temperatures are higher compared with that of cocoyam (Oke & Bolarinwa, 2012) and cassava (Nwokocha, Aviara, Senan, & Williams, 2009), indicating a weaker granular structure of the latter compared with jackfruit seed flour. The differences of this granular structure may occur because of the major contribution of the enzyme work released by controlled *L. plantarum* only during fermentation of the seed. Meanwhile, other researchers used spontaneous fermentation techniques where the growth of microorganisms on the seed were uncontrolled, which might produce different and inconsistent properties of the flour. The detailed effects of fermentation on the pasting properties of jackfruit seed flour described shown in Table 1.

Table 1
Unfermented and fermented jackfruit seed flour pasting profile

Treatment	Peak viscosity (cP)	Minimum viscosity (cP)	Breakdown (cP)	Final viscosity (cP)	Set back (cP)	Pasting temperature (°C)	Peak time (min)
Unfermented	1757	1071	686	1394	323	88.90	5.13
Fermented	2340	1590	750	2412	822	88.00	5.40

The peak viscosity of fermented jackfruit seed flour was higher (2340 cP) compared with that of unfermented flour (1757 cP) as seen in Figure 3. Likewise, a similar trend was observed for setback and breakdown viscosity. This may indicate that fermented flour will have a higher thickening power and more susceptible to heat and mechanical shear. Higher viscosity profile of flour has been reported to have a higher potency as a material for biodegradable film. This can increase the Young's modulus and tensile strength of the film (Retnowati et al., 2015).

Controlled fermentation using *L. plantarum* as the only inoculum on jackfruit seed produce the higher pasting properties of the flour, in contrast with the spontaneous fermentation work on other seed as what have been observed by Oke and Bolarinwa (2012) in cocoyam flour, where the fermented flour appeared to have lower peak viscosity. The reason for this contradictory phenomenon is still unclear. It could be due the different type of amylase released by the microorganism used for fermentation. In the case of cocoyam flour, the fermentation processes increase the amylose content of the flour and lower the peak and the final viscosity observed. Meanwhile, similar enzyme of *L. plantarum* fermentation on jackfruit seed may not be able to degrade the starch into a higher proportion of amylose and increase the amylopectin proportion instead. Hence, the

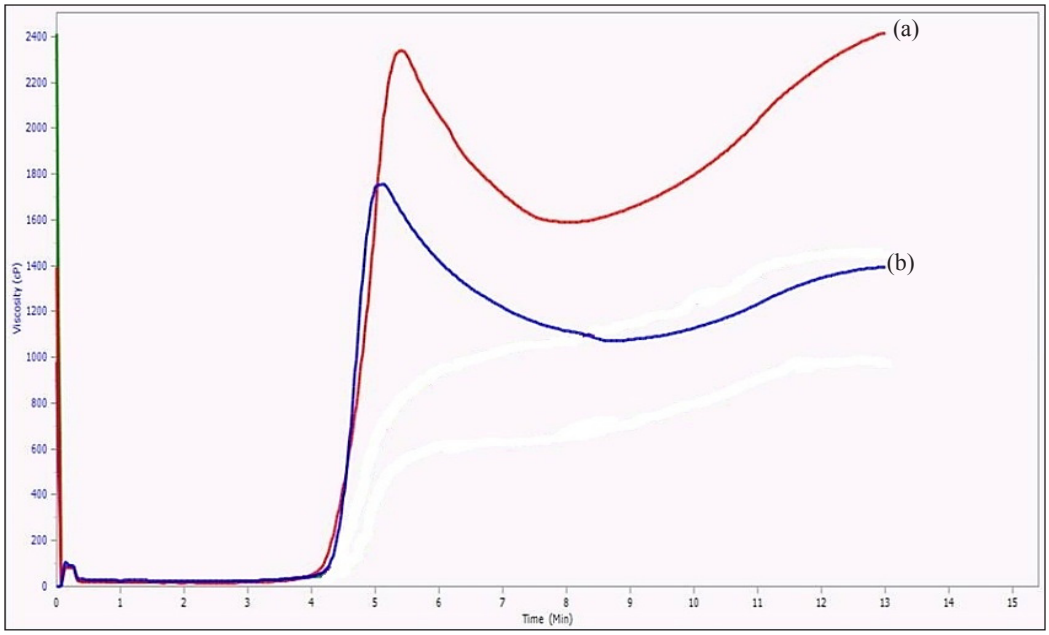
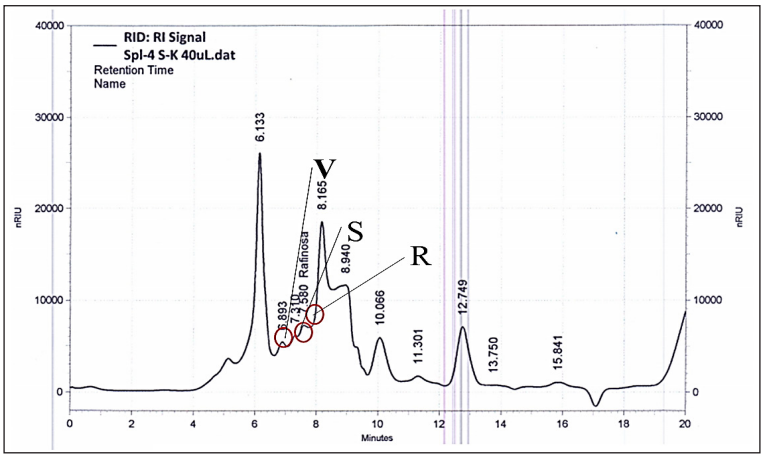


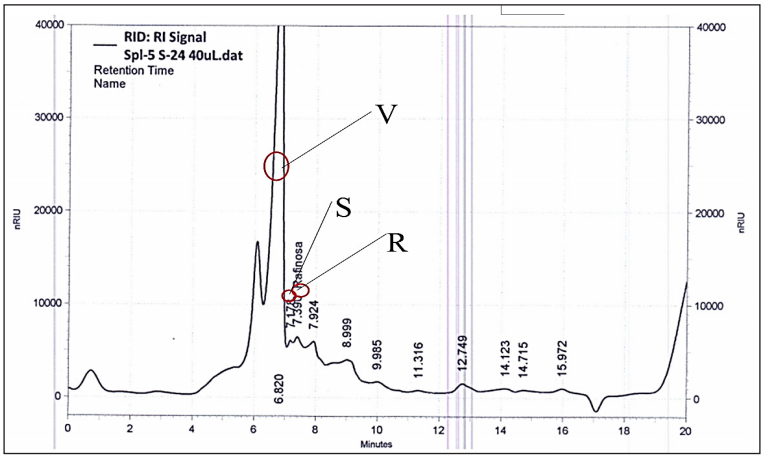
Figure 3. The viscosity profile of fermented (a), and unfermented (b) jackfruit seed flour.

final viscosity of the flour may increase. This reason cannot be confirmed until the content of amylose in jackfruit seed flour is determined. In this study, the amount of the compound was not measured. However, *L. plantarum* fermented jackfruit seed flour has been reported to contain higher water-dissolved shorter oligosaccharides, such as raffinose (Jayus et al., 2016), but not glucose, as the hydrolysis product of enzyme released by *L. plantarum* to degrade α-1,4- and α-1,6- glyosidic linkages (Olympia, Fukuda, Ono, Kaneko, & Takano, 1995). The presence of raffinose and slightly higher molecular weight of oligosaccharides including stachiose and verbaschose (as reported in this study) may change the viscosity profile of the flour as shown in Figure 3.

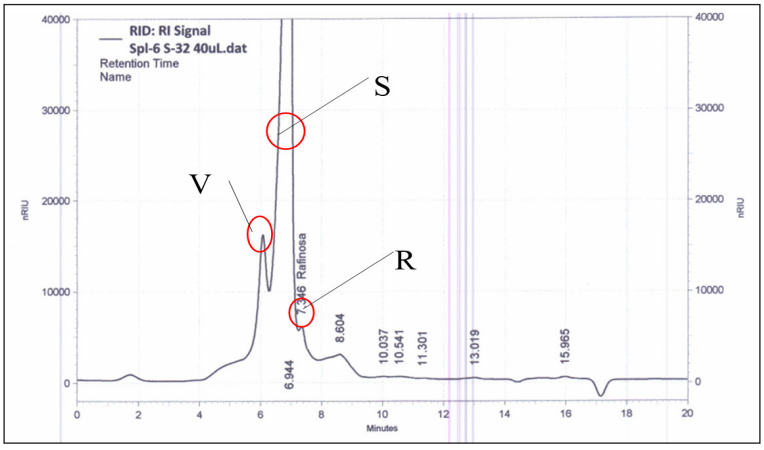
Differences of oligosaccharide content of unfermented and fermented seed flour were detected in the HPLC chromatogram (Figure 4). The raffinoses content were slightly increased over the 24-hour fermentation time (0.36 to 0.44 µg/g of flour), but the stachiose and verbaschose content noticeably increased. In the unfermented seed flour, stachiose and verbaschose content were almost undetected, 0.44 and 0.42 µg/g of flour respectively, but in 24 h fermented seed flour, verbaschose was increased sharply (4.95 µg/g of flour) and no apparent stachiose was detected at this time. Interestingly, the stachiose was noticeable in the 32 h fermented seed flour (5.21 µg/g of flour) followed by the reduction of the amount of verbaschose, indicating that enzymes released by *L. plantarum* were most probably endolytic action enzyme capable to degrade polysaccharide of the flour randomly to produce shorter oligosaccharide.



(a)



(b)



(c)

Figure 4. The HPLC chromatogram of: (a) unfermented; (b) 24 h fermented; and and 32 h fermented (c) jackfruit seed flour. V = Verbaschose, S = Stachiose and R = Raffinose

CONCLUSION

The *L. plantarum* fermentation of jackfruit seed appeared to influence WHC and OHC of the flour obtained, indicating that shorter amyloses were released during fermentation. The peak viscosity, setback and breakdown of fermented jackfruit seed flour were higher than that of the unfermented one, indicating that fermented flour would have a higher thickening power and possibly be more susceptible to mechanical shear and heat, and hence has a potential as a thickening agent. The enzymic activity of *L. plantarum* on the preparation of the flour contributed to changes of the soluble saccharide, and the differences in oligosaccharides content, which include stachiose and verbaschose, indicate enzymes secreted by *L. plantarum* during fermentation capable to hydrolyse the starch randomly, releasing shorter oligosaccharide but not monosaccharide, in which this enzyme character will produce the flour containing more oligosaccharides which may have a potential as a material for biodegradable film.

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Sensory and Chemical Characteristics of Bar Cookies Made from Mung Bean Flour and Ripe Plantain var Raja as Emergency Food

Nurhayati[1,2]*, Maryanto[1] and Larasati Gandaningarum[1]

[1]Department of Agricultural Product Technology, Faculty of Agricultural Technology, University of Jember, East-Java, 68121, Indonesia
[2]Center for Development of Advanced Science and Technology, University of Jember, Jember, East-Java, 68121, Indonesia

ABSTRACT

Bar cookies are generally consumed as snacks. This product is considered as emergency food that is easy to consume, provides sufficient calories and adequate nutrients. This study evaluates the quality of bar cookies based on their sensory and chemical characteristics. The cookies made from mung bean flour and ripe plantain var. Raja. There were four ratios of formulation i.e. 10 % mung bean flour with 90 % ripe plantain puree (P1), 20 % mung bean flour with 80 ripe plantain puree (P2), 30% mung bean flour with 70 % ripe plantain puree (P3) and 40 % mung bean flour with 60 % ripe plantain puree (P4). The cookie bars were evaluated based on their sensory attributes by using preference test. Chemical characteristics were based on the proximate analysis (water, ash, protein, lipid and carbohydrate content). The preferred formula of the bar cookies was 20 % mung bean flour with 80 ripe plantain puree (P2). Sensory characteristics of the preferred bar cookies were 3.29 colour; 2.93 aroma; 3.39 flavour; 2.89 texture; and 3.29 overall with the scale dislike (1) to like (5). Chemical characteristics of preferred bar cookies (P2) were 36.53 % water, 2.60 % db ash, 10.86 % db fat, 11.69 % db protein, 36.06 % db carbohydrate. The proximate composition for formulation of the bar cookies is significant.

Keywords: Bar cookies, emergency food, mung bean flour, plantain

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E-mail addresses:
nurhayati.ftp@unej.ac.id (Nurhayati)
maryanto.ftp@unej.ac.id (Maryanto)
larasatiganingarum@gmail.com (Larasati Gandaningarum)
* Corresponding author

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INTRODUCTION

Bar cookies are a food product that is commonly made from soy flour with the addition of fruits and food additives, such as syrup, caramel and chocolate (Sarifudin, Ekafitri, Surahman, & Putri, 2015). Marketable bar cookies are made from soy flour mixed dry fruits and generally consumed as snacks in the bars form (Setyaningtyas, 2008). The bar cookies do not require a high gluten flour (Marissa, 2010). Nowadays, consumers are wiser and they aim for good quality food which is also affordable, nutritious and tasty (Adriani & Bambang, 2012). Ladamay and Yuwono (2013) found bar cookies can be made using mung bean flour which are high in nutrition and taste good.

Mung beans (*Phaseolusradiatus*, L) are widely cultivated in Indonesia. Mung beans contain 62.5% carbohydrate (Kusharto, 2006), 22.2% protein (Setyaningtyas, 2008) and 1.3% fat (Rukmana, 1997). Mung beans are used as composite flour or hunkue.

Musa sp was classified into two groups: plantain and banana. Plantain belongs to a cooking banana subgroup (AAB, ABB, or BBB) while banana belongs to dessert banana subgroup. Plantains are longer, have a thicker skin, and contains more starch than bananas. Dessert banana cultivars in the world are AA or AAA, this last group includes almost all the cultivars for the export market (Aurore, Parfait, & Fahrasmane, 2009; Ploetz, Kepler, Daniells, & Nelson, 2007).

Banana and plantain are important staple foods in many developing countries, especially in Indonesia. *Musa* are rich in vitamin C, B6, minerals, and dietary fibre. They are also a rich energy source, with carbohydrates accounting for 22% and 32% of fruit weight for banana and plantain, respectively. People consume 28kg of banana and plantain per capita, or 155kg per year, or almost half kg per day (Robinson & Sauco, 2010).

Unripe plantain contains a high proportion of indigestible compounds, such as resistant starch and non-starch polysaccharides (Nurhayati, Jenie, & Kusumaningrum, 2014), included in the dietary fibre content (Juarez-Garcia, Agama-Acevedo, Sáyago-Ayerdi, Rodriguez-Ambriz, & Bello-Perez, 2006). Ripe banana chips can stimulate probiotics in the colon resulting in short chain fatty acids especially butyric acid which as anti-colon cancer properties (Nurhayati & Rahmanto, 2017). This study evaluates the sensory and chemical characteristics of bar cookies made from mung bean flour and ripe plantain var. Raja as emergency food.

MATERIALS AND METHODS

Materials

Materials for making bar cookies were mung bean flour and plantain (cooking banana) var Raja with maturity level at 5 - 6. Complementary materials were eggs, sugar, salt, margarine, skim milk and flour.

Preparation of Bar Cookies

The mung beans were soaked for 7 hours and its water replaced every 3 hours. They were later dried in the sun and crushed and sieved using an 80-mesh sieve. Sarifudin et al.'s (2015) method was used to make the bar cookies.

There were four ratios of formulation: 10 % mung bean flour with 90% ripe plantain puree (P1), 20% mung bean flour with 80 ripe plantain puree (P2), 30 % mung bean flour with 70% ripe plantain puree (P3) and 40% mung bean flour with 60% ripe plantain puree (P4). Complementary materials were eggs 10%, wheat 20%, sugar 10%, salt 0.25%, and margarine 10% and 5% skim milk. The first step is to mix eggs, margarine and sugar until the dough is consistent and creamy and after that flour, skim milk, salt and plantain puree are added to the dough. The dough is based on 100 g per treatment of flour and plantain.

Analysis of Bar Cookies Characteristic

The sensory characteristics of bar cookies were determined based on a preference test (Meilgaard, Civille, & Thomas, 1999). The chemical characteristics of bar cookies were water content (Association of Official Analytical Chemists International [AOAC], 2005), ash (AOAC, 2005), and total protein and fat content was measured using Kjehdahl and soxhlet methods respectively (AOAC, 2005). Data was analysed using Effectiveness Index (De Garmo, Sullevan, & Canana, 1984) based on the proximate and sensory values.

Statistical Analysis

Results were expressed as the mean ± standard error of three separate determinations. The statistical significance of the generated data was further analysedd by employing one-way analysis of variance (ANOVA) along with the least significant difference (LSD) test. The level of significance of the mean values was assigned at $P < 0.05$.

RESULTS AND DISCUSSION

Sensory Characteristic of Bar Cookies

The sensory characteristic of bar cookies formulation, such as colour, flavour, taste, texture and preference, were evaluated by 28 panellists.

Figure 1 shows the preferred bar cookies was based P2 formula. It had a yellow-brownish colour. The high addition of ripe plantain causes the bar cookies to have darker shade. The cooking banana (plantain) contains phenolic compounds susceptible to enzymatic browning reactions (Sarifudin et al., 2015). Stripping and cutting up of the banana can accelerate the oxidation reaction, either by oxygen and polyphenol oxidase to produce a brown colour. The brown colour on the bar cookies can also be affected by the reaction of reducing sugars with primary amine group during the baking process to form a melanoidin compound (brown colour) in cookies.

Flavour is an important determinant of quality associated with the smell sense. Meilgaard et al. (1999) described flavour in the food industry as important because it

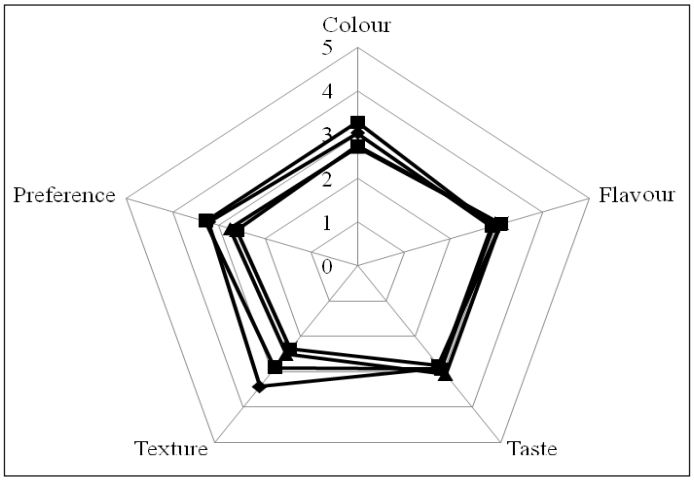

Figure 1. Sensory characteristics of *bar cookies* made from: 10% mung bean flour and 90% plantain puree (P1--■--), 20% mung bean flour and 80% plantain puree (P2--●--), 30% mung bean flour and 70% plantain puree (P3--▲--), 40% mung bean flour and 60% plantain puree (P4--×--).

could influence consumer acceptance of the product. Flavour is caused by the volatile substances.

The flavour values of bar cookies ranged between 2.89 and 3.11. The preferred flavour of bar cookies was the P4 formula (3.11) consisting of 40% mung bean flour and 60% plantain puree. The P1 formula resulted in the lowest flavour (2.89) consisting of 10% mung bean flour and 90% plantain puree. Sidabutar, Rona and Ridwansyah (2013) explained that mung beans contained essential amino acids i.e. leucine, arginine, isoleucine, valine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The amino acids caused the Maillard reaction with the sugar component that was supported by the heating process.

The taste value of bar cookies ranged from 2.82 to 3.43. The higher taste value was P1 formula (3.43), while the lowest taste value was P4 (2.82). Ferawati (2009) explained the banana var Raja had a distinctive sweet taste as a result of the starch degradation to reduce sugars during the ripening process of bananas. According to Soltani, Alimardani and Omid (2010), the sugar content in the unripe bananas was between 1-2% and 15-29% if the fruit is ripe.

Texture is an important determinant of bar cookies quality associated with the touch sense. Tan, Kanyarat and Azhar (2012) reported that the texture determined consumer acceptance of the product. The texture value of bar cookies ranged between 2.36 and 3.43. The preferred texture of bar cookies was P1 formula (3.43) consisting of 10% mung bean flour and 90% plantain puree, while P4 formula (2.36) consisted of a 40% mung bean flour and 60% plantain puree.

Adding plantain puree softens the texture and increases its moisture content. The texture of cookies bars is influenced by the starch content, especially amylose component of mung beans. Akubor (2003) explained that the amylose content of the mung bean flour results in firmer and harder texture of cookies. Therefore, increasing the proportion of green bean flour leads to increased hardness of the texture of bar cookies.

The preference value of bar cookies ranged from 2.61 to 3.29. The most preferred value was P2 formula (3.29) consisting of 20% mung bean flour and 80% plantain puree; P4 formula resulted in the lowest value of preference. Increasing the proportion of mung bean flour decreased the preference value of bar cookies. Ripe plantain puree improves texture of cookies to become softer and sweeter while increasing the proportion of mung bean flour hardens the texture.

Chemical Characteristics of Bar Cookies

Water Content of Bar Cookies. Water content of bar cookies (Figure 2) was 40.97% for P1 db (10% mung bean flour and 90% ripe plantain puree), 36.62% db to P2 (20% mung bean flour and 80% ripe plantain puree), 34.18% db for P3 (30% mung bean flour and 70% ripe plantain puree) and 26.52% db for P4 (40% mung bean flour and 60% ripe plantain puree). Statistical analysis showed the main materials (mung bean flour and ripe plantain puree) affected the moisture content of bar cookies. Higher proportion of plantain puree can increase the water content of bar cookie.

Nio (2012) reported the water content of ripe bananas was high, about 66.0% and which could affect texture of the dough. Akubor (2003) stated that the amylose content of mung beans was about 33%, higher than wheat (28%). Amylose was easier to absorb than amylopectin which

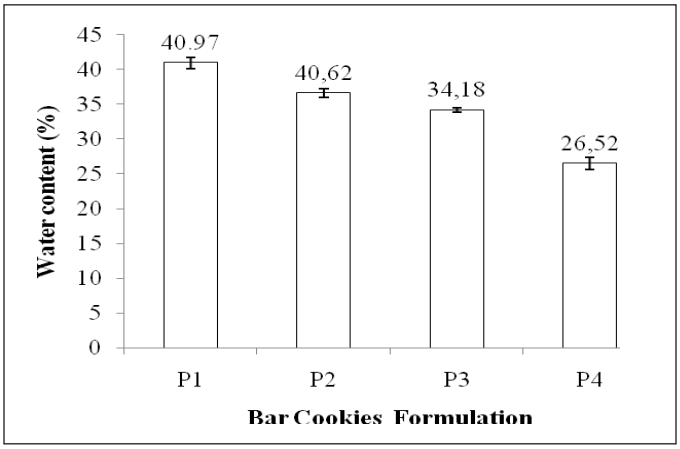

Figure 2. Water content of bar cookies made from:10% mung bean flour and 90% plantain puree (P1), 20% mung bean flour and 80% plantain puree (P2), 30% mung bean flour and 70% plantain puree(P3), 40% mung bean flour and 60% plantain puree (P4).

affects the evaporation of water during baking process. The egg addition on the dough also affects the water content of the snack bar. It causes the lecithin content of egg yolk to absorb water in the dough.

Ash Content of Bar Cookies. The ash content is the amount of inorganic compounds remaining after the combustion process l in the form of ash, while the organic material is burned into water (H_2O) and carbon dioxide (CO_2).

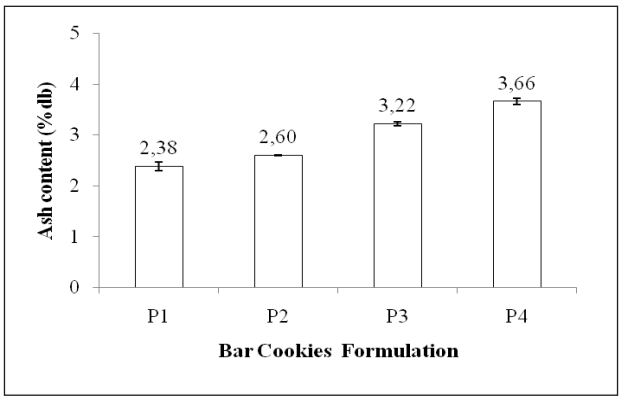

Figure 3. Ash content of *bar cookies* made from:10% mung bean flour and 90% plantain puree (P1), 20% mung bean flour and 80% plantain puree(P2), 30% mung bean flour and 70% plantain puree(P3), 40% mung bean flour and 60% plantain puree (P4).

The ash content of bar cookies (Figure 3) ranged from 2.38% -3.66% db; and 2.38% db for formula P1; 2.60% db for formula P2; 3,22% db for P3 formula; and 3.66% db for formula P4. The composition of the main materials (mung beans flour and ripe plantain puree) causes a significant difference in the ash content of the bar cookies.

The ash content can be influenced by the mineral content in mung bean flour. The higher proportion of mung bean flour leads to higher level of ash content in the bar cookies. Ratnasari, Yunianta and Maligan (2015) reported that adding of mung bean flour into biscuits increased its ash content while Soebito (1988) found the ash content of mung beans was higher (3.7%) than ripe plantain (1.0%).

Fat Content of Bar Cookies. The fat content of bar cookies (Figure 4) was 10.65% db for formula P1; 10.86% db for formula P2; 12.08% db for P3 formula; and 12.56% db for formula P4. The fat content of bar cookies was significantly different as they were made from different composition of the main flour. Kusnandar (2010) reported that mung bean flour contained 3.9% of fat content while banana puree contains 1.2% fat content.

Fat content can improve the texture of cookies. Eggs and margarine are rich in essential fatty acids and are the main ingredients in bread making to improve its physical properties and enhance the nutritional value of bread. Egg yolk contains high fat (31.9%).

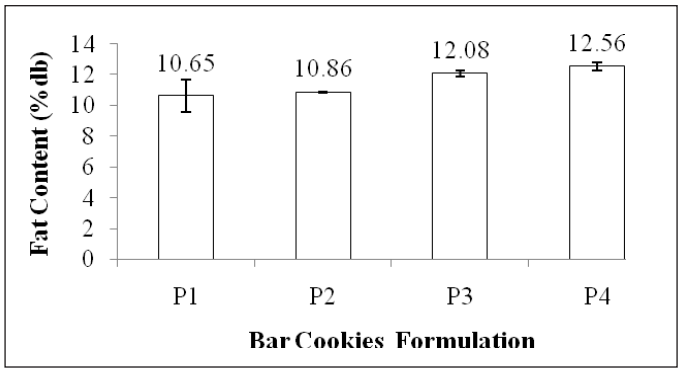

Figure 4. Fat content of bar cookies made from: 10% mung bean flour and 90% plantain puree (P1), 20% mung bean flour and 80% plantain puree(P2), 30% mung bean flour and 70% plantain puree(P3), 40% mung bean flour and 60% plantain puree (P4).

Protein Content of Bar Cookies. The protein content of bar cookies (Figure 5) ranged from 8.51% -20.64% db. The protein content of bar cookies was 8.51% db for formula P1; 1.69% db for formula P2; 14.15% db for P3 formula; and 20.64% db for formula P4. The composition of the main ingredients (mung beans flour and ripe plantain puree) affected the protein content of bar cookies. Increasing mung bean flour increased the protein content of bar cookies. Ladamay and Yuwono (2013) reported that the addition of mung bean flour could increase the protein content in barfood.

The addition of mung bean flour into food bars aims to increase the protein in solid foods that cannot be substituted with tapioca flour. Setyaningtyas (2008) explained the protein content of mung beans was 22.2% and 1.2% for ripe plantains. The mung beans are rich in essential amino acids, among others, the amino acid

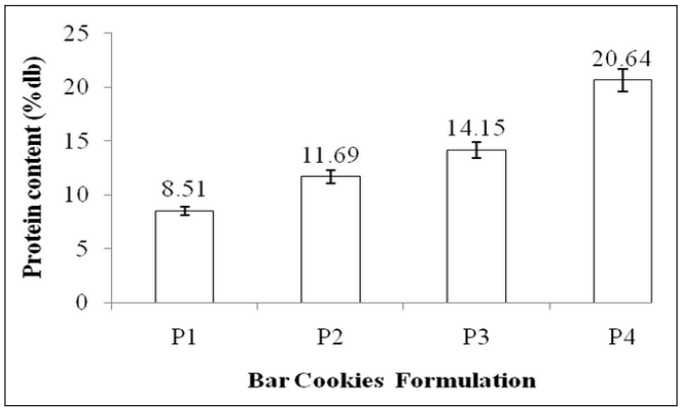

Figure 5. Protein content of bar cookies made from:10% mung bean flour and 90% plantain puree (P1), 20% mung bean flour and 80% plantain puree(P2), 30% mung bean flour and 70% plantain puree(P3), 40% mung bean flour and 60% plantain puree (P4).

leucine, arginine, isoleucine, valine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

Carbohydrate content of Bar Cookies. Carbohydrate content of bar cookies (Figure 6) ranged between 36.06% (db) and 38.11% (db). Carbohydrate content of bar cookies was 36.06% db for formula P1; 36.53% (db) for formula P2; 37.81% db for P3 formula; and 38.11% db for formula P4. Increasing the proportion of mung bean flour increased the carbohydrate content of bar cookies. Lumiar (2010) reported the carbohydrate content of mung beans was 62.5%, composed of starch, sugar and fibre. Mung bean starch consists of 28.8% amylose and 71.2% amylopectin.

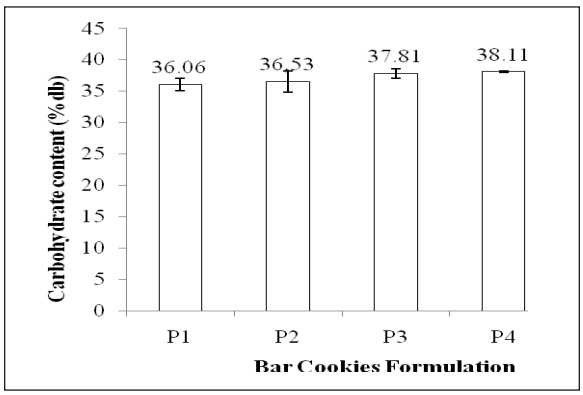

Figure 6. Carbohydrate content of *bar cookies* made from: 10% mung bean flour and 90% plantain puree (P1), 20% mung bean flour and 80% plantain puree(P2), 30% mung bean flour and 70% plantain puree(P3), 40% mung bean flour and 60% plantain puree (P4).

Effectiveness Value of Cookies Bars. The best treatment can be determined by assigning weight to some parameters that have been tested, such as water content, ash content, fat content, protein content, carbohydrate content, and sensory values. The effectiveness value of the bar cookies are shown in Table 1. It can be seen the best formulation of bar cookie is P2 made from 20% mung bean flour and 80% ripe plantain puree. The P4 formulation resulting in the bar cookies being bright in colour and having a good aroma, tastier and softer texture. The P2 bar cookies had a 40.97% of water content, ash content 2.60%, fat content 10.86%, protein content 11.69% and carbohydrate 36.53%.

Table 1
The Effectiveness value of bar cookies formulation

Formulation	Effectiveness Value
P1 (10% green bean flour, 90% ripe plantain puree)	0.45
P2 (20% green bean flour, 80% ripe plantain puree)	0.55
P3 (30% green bean flour, 70% ripe plantain puree)	0.51
P4 (40% green bean flour, 60% ripe plantain puree)	0.49

CONCLUSION

Sensory properties of preferred bar cookies include their colour, aroma, flavour and texture at 3.29; 2.93; 3.39; 2.89 respectively; and 3.29 on the scale range values really dislike (1) to like (5). Formulation of the bar cookies has a significant effect on their chemical characteristics. The preferred formulation of bar cookies was P2 made from main ingredient i.e. 20% mung bean flour and 80% ripe plantain puree. Chemical characteristics of preferred bar cookies (P2) were 40.62 % water, 2.60 % db ash, 10.86 % db fat, 11.69 % db protein, 36.53 % db carbohydrate.

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Isolation and Identification of *Bacillus thuringiensis* from *Aedesaegypti* Larvae as Potential Source of Endotoxin to Control Dengue Vectors

Maria Goretti Marianti Purwanto*, Renardi Gunawan, Ida Bagus Made Artadana, Mangihot Tua Goeltom and Theresia Desi Askitosari

Faculty of Biotechnology, Universitas Surabaya, Raya Kalirungkut, Surabaya 60293, Indonesia

ABSTRACT

Dengue is an emergent disease transmitted by *Aedes aegypti* mosquitos prominent in tropical countries. Numerous methods have been used to prevent the spread of Dengue fever, such as fogging and treatment using anti-larvae chemicals, yet these methods are harmful. *Bacillus thuringiensis* found in *Aedes aegypti* larvae is capable of producing endotoxin that able to kill insects without any side effect on humans, thus it is able to control Dengue vectors without any adverse effects to the environment. *Aedes aegypti* larvae were crushed and mixed with saline solution to isolate the bacteria in the larvae. From all bacterial colonies extracted from the larvae, 13 colonies with appearance closest to *Bacillus* colonies were screened using gram staining, spore staining, and biochemical testing. From 13 colonies, 8 of them were further analysed using ARDRA and *cry1A* gene amplification. These analyses showed one of the colonies had *cry1A* gene, which indicated the colony was *Bacillus thuringiensis*. The isolated *Bacillus thuringiensis* was used for endotoxin production and efficacy assays.

Keywords: Aedes aegypti, *Bacillus thuringiensis*, Dengue vectors, endotoxin

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E-mail addresses:
maria_gmp@staff.ubaya.ac.id (Maria Goretti Marianti Purwanto)
renardig90@hotmail.com (Renardi Gunawan)
arta@staff.ubaya.ac.id (Ida Bagus Made Artadana)
ihot_gultom@staff.ubaya.ac.id (Mangihot Tua Goeltom)
desy_askito@staff.ubaya.ac.id (Theresia Desi Askitosari)
* Corresponding author

INTRODUCTION

Aedes aegypti and *Aedes albopictus* mosquitoes are the main vector of several viruses such as Dengue virus, Yellow fever virus, Chikunguya virus (Kraemer et al., 2015) and Zika virus (World Health Organization [WHO], 2016). These viruses have become major public health concerns, with increasing disease incidence and

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prevalence of dengue and other associated fevers. *Ae. aegypti* and *Ae. albopictus* distributions are predicted to occur in tropical and subtropical areas, with *Ae. albopictus* having wider area due to its higher tolerance to lower temperatures (Kraemer et al., 2015). In Indonesia, annual dengue haemorrhagic fever occurrence had increased from 0,05 per 100000 people in 1968 to 35-40 per 100000 people in 2013 (Karyanti et al., 2014).

Aedes aegypti and *Aedes albopictus* lifecycle is the following: Egg-larva-pupa-adult and require still water to live their non-adult stages of life. An adult female mosquito can produce 100-200 eggs per batch, and each egg can develop within 2 days in tropical climate. In larva stage, they undergo several changes in size called instars. Initially, a newly hatched larva has around 1.7 mm length, and can reach up to 7.2 mm at the 4th instar (Bar & Andrew, 2013). They feed on organic matters in the water, and if undisturbed, can be found floating in the water surface. After 4th instar, larvae will enter pupal stage. In this stage, they no longer eat and after a couple of days will emerge as an adult mosquito (Zettel & Kaufman, 2012).

To control its growth and population, several methods have been undertaken, such as treatment with DDT, Malathion or Temephos (Abate powder). However, some chemicals used to control mosquito growth have been found to be harmful for humans, especially DDT which was banned in several countries. Temephos are non-harmful to human, but several researches have reported increasing resistance of *Aedes aegypti* larvae against it (Diniz, Henriques, Leandro, Aguiar, & Beserra, 2014). *Bacillus thuringiensis* is capable of producing crystal protein consisting of δ-endotoxins, which possess insecticidal activity. The crystal proteins, or Bt toxins, are produced during sporulation stage of *B. thuringiensis* as a 130-140 kDa protoxins in bipyramidal shaped crystals. The crystal proteins are cleaved into an active toxin that can kill the insect (Regev et al., 1996). Cry toxin, one of the proteins, comprises crystal protein and has specific targets (Bravo, Gill, & Soberón, 2007). Thus, by finding a *B. thuringiensis* that is able to produce crystal proteins specific for *Aedes* larvae, we could make a specific larvicide for *Aedes* larvae.

Dengue fever is a major health concern 126.675 being infected with it resulting in 1,229 deaths in 2015 (Kementerian Kesehatan Republik Indonesia [Kemenkes], 2016). It is an endemic problem requiring an urgent solution. Proper pest controls to curb *Aedes aegypti* and *Aedes albopictus* is a must. Prevention is more economical than treating the disease. Using Temephos has been proven to completely eradicate the larvae, and there are indications the larvae has developed resistance to Temephos (Mulyatno, Yamanaka, Ngadino, & Konishi, 2012). This research attempts to isolate *Bacillus thuringiensis* from larvae with movement impairment and sign of sickness. The frailty and sickness may be caused by *B. thuringiensis* crystal protein, thus by isolating the bacteria, we can produce the crystal protein as anti-larvae. Production

of crystal protein from *B. thuringiensis* for this purpose has never been attempted in Indonesia.

MATERIALS AND METHODS

Materials

Aedesaegypti larvae were obtained from water containers within the vicinity of Universitas Surabaya, Surabaya, Indonesia. Restriction enzymes were purchased from Thermo Fischer.

Collecting *Aedesaegypti* Larvae

Aedesaegypti larvae were taken from water containers and still water from unused tires in Universitas Surabaya. Collected larvae were put in glass jars, and each species was confirmed by observing larva's comb under microscope, and later stored in a small plastic container for bacterial isolation.

Bacterial Isolation from Larvae

Aedes aegypti larvae were selected for bacterial isolation. Larvae with impaired movement and slow response were transferred into sterile test tubes. Each test tube was filled with 4-5 larvae before they were crushed. 4 ml of 0.85% NaCl was added and incubated at room temperature for 5 minutes. A serial dilution was prepared up to 10^{-2} using 0.85% NaCl sterile saline solution, and then each dilution was incubated in 70°C for 15 minutes. After incubation, each diluted sample was plated into Nutrient agar with 0.3% w/v Yeast extract. Each petri dish was incubated at 37°C for 16-24 hours.

Bacterial Selection

Colonies were screened for *Bacillus*-like characteristics, such as white to yellowish colonies, entire or undulate arborescent colony, motile and smooth (for yellowish colonies) or dull (for white colonies). Every colony with similar characteristics were inoculated at pH 6.8 LB (Luria Bertani)-Acetate broth selective medium. LB-Acetate medium was made based on Travers, Martin, & Reichelderfer (1987). LB-Acetate medium was incubated at 37°C, 150 rpm for 24 hours, and then transferred to water bath at 80°C for 5 minutes. 100 µL of each LB-Acetate medium was inoculated in LB agar medium (pH 7,2) and incubated at 37°C for 16-24 hours.

Biochemical Assay and Staining

Colonies grown in LB agar pH 7.2 were screened for biochemical tests and staining. Each colony was tested in the following: starch hydrolysis, motility, and nitrate. A starch agar with addition of 2% amylum was prepared for starch hydrolysis analysis of samples. Each suspected colony was inoculated into the agar and incubated at 37°C for 16 hours. Then iodine solution was added into the plates and spread evenly. Positive results can be inferred from the formation of white area surrounding the colonies (modified from Clarke & Cowan, 1952). For motility assay, a bacterial colony was taken using a toothpick, and then inoculated into an agar medium by stabbing. The medium then was incubated at 37°C for 16-24 hours (modified from Bergey, 2005).

Nitrate reduction test was performed based on Zobell (1932) with modifications. Agar medium was prepared by adding 1 gram of KNO_3 per litre of nutrient agar medium. Each colony was then inoculated into the medium and incubated at 37°C for 24-48 hours. A drop of α-naphthylamine and sulfanilic acid was added into the medium and the colour change was observed. A toothpick of zinc powder was added into the medium after observing the change in colour. Citrate utilisation was analysed using Simmons Citrate Agar. The SCA slant medium was prepared, and bacterial sample was inoculated into the medium. After 16-24 hours of incubation at 37°C, the alteration in the colour of the medium would indicate the result (modified from Hemraj, Diksha, & Avneet, 2013).

Gram staining was done based on a method modified from Gram (1884), and spore staining (modified from Schaeffer & Fulton, 1933) of colonies were used to analyse cell characteristics of each colony. Gram positive and spore producing colonies were used for further molecular tests.

DNA Extraction from Bacterial Colonies

Bacterial colonies were transferred using tip of toothpicks to PCR tubes filled with 100 µL ddH20 and mixed thoroughly. They were incubated at -80°C for 20 minutes and then immersed in boiling water for 10 minutes to lyse the cells. Later, they were centrifuged at 10,000×g for 10 seconds and the supernatant collected and checked for DNA concentration using Nanodrop (modified from Bravo, 1998).

16S rRNA Amplification

Primers used for PCR amplification of 16S rRNA were 27Fprimer (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R primer (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR was carried out in 50 µL tubes. 1 µL DNA template was mixed with 10 µL Master Mix 2x concentration and 0.5 µL 10 pmol primers, then ddH_2O was added until total volume is 20 µL. PCR cycles are the following: 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min. PCR results were visualised in a 1% agarose gel electrophoresis in 100V for 30 minutes.

ARDRA Analysis

PCR amplicons were treated with restriction enzymes PstI, HindIII, EcoRI and HaeIII. 5 µL of PCR results were mixed with 1 µL 10x Restriction buffer, 0.5 µL Restriction enzyme and 8.5 µL ddH_2O then incubated at 37°C for 15 minutes (modified from Thermo Fisher protocol). The result was visualised in 1.5% agarose gel electrophoresis in 100V for 45 minutes.

***Cry1* Gene Amplification**

DNA sample was amplified by Lep1A (5'-CCGGTGCTGGATTTGTGTTA-3') and Lep1B (5'-AATCCCGTAT TGTACCAGCG-3') primers to detect *Cry1* gene on *Bacillus thuringiensis*. For 10 µL total PCR reaction, 1 µL DNA template, 5 µL Master Mix and 0.5 µL for each primer was mixed in a PCR tube and mixed thoroughly. PCR reaction was performed at

94°C initial denaturation for 2 minutes, then 35 cycles of 94°C for 30 second, 50°C for 30 second, and 72°C for 2 minutes then 72°C for 10 minutes final elongation (modified from Rijzaani & Bahagiawati, 2003).

RESULTS AND DISCUSSION

Bacterial Isolation from *Aedes aegypti* Larvae Collection and Selective Medium Treatment

Aedes aegypti larvae were collected from water containers in Universitas Surabaya's greenhouse and static water contained in unused tires around the vicinity of the campus. Larvae were identified using their comb spine, observed under microscope (Figure 1). *Aedes aegypti* has comb spines arranged in a row, with each comb spines pointed and curved alongside with smaller denticles (Bar & Andrew, 2013). Larvae was collected twice, and bacteria extracted from the first larvae collection was numbered I, and the second larvae collection were numbered II.

Preliminary examinations of morphology showed numerous types of bacterial colonies, and mostly growing close to each other. Colonies were transferred into a new medium to separate each of them. Each colony was inoculated in LB-Acetate selective medium. Spore-forming *Bacillus thuringiensis* can be distinguished from other bacteria by inoculating in medium with sodium acetate. *Bacillus thuringiensis* spores were unable to germinate in presence of sodium acetate, and heat applied after incubation destroys other unwanted vegetative bacteria still alive in the medium (Travers et al., 1987). This treatment should eliminate most unwanted bacteria. Colonies that were able to grow after LB-Acetate medium treatment were screened using biochemical tests and staining.

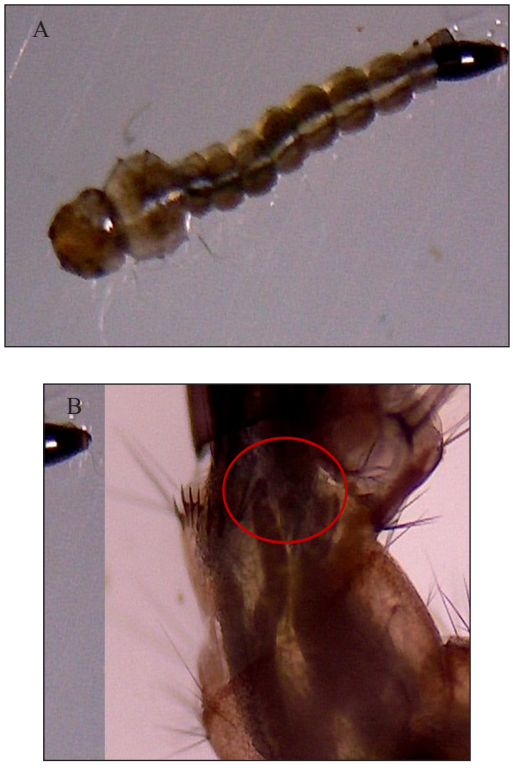

Figure 1. Aedes aegypti larvae (A) and larvae comb (B with 40× magnification). The comb spine of *Aedes aegypti* is circled.

Biochemical Assay and Staining

Gram staining of samples (Figure 2) showed various bacterial types; however, only rod-shaped gram-positive bacteria were used for further experiment. Some bacteria have endospores, which can be seen gram stain as hollow point in the cells. Spore staining

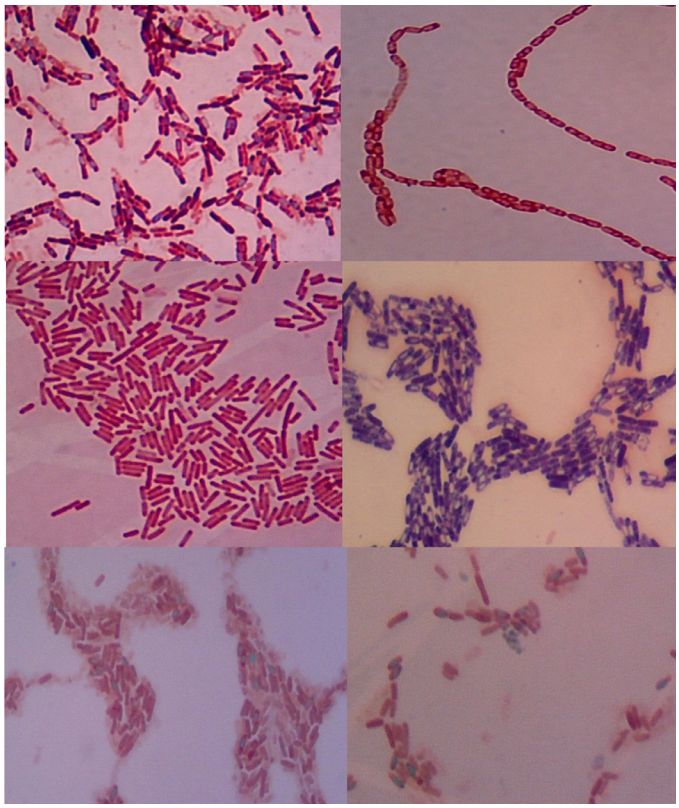

Figure 2. Several bacterial staining results. Top left and middle right are gram positive, spore-producing *Bacillus*. Bottom pictures show spore staining result, with both having greenish spore in red cells.

using malachite green was also performed to visualise the spores. *B. thuringiensis* from the previous unpublished research was used as positive control.

Non-spore forming, gram positive bacteria were eliminated, while the *B. thuringiensis* candidate cultures were analysed further. All strains were stained to observe their cell morphology after LB Acetate treatment. There were 13 bacteria samples, and 10 were Gram positive while 3 of them were Gram negative. The observed cell shape were mostly bacilli, with 2 strains streptobacilli, and 7 out of 13 samples have endospore (Table 1). Samples taken from first bacterial extraction (I) were all gram-positive bacteria, and from the second bacterial extraction, there were 3-gram negative bacteria among 8 samples observed.

These colonies were inoculated in biochemical assay medium. Colonies were tested on several biochemical characteristics displayed in Bergey's Manual of Systematic Biology (De Vos et al., 2009). Nitrate reduction test, starch hydrolysis, citrate fermentation and bacterial motility were analysed using respective medium for each assay. Based on Bergey's manual, *B. thuringiensis* would display positive result

Table 1
Gram staining, cell shape and *endospore* of *bacterial sample isolated from* A. aegypti *larvae*

Sample	Gram	Cell shape	Endospore
Positive Control	Gram positive (+)	Bacilli	Yes
I-2B	Gram positive (+)	Bacilli	Yes
I-4B1	Gram positive (+)	Bacilli	-
I-4B2	Gram positive (+)	Bacilli	Yes
I-4D1	Gram positive (+)	Streptobacilli	Yes
I-4D2	Gram positive (+)	Bacilli	Yes
II-1A16B	Gram negative (-)	Bacilli	-
II-1A2	Gram positive (+)	Streptobacilli	-
II-1C	Gram positive (+)	Bacilli	Yes
II-224	Gram negative (-)	Bacilli	-
II-1F1	Gram positive (+)	Bacilli	-
II-2C	Gram negative (-)	Bacilli	-
II-1F2	Gram positive (+)	Bacilli	Yes
II-212	Gram positive (+)	Bacilli	Yes

in starch hydrolysis, nitrate reduction, citrate fermenting and motile. Citrate fermentation on *B. thuringiensi* positive control should give positive result, yet it showed negative result along with all citrate tests. This may be due to the fact citrate media (Simmons Citrate Agar) pH indicator was already degraded. Biochemical assay result (Table 2) showed that all samples had diverse assay results, with only 2 showed similarities

Table 2
Biochemical assay results of bacteria isolated from A. aegypti *larvae*

Sample	Starch hydrolysis	Nitrate reduction	Motility	Citrate
Positive Control	+ (Positive)	+ (Positive)	+(Positive)	-(Negative)
I-2B	+(Positive)	+(Positive)	- (Negative)	- (Negative)
I-4B1	- (Negative)	- (Negative)	- (Negative)	- (Negative)
I-4B2	+(Positive)	- (Negative)	- (Negative)	- (Negative)
I-4D1	+(Positive)	+ (Positive)	- (Negative)	- (Negative)
I-4D2	+(Positive)	+(Positive)	+ (Positive)	- (Negative)
II-1A16B	- (Negative)	- (Negative)	- (Negative)	- (Negative)
II-1A2	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-1C	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-224	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-1F1	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-2C	- (Negative)	-(Negative)	- (Negative)	- (Negative)
II-1F2	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-212	+(Positive)	+(Positive)	+ (Positive)	- (Negative)

with positive control, sample I-4D2 and II-212.The diverse biochemical assays results obtained might indicate that samples different from positive control were not *B. thuringiensis*, but the variety of results have been observed before. Gonzalez et al. (2011) discovered several *B. thuringiensis* that had slight variance in their biochemical characteristic. From biochemical point of view, these 2 samples were the promising candidates for *B. thuringiensis*. However, all gram-positive samples were further analysed by PCR and ARDRA to confirm their species.

Sample II-212 biochemical assay and staining were done after all sample had undergone ARDRA analysis. This sample was taken to consideration due to its colony shape that closely resembles *B. thuringiensis*. The result of biochemical assay and staining confirmed the similarity with the positive control, and thus, were analysed further.

16S rRNA Amplification

DNA of bacterial samples was extracted using heat-shock method to make the cells undergo lysis and DNA can be extracted from supernatant. The bacterial DNA samples were amplified using 27F and 1492R primers. Bacterial samples from first batch extraction were all tested, and all samples showed band with similar size (Figure 3A). Positive control band was around 1500 bp in size, similar to sample

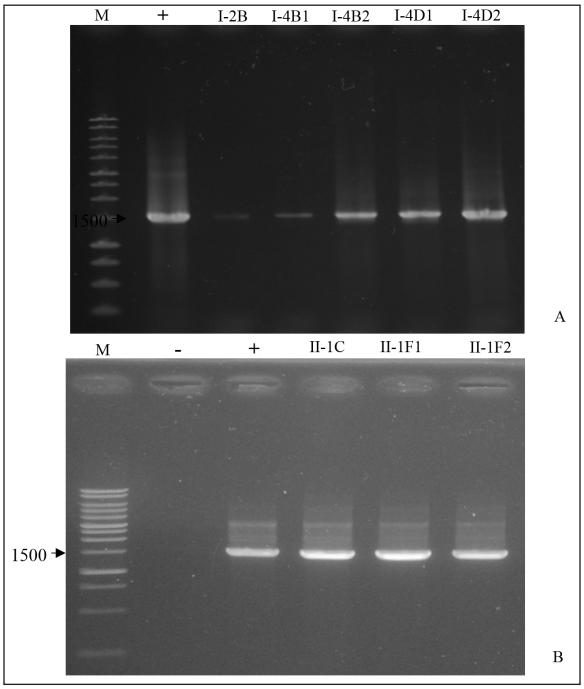

Figure 3. PCR result of 16S rRNA amplification for samples from first batch extraction (A) and second batch extraction (B). DNA ladder (M) used was 1000 bp ladder.

bands which around 1500 bp as well. Both first and second batch samples showed similar band size. Figure 3A showed I-2B and I-4B1 had smaller bands compared to other 3 samples, which might be caused by low DNA concentration prior to PCR.

Samples from the second batch were selected based from staining results. 3 gram positive, bacilli-shaped bacteria (II-1C, II-1F1, and II-1F2) had their DNA extracted and used for 16S rRNA amplification. Figure 3B shows the PCR amplification result for 3 samples, having bands in the same size as positive control, and all were around 1500 bp in size. 16S rRNA amplification in previous research (Atallah, El-Shaer, & Abd-El-Aal, 2014; Shishir et al., 2014) showed similar 16S rRNA size of 1500 bp.

ARDRA Analysis

Based on staining and biochemical activity assay, only several samples which showed similar characteristics to *B. thuringiensis* were tested for ARDRA. All first batch samples (I-2B, I-4B1, I-4B2, I-4D1, I-4D2) were tested using *Pst*I, *Eco*RI, and *Hind*III restriction enzymes. Figure 4 shows the restriction results of the samples. *Hind*III and *Eco*RI restriction enzymes cleaved both the positive control and samples into similar restriction patterns, but *Pst*I restriction shows a different result. Positive control *B. thuringiensis* was not cleaved by *Pst*I, giving a band size of 1500 bp while all samples were cleaved into 2 bands around 900 and 700 bp.

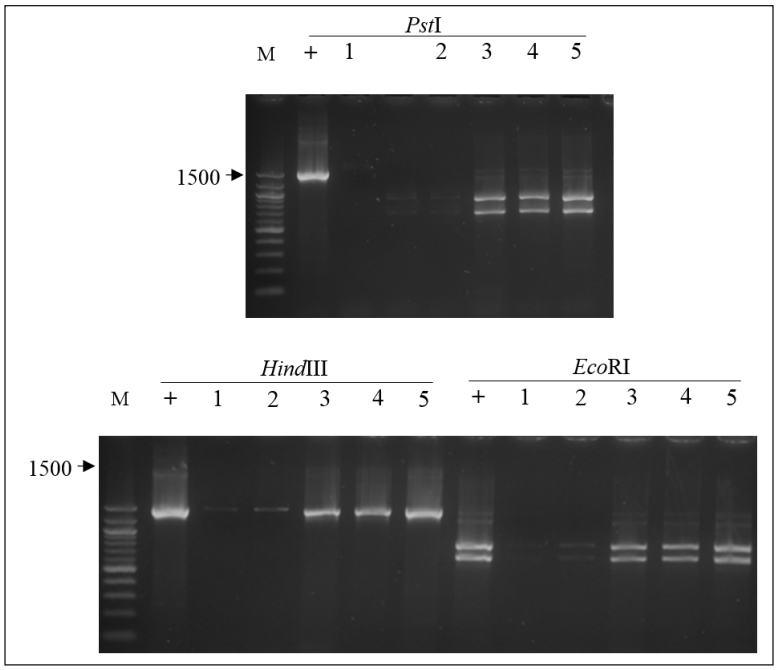

Figure 4. ARDRA analysis result using *Pst*I, *Hind*III, *Eco*RI restriction enzyme. Marker used is 100 bp ladder. Sample 1 (I-2B), 2 (I-4B1), 3 (I-4B2), 4 (I-4D1), and 5 (I-4D2).

ARDRA result from second batch bacteria showed a different restriction pattern on sample 2 (II-1F1). As can be inferred from Figure 5, sample II-1F1 was cleaved into 2 fragments of 900 and 700 bp in size respectively. In this ARDRA analysis, *Eco*RI enzyme (data not displayed) gave no distinct restriction patterns, so *Hae*III was used instead. *Hae*III restriction of II-1F1 sample yielded different patterns (indicated by white arrow), albeit less profound compared with *Pst*I.

This result indicated the difference between positive controls with bacterial samples, namely 16S rRNA of *Bacillus thuringiensis*. All first batch samples restriction patterns were different from positive control; therefore, the bacterial samples may not *B. thuringiensis*. In second batch bacterial samples, only II-1F1 sample had different restriction patterns. All samples with different restriction patterns were omitted from next process, except sample I-4D2 which have similar biochemical assay result with positive control.

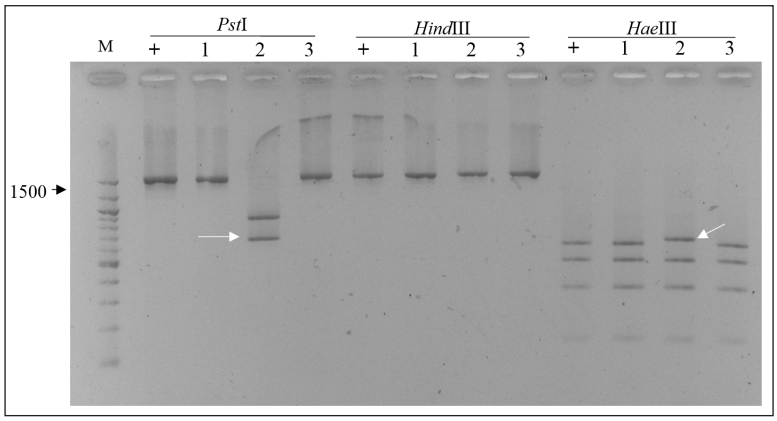

Figure 5. ARDRA analysis result using *Pst*I, *Hind*III, and *Hae*III restriction enzyme. Sample 1(II-1C), 2 (II-1F1), 3 (II-1F2) is each cleaved by 3 restriction enzymes. Different restriction bands are indicated by white arrows.

Cry1 Gene Amplification

Inconclusive results of ARDRA meant it was difficult to identify *B. thuringiensis* sample. Therefore, another set of primers Lep1A and Lep1B were used to amplify *cry1* gene of *B. thuringiensis*. Therefore, these pairs of primers can detect *B. thuringiensis* based on their *cry1*gene. From the result of previous biochemical assay and ARDRA, 4 samples were amplified using Lep1A and Lep1B primers. Sample I-4D2 was tested for biochemical characteristic with positive control *B. thuringiensis*. Another two samples were taken from ARDRA result (II-1C and II-1F1), with sample II-212 tested because it has similar biochemical characteristics.

Lep1A and Lep1B amplify Lepidopteran-specific crystal toxic genes, which produce crystal proteins to kill

Leptidoptera insects (Bravo et al., 2007). PCR amplification of *cry1* gene using Lep1A/Lep1B primers showed a 357 bp band, similar to 363 bp*B. thuringiensis* positive control (Figure 6). Other samples didn't produce any amplification bands, and II-1F2 showed a faint band, but it was too little to be considered.

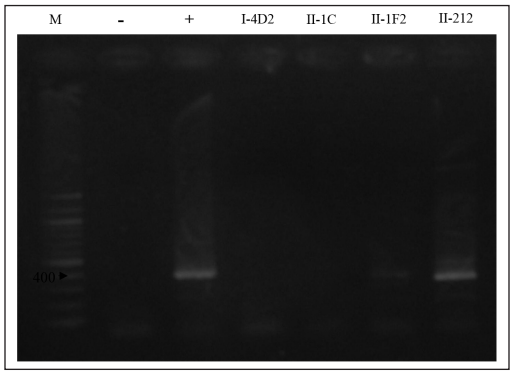

Figure 6. cry1A gene amplification. Sample II-212 (white arrow) shows a band similar to positive control. Marker used is 100 bp ladder.

Abdulreesh, Osman and Assaeedi (2012) found that Lep1A/Lep1B primers amplified *cry1Aa, cry1Ab*, and *cry1Ac* genes in *B. thuringiensis*, resulting in a 490 bp band. Ammouneh, Harba, Idris and Makee (2011) also had 490 bp band using Lep1A/Lep1B primers. Rijzaani and Bahagiawati (2003) showed Lep1A/Lep1B primers produced several kinds of bands aside from 490 bp, depending on the *B. thuringiensis* isolate. In this research, Lep1A/Lep1B amplification resulted in 363 bp for positive control and 357 bp for sample II-212.

According to Abdulreesh et al. (2012), 490 bp indicated amplification of *cry1Aa, cry1Ab*, and *cry1Ac* gene. The smaller amplicon band in this research might because the bacteria don't possess all 3 *cry1Aa, cry1Ab*, and *cry1Ac* genes, for both positive control and sample II-212. This indicated that crystal protein produced by sample II-212 might had reduced efficiency in killing Lepidopteran insects, since it did not have a complete 3 genes. But its efficacy towards mosquito still requires further testing.

The RFLP was performed to analyse similarity between sample and positive controls. Both were analysed using 4 restriction enzymes to detect any differences in gene. The restriction on both the sample and control yielded only 1 band size of 360 bp as can be seen on Figure 7. From this result it can be inferred that II-212 is closely related to positive control *B. thuringiensis*.

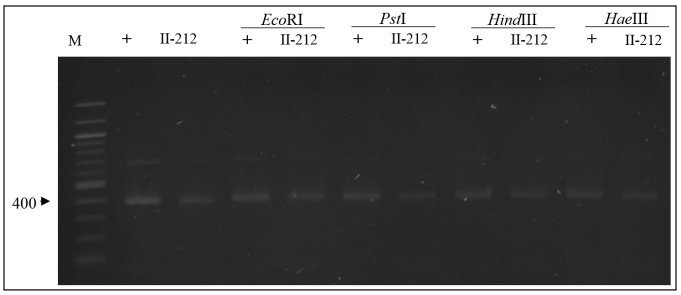

Figure 7. *cry1* gene amplicons RFLP. All samples show uniform sized bands to untreated PCR result (lane 2 and 3). Marker used is 100 bp.

CONCLUSION

The bacterial isolation from *Aedes aegypti* larvae with impaired movement and slow response was performed to find *B. thuringiensis* using several biochemical assays, staining and molecular analysis. Bacterial samples were placed in selective medium to eliminate non-spore-forming gram-positive bacteria. Biochemical assay and staining were used to narrow the bacteria candidate for *B. thuringiensis* and as cross-reference for molecular analysis. ARDRA and *cry1* RFLP analysis indicated sample II-212 was *B. thuringiensis*. The efficacy of the crystal protein needs to be tested further to detect II-212 toxicity towards *Aedes aegypti* larvae.

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Isolating and Characterising Chitinolytic Thermophilic Bacteria from Cangar Hot Spring, East Java

Ruth Chrisnasari*, Devi Verina, Aime Clorinda Tapatfeto, Stefan Pranata, Tjandra Patjajani, Mariana Wahjudi and Maria Goretti Marianti Purwanto

Department of Biology, Faculty of Biotechnology, University of Surabaya, Jalan Raya Kalirungkut, Surabaya 60293, Indonesia

ABSTRACT

In the present study, chitinolytic thermophilic bacteria were collected from Cangar hot spring, East Java, Indonesia and screened. The 16S rRNA gene sequencing was used to identify the isolated bacterium which showed highest chitinolytic activity. The identified isolate was then characterised based on morphological and physiological analyses. The results showed the isolated bacterium belonged to *Bacillus licheniformis*. This isolate produced large amounts of chitinase on 0.9% (w/v) colloidal chitin (pH 7.0) at 52°C in a very short time (24 hours). Two pairs of primer were designed to detect the presence of glycosyl hydrolase (GH) 18 chitin domain sequences in the isolated bacterium. Two amplicons sized ~250 bp and ~1000 bp were obtained from PCR process. Then the amplicons were sequenced and analysed. The sequencing results showed the isolated *Bacillus licheniformis* was proven to have genes encoding *ChiA* and *ChiC* domain.

Keywords: Bacillus licheniformis, ChiA, ChiC, thermophilic bacteria, thermostable chitinase

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E-mail addresses:
ruth_c @staff.ubaya.ac.id (Ruth Chrisnasari)
devi.verina@yahoo.com (Devi Verina)
aimee_tjung@yahoo.com (Aime Clorinda Tapatfeto)
stefanp192@gmail.com (Stefan Pranata)
tjandra@staff.ubaya.ac.id (Tjandra Patjajani)
mariana_wahyudi@staff.ubaya.ac.id (Mariana Wahjudi)
maria_gmp@staff.ubaya.ac.id (Maria Goretti Marianti Purwanto)
* Corresponding author

INTRODUCTION

Chitinases (EC 3.2.1.14) are grouped into either Family 18 or Family 19 under glycosyl hydrolases superfamily which is capable of degrading chitin into its derivates by hydrolysing the β-1,4-glycosidic bonds between the N-acetylglucosamine residues (Shaikh & Deshpande, 1993). Nowadays, the demand for chitinase with new or desirable properties has increased due to a wide-range of industrial application of chitin derivates, such as chitooligosaccharides and

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N-acetylD-glucosamine (Ramirez-Coutino, Marin-Cervantes, Huerta, Revah, & Shirai, 2006). Chitooligosaccharides produced by enzymatic hydrolysis of chitin has been especially used in pharmaceuticals fields as antioxidant, immunostimulant (Shahidi, Arachchi, & Jeon, 1999), antihypertensive, antibacterial, antifungal, and as a food quality enhancer (Bhattacharya, Nagpure, & Gupta, 2007).

Chitinases are produced by various microbes and recognised as extracellular inducible enzymes. Most bacteria secrete Family 18 chitinases to degrade chitin and utilise it as an energy source (Hart, Pfluger, Monzingo, Hoihi, & Robertus, 1995). The superiority of chitinase-producing bacteria is one of the key factors in the enzyme production. The high biodiversity in Indonesia presents a great opportunity to get potential bacteria with special characteristic to be used as enzymes producer. Therefore, the exploration of the chitinase-producing bacteria is vital Indonesia. Chitinolytic thermophilic bacteria can be isolated from both soil and aquatic thermophile habitats i.e. hot spring and crater. The advantage of using thermophilic bacteria is their ability to synthesise the heat stable molecule, including enzymes. Thermostable enzymes produced by thermophilic bacteria are very effective and beneficial for industrial processes that need high temperature — e.g. chitin degradation in pharmaceutical industries and waste processing in seafood industry. High temperature can improve reaction speed, increase the solubility of the reactants and non-volatile products as well as reducing mesophilic microbial contamination (Martin, Delatorre, & Camila, 2007).

The aim of this study was to isolate the most prominent local chitinolytic thermophilic bacteria from Cangar Hot Spring, East Java for thermostable chitinase production. The obtained isolate then was identified based on molecular, morphological and physiological analyses. The identified isolate was used to produce chitinase under specific condition. The isolate was then further characterised by detection of glycosyl hydrolase (GH) 18 chitin domain sequences in the isolate genome using PCR based method.

MATERIALS AND METHODS

Enrichment and Cultural Medium

Nutrient Broth (NB) (Merck) and Luria Bertani (LB) broth (Scharlou) were used as enrichment medium. Thermus colloidal chitin (TCC) broth containing 0.7% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) K_2HPO_4, 0.1% NaCl, 0.01% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) yeast extract, 0.1% (w/v) bactotryptone and 0.5% colloidal chitin (Yuli, Suhartono, Rukayadi, Hwang, & Pyun, 2004) was used as culture medium. The TCC agar medium for screening process was made by adding 15 g L^{-1}bacto agar in the TCC broth medium. The chitin was produced from shrimp shell and the colloidal chitin was made based on Hsu & Lockwood (1975).

Bacterial Isolation, Screening and Identification

A total of four different soil and water mixture samples were aseptically collected from different regions of Cangar Hot Spring, East Java, Indonesia. The four samples were enriched in NB and LB broth solution respectively with sample and medium ratio 1:3. The enriched samples were incubated for 24 hours at 52°C with 150 rpm of shaking speed. Bacterial strains were isolated and screened from enriched medium following standard procedures using spread plate technique on TCC agar plates. Morphologically distinct colonies were sub-cultured in TCC broth and purified to single species level using streak plating repeatedly on TCC agar plates. Pure isolates were maintained by sub-culturing on TCC slants and stored at 4°C.

The pure isolates were screened for chitinase activity in TCC broth. The isolates were previously grown in LB broth at 52°C until each isolate reach 0.5 of OD_{600}. As much as 1 mL of each isolate taken and added to 9 mL of TCC broth and incubated for 36 hours at 52°C. The samples were then centrifuged at 4000 rpm for 3 minutes. The supernatant was used for N-acetyl D-glucosamine detection using Nelson–Somogyi assay (Nelson, 1944).

The selected isolate was identified through partial 16S rRNA gene sequencing analysis. Chromosomal DNA of the isolate was extracted from the pure culture using Fungal/ Bacterial DNA MiniPrep Kit (Zymo Research) and amplified using a pair of 16S universal primer (Botha, Botes, Loos, Smith, & Dicks, 2012) ordered from Macrogen, Korea (Forward: 5'-CACGGATCCAGACTTTGATY MTGGCTCAG-3' and Reverse: 5'-GTGAAGCTTACGGYTAGCTTGTTA CGACTT-3'). The amplification reaction mixture contained 5 µl of 16S forward primer 10 µM/µl, 5 µl of 16S reverse primer 10 µM/µl, 25 µl of GoTaq Green Master Mix 2X (Intron), 2.5 µl of DMSO, and 12.5 µl of double-distilled water (ddH_2O). The amplification was performed with initial denaturation at 94 ºC for 5 min, 30 cycles of denaturation at 94 ºC for 45 sec, annealing at 55 ºC for 1 min, and elongation at 72 ºC for 1.5 min followed by final elongation at 72 ºC for 5 minutes. The preparation of samples for sequencing analysis was as follows: (1) the PCR products were purified using PCR Purification Kit (Roche), cloned into pGEMT-Easy (Promega) and transformed to *E. coli* DH5α competent, (2) the transformed cells were confirmed by colony PCR method, (3) DNA plasmid was extracted from the transformed cells using Plasmid Isolation Kit (Roche) and analysed for sequencing (Macrogen, Korea). The homology analysis of 16S rRNA gene sequence was conducted using BLAST algorithm in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Bacterial confirmation and characterisation through morphological and physiological properties were conducted based on Bergey's Manual of Systematic Bacteriology (De Vos et al., 2009).

Chitinase Production

As much as 10% (v/v) of isolate was inoculated into TCC broth medium and agitated at 180 rpm (Yin Der shaker incubator). The fermentation conditions were 0.9% (w/v) of colloidal chitin concentration, pH 7.0 and a temperature of 52ºC. Sub-sample of the culture (50 mL) at initial and final fermentation was concentrated and analysed for chitinase activity assay (Rahayu, Fredy, Maggy, Hwang, & Pyun, 1999).

Chitin Domain Sequence Detection

Chitin Domain Sequence (CDS) was detected based on PCR method using 2 pairs of primer. The first primer was designed to detect *ChiA* (FChiA: 5'-GGYGTCGATVTSGACTGGGAGTAYCC-3' and RChiA: 5'-TCRTAGGTCATRATATTGATCCARTC-3'). The second primer was designed to detect *ChiB* (FChiB: 5'-CTACGCCGGAATACGAAGGGATCGGATA-3' and 5'-AACTCCGCTTCCTCACCAGGTT-3'). Amplification reaction was made in 100 µl containing 100 ng chromosomal DNA, 10 µM/µl forward and reverse primers respectively, 50 µl GoTaq Green Master Mix 2X, and ddH_2O. Amplification process was performed with initial denaturation at 95°C for 5 min, 35 cycles consist of denaturation 95°C for 45 sec, gradient annealing with varied temperature of 53-66°C for 45 sec, and elongation 72°C for 1 min, followed by final elongation 72°C for 10 minutes. PCR product was visualised using agarose gel electrophoresis. The remaining PCR product was purified and prepared for sequencing analysis.

RESULTS AND DISCUSSION

Soil and water mixture samples were taken from four different location of Cangar Hot Spring. Of the four locations (named as location "A", "B", "C" and "D"), 19 single colonies with chitinolytic activity were obtained, where 4 colonies obtained from location B, 12 colonies at locations C and 3 colonies at locations D. None of the colony obtained from location A. The 19 colonies then were screened for chitinolytic activity in TCC broth medium based on amount of N-acetyl D-glucosamine produced as presented at Figure 1. From the data, colony D11 showed highest chitinolytic activity compared to the other colonies, although it is not significantly different with colony C14 and D10 (p-value > 0.05). The D11 colony was then identified, characterised and used for further experiments.

Colony D11 was identified based on the homology of the partial 16S rRNA gene analysis. The homology analysis of gene sequence showed that colony D11 was 99% identical with *Bacillus licheniformis* strain ATCC 14580. *Bacillus licheniformis* have been reported to have multiple and thermostable chitinase (Takayanagi, Ajisaka, Takiguchi, & Shimahara, 1991; Tantimavanich, Pantuwatana, Bhumiratana, & Panbangred, 1998; Trachuk, Revina, Shemyakina, & Stepanov, 1996), making this species commonly used as antifungal biocontrol agents and suitable for industrial

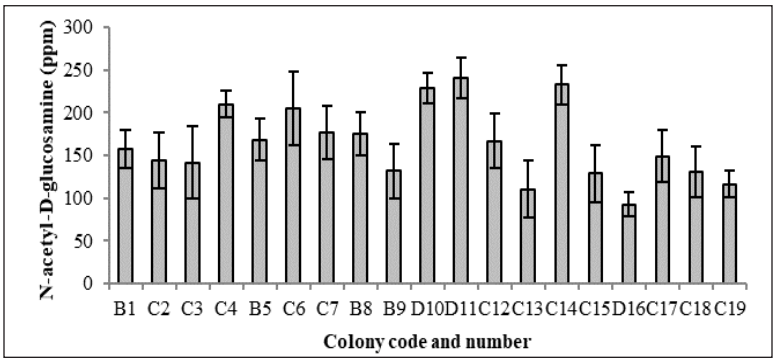

Figure 1. The screening based on chitinolytic activity of 19 isolates obtained from Cangar Hot Spring

chitin waste degradation (Kamil, Rizk, Saleh, & Moustafa, 2007; Veith et al., 2004).

The characterisation assay on morphological and physiological analysis based on Bergey's *Manual of Systematic Bacteriology* is presented in Table 1. *Bacillus licheniformis D11* showed a positive result in the following tests: catalase, amylase, oxidase, and gelatinase production; acid production from glucose, mannitol, arabinose, sucrose and glycerol; growth in 2-7% (w/v) NaCl; Voges-Proskauer test; nitrogen fixation; nitrate reduction, motility and anaerobic growth. *Bacillus licheniformis D11* showed a negative result in the following tests: acid production from lactose and xylose, hydrolysis of urea, utilization of acetate and citrate; indole formation; methyl red test and indole formation. The growth of *Bacillus licheniformis* D11 on TCC broth medium showed the lag (0-4 h), log (4-16 h), stationary (16-28 h) and the death phase (28-48 h) during incubation time (Figure 2).

In correlation to the cell growth curve of Figure 2, chitinase had been produced since the log phase and achieved the optimum at the middle of stationary phase (24 h). The enzyme production was then decreased at 36-48 hours due to lack of nutrients or secretion of toxic substances which inactivated the enzymes (Saima, Roohi, & Ahmad, 2013). *Bacillus licheniformis* D11 achieved optimum amounts of chitinase in a very short time (Figure 3), 24 hours, compared with the other chitinase producer bacteria. *Microbispora* sp. (Nawani, Kapadnis, Das, Rao, & Mahajan, 2002), *B. cereus*, *B. sphaericus* and *B. alvei* (Wang & Hwang, 2001), as well as *Aeromonas punctata* and *Aeromonas hydrophila* (Saima et al., 2013) produced the highest chitinase after 48 h. *Bacillus* sp. HSA,3-1a had been reported to produce the highest chitinase at the end of the stationary phase after 72 h incubation time (Natsir, Patong, Suhartono, & Ahmad, 2010). The short production time revealed *Bacillus licheniformis* D11 to be one of the prominent chitinase producers.

Detecting the presence of glycosyl hydrolase (GH) 18 Chitin Domain Sequence (CDS) in *Bacillus licheniformis D11* genome was done by PCR method using 2 pairs of primer. The first primer was designed to

Table 1
Morphological and physiological characteristic of d11 isolate

Characteristic	Colony Properties	Reference*
Colony shape	Irregular	Irregular
Elevation	Flat	Flat
Margin	Undulate	Undulate
Colony colour	White	White
Cellular morphology	Rod-shaped	Rod-shaped
Gram staining	Gram positive	Gram positive
Spore	Oval endospore	Oval endospore
Catalase	+	+
Amylase	+	+
Urease	-	-
Oxidase	+	+
Gelatinase	+	+
Acid from:		
- Glucose	+	+
- Lactose	-	-
- Mannitol	+	+
- Xylose	-	-
- Arabinose	+	+
- Sucrose	+	+
- Glycerol	+	+
Utilisation of:		
- Acetate	-	-
- Citrate	-	-
Growth in salinity		
- 2 % NaCl	+	+
- 5% NaCl	+	+
- 7% NaCl	+	+
Indole formation	-	-
Methyl red test	-	-
Voges-Proskauer test	+	+
Nitrogen fixation	+	+
Nitrate reduction	+	+
Motility	+	+
Anaerobic growth	+	+

*Data compiled from De Vos et al. (2009); Oziengbe & Onilude (2012); Sankaralingam, Shankar, Ramasubburayan, Prakash and Kumar (2012); Waldeck, Daum, Bisping and Meinhardt (2006).

detect *ChiA*. Amplification using this primer by gradient thermocycler in variation of annealing temperature (T_a47-60°C) produced one amplicon sized ~250 bp (Figure 4) which was later sequenced and analysed. Based on sequence alignment (BLASTn) result, this primer was able to detect *ChiA* domain sequence in *B. licheniformis* (Table 2). *ChiA* domain sequence can be found in some strains of *Bacillus* sp. i.e *B.*

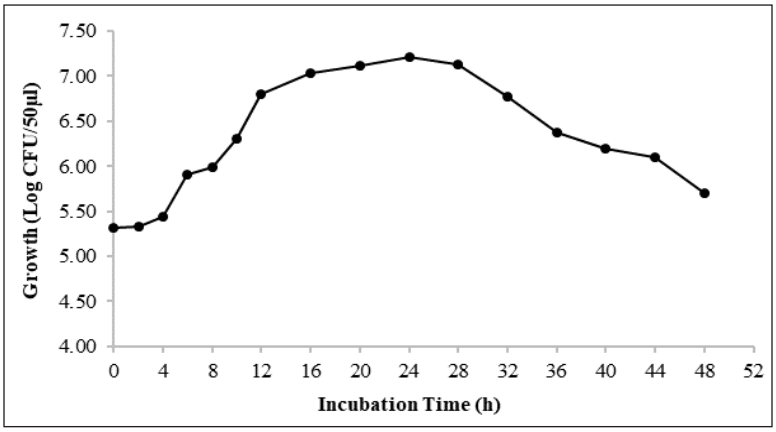

Figure 2. The growth of *Bacillus licheniformis* D11in thermus colloidal chitin broth medium pH 7.0 at 52ºC for 48 hours

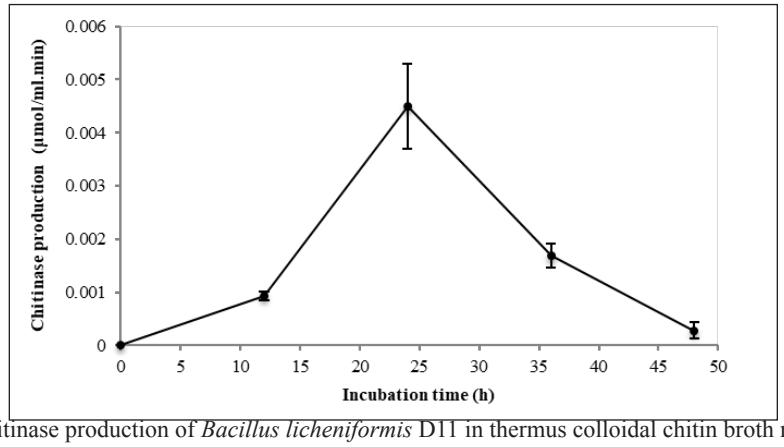

Figure 3. Chitinase production of *Bacillus licheniformis* D11 in thermus colloidal chitin broth medium (pH 7.0) at 52ºC

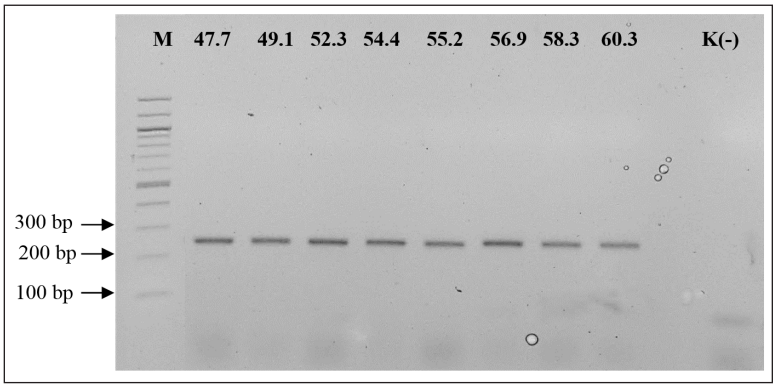

Figure 4. Visualisation of PCR product using *ChiA* primer in variation of 47.7-60.3°C annealing temperature on 2% agarose gel electrophoresis. M= marker 100 bp, 47.7-60.3= annealing temperature in °C, K(-)= negative control (without DNA template).

licheniformis, *B. cereus*, *B. thuringiensis*, and *B. pumilus*. In bacteria, the function of this gene is to degrade insoluble chitin into its derivates and plays an important role in the defence mechanism against pathogens (Funkhouser & Aronson, 2007). *ChiA* domain sequence consists of catalytic domain (GH18), fibronectin domain III (Fn3), and chitin binding domain (CBD) (Herdyastuti, Tri, Mudasir, & Sabirin, 2009; Islam et al., 2010). Amplification using *ChiB* primer by gradient thermocycler in variation of annealing temperature (T_a 53-66°C) produced one amplicon sized ~1000 bp (Figure 5) which was sequenced and analysed. Based on sequence alignment (BLASTn) result, this sequence had high levels of similarities with *ChiA* and *ChiC* domain sequence in *B. licheniformis* (*B. licheniformis* strain HRBL-15TDI7, *B. licheniformis* WX-02, dan *B. licheniformis* chiB gene strain F11) (Table 3). This result confirmed *ChiB* primer can detect the presence of *ChiA* and *ChiC* domain sequence in *B. licheniformis* D11 due to high level of similarity between the domains.

ChiA, *ChiB*, and *ChiC* belong to the group GH18. From the amino acid sequence, *ChiC* has different amino acid sequence compared with *ChiA* and *ChiB*. *ChiB* has a lower specific activity than *ChiA* because of the absence of fibronectin domain III. In addition, *ChiB* cuts GlcNAc oligomers shorter than *ChiA* (Brurberg, Nesl, & Eijsink, 1996). *ChiB* can be found in *Aspergillus fumigatus*, *Photorhabdus themperata*, and some strains of *B. licheniformis*. ChiC has three functional domains, namely N-terminal domain, fibronectin domain III, and catalytic domain. N-terminal domain in

Table 2
Sequence alignment result of ChiA amplicon using BLAST-n NCBI

Subject description	Query cover	Ident	Protein name	Domain
B. licheniformis strain LHH 100 chitinase (*ChiA*-65) gene, complete cds	76%	70%	ChiA-65	*ChiA*
B. licheniformis strain HRBL-15TDI7, complete genome	79%	69%	Chitinase A	*ChiA*
B. licheniformis WX-02 genome	79%	69%	GH18	*ChiA*
B. licheniformis strain UTM104 chitinase gene, partial cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain KNUC 213 chitinase, partial cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain DSM13 chitinase gene, partial cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain N1 chitinase gene, complete cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain CBFOS-03 chitinase (chi 18B), complete cds	76%	69%	Glycosyl Hydrolase	*ChiA*
B. licheniformis strain DSM 8785 chitinase (chiA) gene, partial cds	76%	69%	Chitinase A	*ChiA*
B. licheniformis strain A1 chitinase B gene, complete cds	76%	69%	Chitinase B	*ChiA*
B. licheniformis ATCC 14580, complete genome	79%	69%	GH18/Chitinase A	*ChiA*

Table 3
Sequence alignment result of ChiB amplicon using BLAST-n NCBI

Subject description	Query cover	Ident	Protein name	Domain
B. licheniformis strain HRBL-15TDI7, complete genome cds	100%	99%	Chi C, GH18, Chi A	*ChiC, ChiA*
B. licheniformis WX-02 genome	100%	99%	Chi C, GH18, Chi A	*ChiC, ChiA*
B. licheniformis chiB gene, chiA gene, mpr gene and ycdF gene, strain F11	100%	99%	Chi C (*binding domain*), Precursor ChiB, Putative Dehidrogenase	*ChiA, ChiC*
B. licheniformis ATCC 14580, complete genome	100%	99%	Chi C, GH18, Chi A	*ChiC, ChiA*
B. licheniformis strain SK-1 chitinase precursor (chiB) and putative chitinase precursor	100%	99%	Putative Chitinase	*ChiA*
B. licheniformis DSM13 = ATCC 14580, complete genome	100%	99%	Chi C, GH18, Chi A	*ChiC, ChiA*
B. licheniformis chiB gene, chiA gene, mpr gene and ycdF, strain F5	100%	99%	Putative Chitinase Precursor ChiB	*ChiB*
B. paralicheniformis strain BL-09, complete genome	100%	99%	Glycosyl Hydrolase	*ChiA*
B. paralicheniformis strain ATCC 9945a, complete genome	100%	94%	Putative Chitinase Precursor	*ChiA*
B. licheniformis strain MS-3 chitinase A-BL3 (chiA) gene, complete cds	100%	94%	Chitinase A-BL3	*ChiA*
B. licheniformis gh18D gene for glycoside hydrolase, complete cds	100%	94%	Glycosyl Hydrolase	*ChiA*
Bacillus sp. AV2-9 chitinase large (chiL) gene, complete cds	99%	82%	Chitinase L	*ChiA*

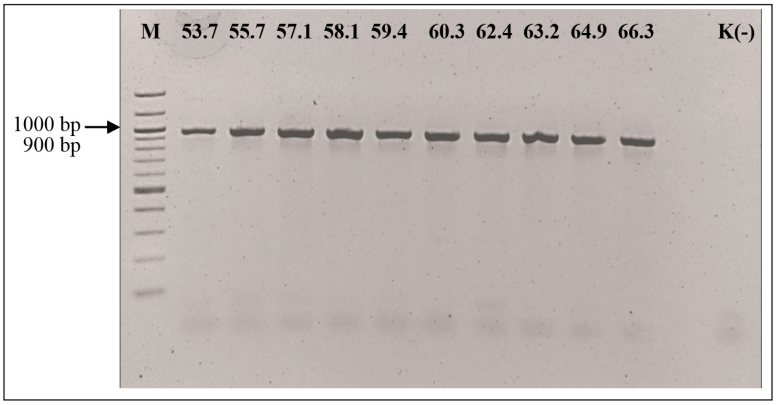

Figure 5. Visualisation of PCR product using *ChiB* primer in variation of 53.7-66.3°C annealing temperature on 1.5% agarose gel electrophoresis. M= marker 100 bp, 53.7-66.3= annealing temperature in °C, K(-)= negative control (without DNA template).

ChiC is similar to the C-terminal extension of *ChiA* (Tsujibo et al., 1998). Chitinase gene with *ChiC* domain can be found in *Streptomyces lividans*, *Paenibacillus* spp., *Pseudomonas* sp., *Serratia marcescens* and *Bacillus weihenstephanensis*.

CONCLUSION

A total of 19 chitinolytic thermophilic bacteria were collected from Cangar hot spring, East Java, Indonesia. From the screening process, D11 isolate had the highest chitinolytic activity. The D11 isolate was identified as *Bacillus licheniformis* through molecular, morphological and physiological analyses. This isolate produced large amounts of chitinase ($4.49x10^{-3}$µmol/ml. minutes) on 0.9% (w/v) colloidal chitin (pH 7.0) at 52 °C in a very short time, 24 hours compared with other *Bacillus sp.* The sequence analysis showed that the isolated *Bacillus licheniformis* was proven to have genes encoding *ChiA* and *ChiC* domain. This isolate can be used for further application on chitinous waste degradation or chitin derivates production in pharmaceutical industries.

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Enzymatic Dehairing of Goat Skin Using Keratinase from *Bacillus sp*. MD24, A Newly Isolated Soil Bacterium

Suharti Suharti*, Maurilla Trisna Riesmi, Arina Hidayati, Umi Faridatuz Zuhriyah, Surjani Wonorahardjo and Evi Susanti

Department of Chemistry, Faculty of Mathematics and Sciences, State University of Malang, East Java, Indonesia

ABSTRACT

A newly keratin-degrading bacterium was isolated from a residential soil at Wajak district, Malang, East Java, Indonesia. Sequence homology analysis showed the 16S rRNA had only 89% sequence identity to the available bacterial 16S rRNA which led to a discovery of a new species of *Bacillus*. The bacterium was named *Bacillus sp* MD24. The isolated bacterium degraded 71% mass of whole chicken feathers within 10 days. Keratinase fermentation using 1% of chicken feathers as sole source of carbon and nitrogen exhibited highest enzyme activity at third day under optimum condition (pH of 8 and temperature of 37^{o}C). At enzyme concentration of 0.3 U/mL, the crude extract keratinase started to exhibit dehairing activity on goat skin after an overnight incubation; the best incubation was achieved at 72 hours. Surface of enzymatically dehaired goat skin was compared to chemically dehaired skin goat. The result showed similar or improved surface of the skin which makes the crude keratinase from *Bacillus sp* MD24 a potential candidate for application in leather industry to avoid pollution problems due to the use of chemicals.

Keywords: *Bacillus*, dehairing, keratinase, dehaired goat skin, keratin-degrading bacterium

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E-mail addresses:
suharti.fmipa@um.ac.id (Suharti Suharti)
maurillatr@gmail.com (Maurilla Trisna Riesmi)
arinahidayati471@yahoo.com (Arina Hidayati)
zuhriyah.kim13@gmail.com (Umi Faridatuz Zuhriyah)
surjani.wonorahardjo@um.ac.id (Surjani Wonorahardjo)
evi.susanti.fmipa@um.ac.id (Evi Susanti)
* Corresponding author

INTRODUCTION

Leather industry contributes greatly to the Indonesian economy but there has been an increasing environmental concern regarding various pollutants as by-products of this industry (Kolomaznik, Adamek, Andel, & Uhlirova, 2008; Lofrano, Meriç, Zengin, & Orhon, 2013; Syed et al., 2010; Zhang, 2007). Biotechnological processes such as enzymatic process offer cleaner technologies

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which minimise production of hazardous waste. Dehairing is an important step during leather processing. Hair is composed primarily of strong fibrous proteins named as keratin (McKittrick et al., 2012) which contains of large amount of cysteine residues. The thiol groups form cysteine residues form strong covalent disulphide bonds that cross links the polypeptide chains together. Sodium sulphide was conventionally applied for hair removal during dehairing process to break the disulphide bonds. Although this process is very effective, it generates hydrogen sulphide gas, obnoxious odour and toxic gas. Single inhalation exposure to hydrogen sulphide in experimental animals can result respiratory, immunological/lymphoreticular, cardiovascular, neurological effects and even death (Selene & Chou, 2003). Short term inhalation studies of hydrogen sulphide showed ocular, neurological, cardiovascular, metabolic, reproductive, and developmental effects at lowest concentration tested at 28 mg/m^3.

Microbial keratinases have been reported to be promising enzymes for application in dehairing process. Various extracellular keratinase showed capability to degrade keratin, such as keratinase produced by *Bacillus subtillis* (Andre & Macedo, 2014), *Bacillus halodurans* strain PPKS-2 (Prakash & Jayalakshmi, 2010), *Brevibacillus brevis* US575 (Jaouadi et al., 2015) and *Bacillus licheniformis* ER-15 (Tiwary & Gupta, 2010). Keratinase application in dehairing step accomplishes many advantages especially to overcome environmental pollution and leather quality (Ismail, Housseiny, Abo-Elmagd, El-Sayed, & Habib, 2012) Biochemical and biophysical properties of keratinases vary greatly among microorganisms isolated from different environment. Hence, isolation of a new microbe might be directly correlated to finding a novel keratinase.

A new *Bacillus* sp. was isolated from decomposed chicken feather containing soil. The isolate produces keratinase and the enzyme showed activity in dehairing of goat skin. This finding adds to the library of keratinase producing microbial collection for sources of keratinase, a potential replacement agent of the harmful chemicals commonly applied in leather dehairing, as eco-friendly and economically viable alternatives.

MATERIALS AND METHODS

Isolation and Characterisation of Keratinase Producing Bacterial Strain

Chicken feather was decomposed by mixing it with moistened soil and incubated for approximately 1 month. The mixture was mosturized daily with ±10% (v/w) of water. Microbial strains were isolated from decomposed chicken feather containing soil. A soil suspension was prepared by dissolving around 1 g decomposed chicken feather in 100 mL of 0.85% NaCl and serial dilution of 1000-fold was created from the suspension. A series of 100 µL diluted suspension was plated on a skim-milk medium (0.5% NaCl; 0.1% MgSO4; skim milk 5.0%, and 1.5% agar with a pH of 7.5) and incubated at 37^0C overnight.

Colonies with clear zone were picked up and tested for their ability to degrade keratin by placing it on keratin medium (0, 5% keratin powder; 0.03% K_2HPO_4; 0.04% KH_2PO_4; 0,05% NaCl; 0,01 % $MgCl_2.6H_2O$; and 1,5% agar). A colony called MD24 with the highest proteolytic index was selected and maintained for further analysis. Strain identification was done by homology study of 16S rRNA gene sequence. Amplification and sequencing of 16S rRNA gene was performed based on Macrogen (Seoul, Korea) using internal primer 785F ($^{5'}$GGATTAGATACCCTGGTA$^{3'}$). Resulting sequence was used as query sequence to find similar 16S rRNA genes at data base using BLASTN program provided by National Centre for Biotechnology Information homepage. Ten 16S rRNA gene sequences with the highest identity were selected and aligned with the 16S rRNA from the isolate bacterium. Sequence alignment was performed using Clustal X2.1 and phylogenetic tree was constructed using MEGA 6 with the nearest neighbour-joining method.

Chicken Feather Degradation

Chicken feathers are potential carbon and nitrogen sources for keratinase production. The selected isolate was tested for its capability to degrade whole chicken feathers. The isolate was grown in a 100 mL medium containing 5.0 % NaCl; 1.0 % $MgSO_4$; 0.5 % K_2HPO_4; and ± 0,1 g chicken feathers for 10 days at 37^{o}C. Chicken feather degradation was followed by weight loss of feathers and increasing tyrosine concentration.

Optimisation of Keratinase Production

Optimisation of keratin production was done based on carbon source, pH medium, and temperature incubation. Keratin production was examined using 1% chicken feathers as a sole carbon and nitrogen source and combination of 1% chicken feathers with 1% of additional carbon source (glucose, sucrose or glycerol). The physical condition, pH (in the range of 6-9) and temperature (in the range of 35-41°C), were optimised for maximum yield of keratin production.

Keratinase Activity Measurement

Keratinase activity was measured spectroscopically by measuring tyrosine released through enzymatic hydrolysis. A reaction mixture containing 1 mL of 1% keratin and 1 mL of Tris-HCl buffer pH 8 and 1 mL of crude extract keratinase was incubated at 37°C for 30 minutes. The enzymatic reaction was stopped by adding 1 mL of 10% trichloroacetic acid (TCA). Subsequently, the reaction mixture was incubated on ice for 20 minutes. The mixture was then centrifuged at 5000 rpm for 5 minutes and the absorbance of supernatant was measured at 280 nm. Catalytic site of keratinase was tested through inhibition test using phenazine methosulphate (PMSF) at 0.5 and 1 mM and Ethylenediaminetetraacetic acid (EDTA) at 0.5 and 1 mM. Dependency of keratinase on metal ions were tested using 0.5 and 1 mM divalent cations (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, an Ni^{2+}). Optimum temperature and optimum pH were determined under the best divalent ion activity induction. In order to know the

increase of absorbance due to non-enzymatic activities, control reaction was performed using same chemical composition but the enzyme was added after the addition of TCA.

Dehairing of Goat Skin

Sundried goat skin was prepared without adding any chemicals. It was cut into 1 × 1 cm pieces and soaked in a 20 mL of 0.3 U/ml crude extract keratinase at optimum keratinase activity condition (37^{o}C, pH 8, in 1 mM $CaCl_2$) overnight (16 h), 48 h, and 72 h. Skin was then gently scraped to remove hairs. Chemical dehairing was done using a 20 mL solution containing 2% technical grade sodium sulphide (SN) and 2% technical grade lime (this method is used at local tannery industry at Mageta district, East Java, Indonesia). Sodium sulphide and lime were collected from local tanning industry. Chemical dehairing was done for 24 hours. Skin was also gently scraped to remove hairs and washed with demineralised water. Dehaired skin surfaces were examined using Scanned Electron Microscopy (SEM).

RESULTS AND DISCUSSION

Screening of Keratinase Producing Bacterial Strain

A total of 22 colonies exhibited production of extracellular proteases after 24 h incubation. The ratio of clear zone and colony diameters served as a proteolytic index for selection of a strain with highest protease production ability. A selected colony called MD24 with the ratio of 4.7 was subsequently transferred to keratin containing medium (Figure 1A). Figure 1B shows the MD24 isolate after 16 h incubation on keratin containing medium.

Morphological characterisation of MD24 isolate indicated that the isolate was a *rod*-shaped gram-positive *Bacillus* (Figure 2). Sequence alignment of 16S rRNA gene against available bacterial 16S rRNA gene

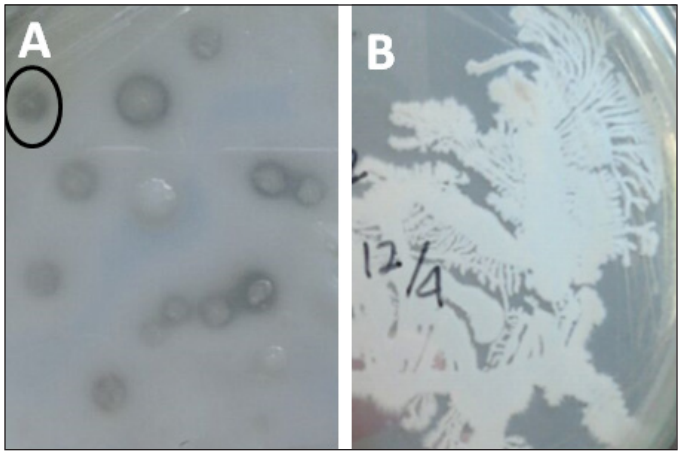

Figure 1. Screening keratinase bacterial strain. A. Selected strain from skim milk medium. B. Selected bacterial strain grown on keratin containing medium.

at the database exhibited 89% maximum of sequence identity. "A prokaryotic species is considered to be a group of strains that are characterized by a certain degree of phenotypic consistency, showing 70% of DNA–DNA binding and over 97% of 16S ribosomal RNA (rRNA) gene-sequence identity" (Gevers et al., 2005; Stackedbrandt & Goebel, 1994). Therefore, it can be concluded that MD24 isolate is a new isolated *Bacillus* strain and named as *Bacillus* sp MD24. Ten of 16S rRNA gene sequences with 89% identity were aligned using Clustal X2 and a phylogenetic tree was constructed using MEGA6. Figure 3 shows a phylogenetic tree of the 10 selected strain and *Bacillus* sp. MD24. Horizontal dimension represents the genetic change. The tree exhibits one common ancestor that broken into two branches. Ten strains fall into one branch and *Bacillus* sp falls into different branch. Therefore, *Bacillus sp* MD24 might develop a different metabolic pathway compared with another branch.

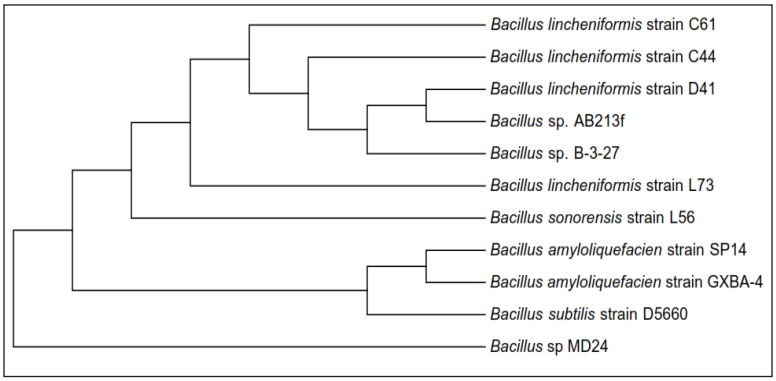

Figure 3. Neighbour-joining phylogenetic tree of *Bacillus* sp. MD24 with closest *Bacillus* strains based on 16S rRNA partial gene sequences

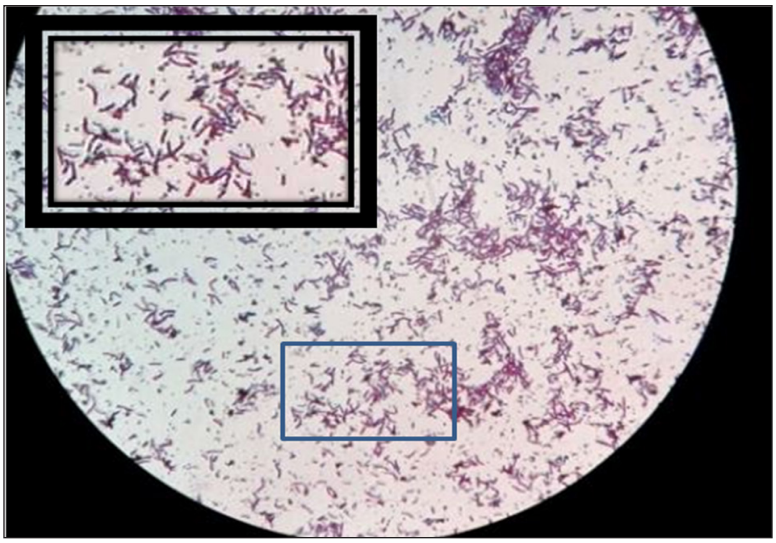

Figure 2. Morphology of *Bacillus* sp MD24. Insert is magnification of the figure signed by a rectangle

Chicken Feather Degradation by *Bacillus sp. MD24*

The poultry industry all over the world resulted in the generation of millions of tonnes of chicken feather waste (Prakash & Jayalakshmi, 2010). Chicken feathers contain 90% or more keratin (Tork, Shahein, El-Hakim, Abdel-Aty, & Aly, 2013) that act as keratinase inducer and cheaper carbon and nitrogen sources for keratinase production (Gurav & Jadhav, 2013). Chicken feathers act as keratinase inducer and it can be applied as medium for keratinase fermentation. Degradation of chicken feathers by *Bacillus* sp. MD24 was examined. Fermentation was done for 10 days using chicken feathers as sole carbon and nitrogen sources. Chicken feather degradation was followed by its weight loss and production of soluble tyrosine. Table 1 shows chicken feather degradation using 1% initial weight of chicken feathers. About 71% of chicken feathers were degraded within 10 days of incubation. Decreasing the weight of chicken feathers was consistent with increasing tyrosine concentration which indicated the enzymatic hydrolysis of keratin into smaller molecule e.g. polypeptides and amino acids. Keratinolytic proteases has been reported from many *Bacillus* strains (Abdel-Naby, El-Araby, & El-Refai, 2015; Macedo et al., 2005; Prakash & Jayalakshmi, 2010). However, *Bacillus* strains are unique and keratinase activity of each strain is unique.

Table 1
Chicken feather degradation by Bacillus sp. MD24

Incubation time (days)	*%Weight loss*	Tyrosine concentration (µmol/mL)	Left over chicken feather
2	37.87%	0.06	
4	45.04%	0.61	
6	48.73%	0.81	

Table 1 *(continue)*

Incubation time (days)	*%Weight loss*	Tyrosine concentration (µmol/mL)	Left over chicken feather
8	63.41%	0.89	
10	71.01%	1.41	

Finding a new keratinase from a new isolate might provide better enzyme for specific applications.

Enzyme Production

Optimum enzyme production was observed at pH 8, 37°C, and 72 h. Although chicken feathers can be degraded up to 71 % within 10 days, optimum enzyme activity was obtained on the third day and decreasing in the following days (Figure 4). In addition of chicken feathers, carbon sources (glucose, sucrose, and glycerol) were examined. Unlike previous finding that found the positive influence of additional carbon sources toward keratinase production (Cavello, Chesini, Hours, & Cavalitto, 2013; Ramnani & Gupta, 2004), *Bacillus* sp. MD24 faithfully using keratin for the best keratinase production when glucose, sucrose or glycerol are present. When cells were grown under keratin as a sole carbon and nitrogen sources, it will produce keratinase to degrade keratin into amino acids and use it for energy as well as building cell chemical. However, although simple carbohydrates might increase cell growth, when keratin is also available in the environment, the cells may prefer to use simple carbohydrates instead of keratin for energy. This will reduce the production of keratinase. In this study, a combination of chicken feathers and complex carbohydrate are used to improve keratinase production. While simple carbohydrates reduce keratinase, complex carbohydrate might increase keratinase production due to a slow-release of simple carbohydrate enzymatically. Cell would balance keratinase and carbohydrate degrading enzyme to provide amino acids and simple carbohydrate for growth.

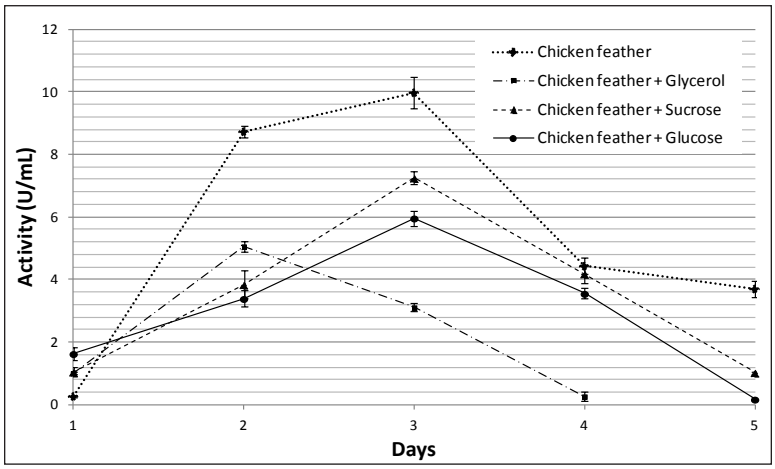

Figure 4. Keratinase production with chicken feathers as sole carbon and nitrogen source and the influences of addition carbon sources on enzyme production

Effect of Inhibitors on Enzyme Activity

The nature of protease was studied using specific class of protease inhibitors, PMSF and EDTA. As shown in Table 2, both PMSF and EDTA inhibit keratinase activity. The results indicated that keratinase from *Bacillus* sp. MD24 is a serine type protease which also depends on the presence of metal. Serine keratinase are reported in several bacteria (George, Chauhan, Kumar, Puri, & Gupta, 2014; Jaouadi et al., 2013; Tiwary & Gupta, 2010).

Table 2.
The effect of PMSF and EDTA on keratinase activity

	Percent Activity	
	0.5 mM	1 mM
Crude extract	100	100
Crude extract + PMSF	32.14	14.29
Crude extract +EDTA	25.00	10.71

The Effect of pH and Temperature on Keratinase Activity

The effect of pH was examined in the range of 7 to 9. The enzymes are active at the pH range of 7-9, but optimum activity was achieved at pH 8, and increasing pH at 9, the activity only remains about 50% of its activity at pH 8 (Figure 5A). Temperature optimum of the enzyme was obtained at 37^{o}C (Figure 5B). At temperature of 25^{o}C, almost no activity was observed for 30 min incubation time.

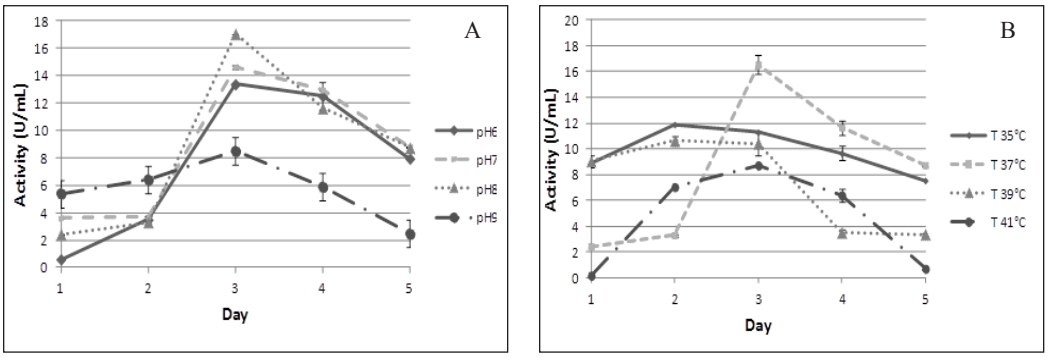

Figure 5. Effect of pH (A) and temperature (B) on keratinase activity

The Effect of Divalent Cations on Enzyme Activity

Table 3 shows the effect of several divalent cations. Addition 1 mM of Ca^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} cations increased enzyme activity by factor of 4.21, 4.21, 3.18, and 2.64 respectively, while Mg^{2+} reduced the activity by a factor of 0.5. Calcium ion has been reported to induce keratinase activity (Kim, 2005; Poopathi, Thirugnanasambantham, Mani, Lakshmi, & Ragul, 2014) Co^{2+}, Mn^{2+}, and Ni^{2+} inhibit keratinase from *Bacillus subtilis* (George et al., 2014; Tork et al., 2013).

Table 3
The effect of divalent cations on keratinase activity

Cation ions	Activity	
	0.5 mM	1 mM
Crude extract	1.00	1.00
Crude extract + Ca^{2+}	2.07	4.21
Crude extract + Co^{2+}	0.89	4.10
Crude extract + Mg^{2+}	0.64	0.50
Crude extract + Mn^{2+}	1.75	3.18
Crude extract + Ni^{2+}	2.00	2.64

Dehairing of Goat Skin

Goat skin hair was removed enzymatically and chemically. Figure 6 shows dehaired skins in 3 consecutive observation days. After an overnight of incubation, the hairs began to fall out. The hairs were easily removed after 72 hours of incubation. Keratinase removes hair skins as good as removal by Na_2S and lime. For 1 × $1cm^3$ of goat skins, hair removal using 20 ml of 2% of Na_2S and 2% lime for overnight can be replaced by enzymatic method using 20 ml of 0.3 U/ml keratinase for 72 h. The incubation time might be shorter by using higher enzyme activity. However, although the enzyme was produced using only carbon and nitrogen, collagenase activity must be measured in the keratinase crude extract.

Increasing keratinase concentration should be done thoroughly to find the best enzyme concentration without damaging collagen structure. Surface quality assessment of dehaired goat skins using SEM showed that hairs were removed effectively using both methods (Figure 7). However, although dehaired skins were washed several times with water, chemical precipitations were observed on chemically dehaired skin surfaces. Enzymatic dehaired skin showed cleaner surfaces. Effective dehairing of goat skin using keratinase has been reported by (George et al., 2014; Paul, Das, Mandal, Jana, & Maity, 2014). This study showed skin surface became more compact and fibre bundle are irregular after enzyme treatment. Keratinase from *Bacillus* sp. MD24 leaves smoother and regular fibre bundle traces.

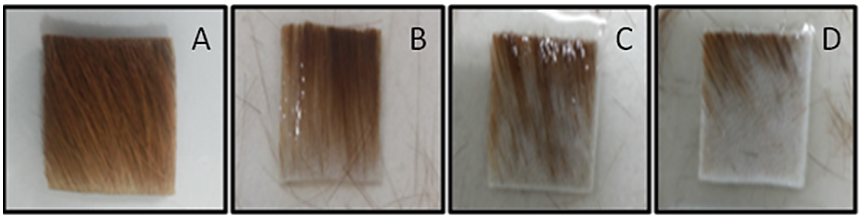

Figure 6. Enzymatic dehaired goat skins. The skin of about 1 cm^2 was soaked in a 20 mL of 0.3 U crude extract keratinase. A. Unhaired goat skin. B. Goat skin after overnight incubation. C. Goat skin after 48 h incubation. D. Goat skin after 48 h incubation.

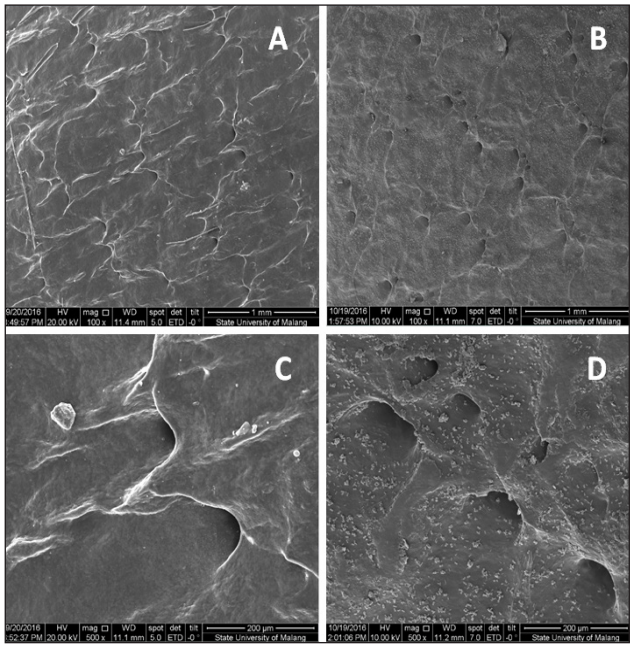

Figure 7. SEM images of surface dehaired skin. A. Enzymatically dehaired skin under 50× magnification. B. Chemically dehaired skin 50× magnification. C. Enzymatically dehaired skin 500× magnification. D. Chemically dehaired skin 500× magnification.

CONCLUSION

Keratinolytic bacterium was isolated from feather contaminated soil. The isolate was identified as a new strain bacterium named *Bacillus* sp. MD24. The keratinase can be produced under chicken feathers as sole nitrogen and carbon sources and the production is repressed by addition of glucose, sucrose, and glycerol. Under experimental condition, maximum enzyme production was obtained at pH 8, temperature of 37°C for incubation time of 3 days. The enzyme activity was also observed at the same pH and temperature conditions. The enzyme is a serine type protease induced by of Ca^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} cations and inhibits by Mg^{2+}. Finally, enzyme application on dehairing of goat skin showed a better quality surface compared with treatment using chemical method. However, further works need to be done such as optimising enzyme and ratio, increasing enzyme yield and scale up fermentation to obtain sufficient enzyme for industrial applications.

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Application of Vetiver (*Vetiveria zizanioides*) on Phytoremediation of Carwash Wastewater

Jovita Tri Astuti, Lies Sriwuryandari and Tarzan Sembiring*

Research Unit for Clean Technology, Indonesian Institute of Science, Jalan Cisitu-Sangkuriang, Bandung 40135, Indonesia

ABSTRACT

Carwash wastewater (CW) contains potentially harmful compounds. Due to water scarcity, its reuse is indispensable. Phytoremediation is one of methods to eliminate pollutants by using plants, such as vetiver grass. This study observed the growth characteristic of vetiver in CW and its capacity in pollutant removal. Treatment with two factors and three replicates was done: Media for vetiver growth was tap water (TW) without carwash wastewater (M_0), mixture of 50% tap water + 50% carwash wastewater (M_1), and 100% of carwash wastewater without tap water (M_2). Media grown without and with vetiver was of V_0 and V_1 respectively. Individual stem of vetiver was planted hydroponically in a chamber. Plant growth was observed once a week. At the harvesting (day 70), root, stem and leaf of the vetiver were collected separately Results showed that vetiver has a capacity to adapt, survive and growing in CW media. Plant generation achieved 70.1-81.8%, 60.6-75.8%, and 71.7-78.5% for stem, leaf and root respectively. Within 70 days, the pollutant that covered of 78.5 and 57.9% N, 83.5 and 69.0% P, 76.0 and 65.3% COD, 68.6 and 64.8% BOD, 81.3 and 59.5% detergent, 98.6 and 95.8% phenol, 73.3 and 61.5% Pb, and 88.5 and 82.8% Zn could be removed by vetiver, i.e. in growth media M_1 and M_2, respectively.

Keywords: Carwash wastewater, phytoremediation, pollutant removal, vetiver grass

INTRODUCTION

The use of industrial wastewater in agriculture is common practice in order to provide a reliable source of water for irrigation and to add valuable nutrients and organic matters to soil. In developed countries, where environmental standards are applied, wastewater is treated firstly before using for irrigation. On the other

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E-mail addresses:
jtri001@lipi.go.id (Jovita Tri Astuti)
lies.sriwuryandari56@gmail.com (Lies Sriwuryandari)
sembiring_t@yahoo.com (Tarzan Sembiring)
* Corresponding author

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hand, in some developing countries, such as Indonesia, untreated wastewater is widely used for irrigation and watering of agriculture lands This is one of the most significant sources of environmental pollution that directly affect human health via crops and soil. Untreated wastewater either industrial wastewater or domestic wastewater contains toxic compounds. Degradation of water quality can pose serious threats due to high population growth and rapid urbanisation. In addition, the urban areas commonly lack wastewater and solid waste treatment facilities (Bomh et al., 2011; Sharma, Agrawala, & Marshall, 2007).

Bandung city, a capital of West Java Province of Indonesia is an urban area with high population growth and rapid urbanisation. Its population density increased from 14,491 to 14,687 p.km^{-2} of which the highest is West Java province. Pollution is with its economic growth which is showed by the vehicle belonging number. The vehicle density increased from 5,323 to 6,618 units.km^{-2}. During 2011 to 2014, the increase of vehicles in Bandung city was much higher than its population as of was 22.93 and 1.3% respectively. The increase in vehicle numbers is followed by the increase in carwash unit establishment. Wastewater is generated directly from carwash unit services directly.

Approximately, 40 L unit^{-1} of water is required for carwash unit service (Zaneti, Etchepare, & Rubio, 2012) whereas in Europe 60-70L unit^{-1} (Boussu, Kindts, Vandecasteele, & Bruggen, 2007). Carwash unit involves the use of detergents, petrol, kerosene and diesel for cleaning. Therefore, carwash wastewater contains potentially harmful compounds and serious damage might result to environment. The discharging of carwash wastewater (CW) directly to watercourses would lead to environmental degradation (Boussu et al., 2007; Zaneti et al., 2012). High level of lead should be considered especially in urban areas with high level of traffic (Paz-Alberto, Sigua, Baui, & Jacqueline, 2007).

Treatment and reuse of wastewater was important due to the increasing of water scarcity (Finley, Barrington, & Lyew, 2009; Jhamaria & Yadav, 2014). By a careful management, the positive aspects of wastewater for irrigation can be achieved (World Health Organization [WHO], 2006). Phytoremediation is an alternative method that uses plants to clean up a contaminated area. Plant species for remediation must be adapted and tolerant to a high concentration of metals. A different plant species shows a different capacity to uptake nutrient and remove the contaminant substances. The selection of appropriate plant as phytoremediator for contaminated area is important Vetiver grass *(Vetiveria zizanioides* L) is an ideal plant species for pollutant removal (Truong & Director, 2006).

The previous study investigated vetiver that can grow in diesel contaminated soil (Nisa & Rashid, 2015). Vetiver system is mainly recommended for wastewater treatment in hydroponic system (Akbarzadeh, Jamshidi, & Vakhshouri, 2015). The use

of vetiver grass in phytoremediation may be cost effective and could be environmentally friendly. Nevertheless, the practical phytoremediation tool for carwash wastewater treatment has not well understood yet. This study was carried out to observe the growth characteristic of vetiver in carwash wastewater and its capacity to remove the existing of pollutant substances.

MATERIALS AND METHODS

Date and Place of Experiment

The study was conducted for 70 days in the field laboratory of Indonesian Institute of Science in Bandung, Indonesia. The position of the place is at 847m ASL (Above Sea Level) with latitude of 06°52'57.5" SL, and longitude of 107°36'39.8" EL. The ambient temperature (T) was recorded in the range of 21-30°C, Relative Humidity (Rh) was 78-87%, and the length of daylight was 38-57%.

Carwash Wastewater (CW), Plant Chamber, and Vetiver Grass used in Study

Wastewater was obtained from outlet pipe of the carwash service unit in Bandung city. It was collected in a 20L-plastic container. Carwash wastewater used during the experiment was from the same carwash service and taken only once. Carwash wastewater parameter specifically are BOD, COD, detergent, phenols (aromatics), phosphates, nitrate, nitrite, ammonia, lead and other minerals, suspended solid and dissolved solid. Prior to use for experiment, CW was filtered using 60-mesh sieve to remove the existence of plastics, papers, rubbers, cigarette etc. The filtered CW was then stocked in plastic container. Glass aquarium of 24 cm length, 20 cm width, and 25 cm height was used for vetiver grass chamber. One side of the aquarium wall was scaled and marked which showed the relation between the heights of liquid surface with volume of media. Each aquarium was completed with aerator (SPA 26). Vetiver grass was placed in media carwash wastewater diluted with tap water at ratio 3:1 (v/v) or equivalent with 25% concentration of the native carwash wastewater. Individual stems of vetiver were separated from its bundle. Individual stems of vetiver with 4-8 pieces of leaf were selected for experiment. Figure 1 shows the stem bundles, individual stems, and hydroponic cultivation of vetiver.

Experiment

In this study, concentration of carwash waste water in growth media (M) and vetiver grass planted in growth media (V) varied. The growth media was without vetiver (V_0) as control. The concentration of carwash wastewater for growth media was 0% of which tap water without carwash wastewater (M_0), mixture of 50% tap water + 50% carwash wastewater (M_1), and 100% of carwash wastewater without tap water (M_2), and with vetiver grass (V_1) respectively. The treatment was carried out with three replications

Aquarium for each treatment was filled with 10 L of growth media (±80% of capacity) and was pumped into the plant

(a) (b) (c)

Figure 1. (a) Stem bundles; (b) individual stems; and (c) hydroponic cultivation of vetiver in media CW

chambers under continuous mixing. From six units of glass aquarium of medium M_0; M_1; and M_2 was then divided into two groups, three units for planted with vetiver grass (V_1) and three units without planted with vetiver (V_0) as a control. Each aquarium consisted of two baskets (B). For treatment V_1, growth media was planted hydroponically with eight individual stems of vetiver grass. Therefore, each aquarium consists of 16 individual stems and in total 48 pieces of individual stems for each treatment. Table 1 presents composition of tap water and carwash wastewater used in study.

Table 1
Composition of tap water (TW) and carwash wastewater (CW) in this study

Parameter	Unit	TW	CW
pH	-	7.29	7.14
BOD_5	mg. L^{-1}	15.9	398
COD	mg. L^{-1}	28.22	812
Total N	mg. L^{-1}	0.08	16.11
Nitrite ($N\text{-}NO_2$)	mg. L^{-1}	0.00	1.27
Nitrate ($N\text{-}NO_3$)	mg. L^{-1}	0.08	3.76
Ammonia ($N\text{-}NH_3$)	mg. L^{-1}	0.00	11.08
Phosphate (PO_4)	mg. L^{-1}	0.24	12.10
Detergent	mg. L^{-1}	1.98	10.29
Phenol	mg. L^{-1}	0.00	0.12
Lead (Pb)	mg. L^{-1}	0.02	0.13

Growth Characteristic

Growth characteristic of vetiver is indicated by its vegetative reproduction, i.e. stem, leaf and root. Three individual stems from each basket were selected randomly as sample for observation (Table 2); they were observed once a week. The number of leaves was calculated and the leaf length was measured using a ruler.

Stem number consisted of initial and new stem generation. At day 70, the whole roots, stems, and leaves were harvested and collected separately. All vetiver roots were cut and sprayed with water, drained, and their root length measured. The root-shoot ratio was calculated by dividing the dry weight of shoot by the dry weight of roots. Dry matter of root and leaf was determined gravimetrically oven-dried at 105°C until; constant weight was achieved.

Table 2
Growth Media, Replication, Baskets, and Individual Stems Planted in Study

Growth media (M)	Replication/ Aquarium (A)	Basket (B)	Code of individual stems of vetiver plant in study*							
M_0	A_1	B_1	**1**	2	3	**4**	5	6	7	**8**
		B_2	**9**	10	11	12	13	14	**15**	**16**
	A_2	B_1	**17**	18	**19**	20	21	22	**23**	24
		B_2	25	**26**	27	28	29	**30**	**31**	32
	A_3	B_1	33	34	**35**	**36**	37	38	39	**40**
		B_2	41	**42**	43	44	**45**	**46**	47	48
M_1	A_1	B_1	1	**2**	3	**4**	5	**6**	7	8
		B_2	**9**	10	11	12	**13**	14	15	**16**
	A_2	B_1	17	18	**19**	20	21	**22**	**23**	24
		B_2	25	26	**27**	**28**	29	30	31	**32**
	A_3	B_1	33	34	**35**	36	37	38	**39**	**40**
		B_2	41	**42**	43	44	**45**	46	**47**	48
M_2	A_1	B_1	**1**	2	**3**	4	5	6	**7**	8
		B_2	**9**	**10**	11	**12**	13	14	15	16
	A_2	B_1	17	**18**	19	**20**	21	22	**23**	24
		B_2	**25**	26	27	28	29	**30**	31	**32**
	A_3	B_1	33	34	**35**	**36**	37	**38**	39	40
		B_2	41	**42**	**43**	44	45	**46**	47	48

Note: Bold and shade of individual stems are randomised selected as samples for observation

Tensile Strength of Vetiver Root

For tensile strength test, as much as 60 of vetiver root of each growth media with L ≥ 50cm were used as specimen. The roots were carefully checked for possible damage before tensile strength test. The diameter of vetiver root was measured in three different positions to obtain a representative value (Mattia, Bischetti, & Gentile, 2005). Tensile Strength measurement was carried out based on ASTM D2101-79 using Universal Testing Machine (Orientec. Model UCT-5T). Tensile strength (T_R) was calculated using Equation 1 below.

$$T_R = F_{max}/\pi.(D/2)^2 \quad (1)$$

where:
T_R = Tensile strength (Mpa)
F_{max} = The maximum registered load (N)
D = Average diameter of root (mm)

Pollutant Removal of Carwash Wastewater

To evaluate the pollutant removal capacity, the growth media were analysed before and after planted with vetiver grass. The analysis was carried out at Water Quality Laboratory, Faculty of Civil and Environment Engineering, Bandung Institute of Technology, which covered of total N, P, COD, BOD, detergent, phenol, lead, and zinc.

RESULT AND DISCUSSION

Growth Characteristic of Vetiver Plant in Carwash Wastewater Media

Response of plant to growth media is reflected by its capacity to adapt, survive, and then generate new stem, leaf, and root. Vetiver can be integrated as wastewater treatment due to the capability of plant in removal of water contaminants (Gupta, Roy, & Mahindrakar, 2012).

Data showed (Figure 2.) the stem generation of vetiver tended to decrease by the addition of carwash wastewater, both at concentration 50% and 100%. At the first two weeks after planting, the stem grew slowly. This period reflected the adaptation phase of vetiver with carwash wastewater as growth media. Afterwards, vetiver showed a capacity to survive and started to generate the new stems. In this study, stem number of vetiver increased linearly with the growth period (Figure 2). Initially, stem number was 1.78±0.19 (M_0); 1.89±0.10 (M_1); and 1.67±0.10 (M_2). It was then increased to 8.56±0.20; 7.00±0.19; and 6.00±0.33 for M_0; M_1; and M_2 respectively. In average, the number of stem at harvesting increased to 4.81 (M_0); 3.70 (M_1); and 3.59 times (M_2) from its initial stage. Stem generation of vetiver in growth media carwash wastewater achieved 81.8% for M_1 and 70.1% for M_2 if compared to control (M_0).

Leaf is the other principal organ of plants, in which photosynthesis and transpiration occurs. Photosynthesis is a sensitised, photochemical, and oxidation-reduction reaction (Meyer & Cusanovich, 2003). The sensitiser is chlorophyll, which captures

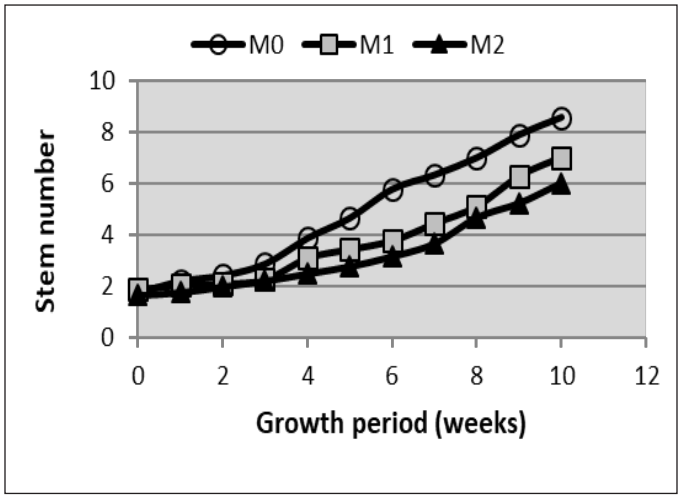

Figure 2. Stem number of vetiver in media with varying concentration of carwash wastewater

light and has functions in transformation of radiant into chemical energy. The main reaction of photosynthesis is fixing of atmospheric carbon dioxide and releasing oxygen to the atmosphere (Equation 2).

$$n\,(CO_2) + 2n\,(H_2O) \xrightarrow[\text{Radiant energy}]{\text{Plant}} (CH_2O)\,n + n\,(O_2) + n\,(H_2O) + n\,(119.6)\ \text{Cal}$$

At the initially, vetiver leaf in this study tend to dry and then fall down. It was not a causal for plant dies due to resistant characteristic. The new leaf was produced slowly in early of cultivation and increased at the 5th weeks after. This condition indicated that vetiver has a capability to grow in media of carwash wastewater, although it needs of several weeks for adaptation.

In this study, the fresh leaf number of vetiver decreased until the first three weeks after cultivation in media CW. After this period, leaf number increased linearly with the plant ages. Initially, the leaf number of vetiver in media M_0; M_1; and M_2 was 7.67±1.15; 7.33±1.15 (M_1); and 6.66±0.58, respectively. Then it increased significantly to 33±2.83 (M_0); 27±2.90 (M_1); and 20±3.03 (M_2) after 70 days of cultivation. Compared to its initial stage, the leaf number of vetiver in all of treatment increased, linear with the plant ages, i.e. 4.30; 3.68; and 3.00 times, for M_0; M_1; and M_2, respectively. Leaf generation of vetiver achieved 81.82% in M_1 and 60.61% in M_2 compared M_0. The leaf generation within ten weeks of cultivation is shown in Figure 3. The study result is in line with previous studies that the maximum leaf number of vetiver planted in diesel contaminated soil was 24 pieces (Darajeh et al., 2014).

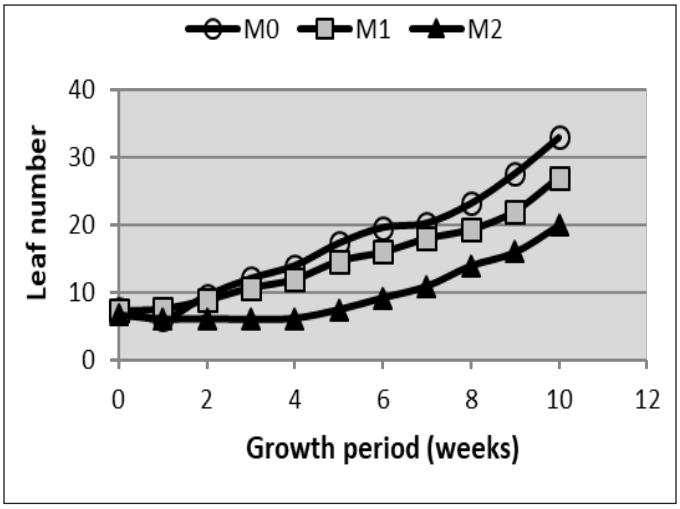

Figure 3. Leaf number of vetiver in media with varying concentration of carwash wastewater

Table 3 shows the vetiver leaf distributed in various length classes. In control media, the leaf length was distributed in all categories, from L< 10 to L ≥ 90 cm. Meanwhile, the vetiver leaf in M_1 and M_2 was dominated by leaf with L ≤3 0 cm at 68.5 and 82.4% respectively. Vetiver leaf with L > 60 cm has not been generated in M_1 and M_2 during 70 days of cultivation. As a result, the leaf area of vetiver in M_1 and M_2 was slightly lower than M_0. Vetiver with higher leaf area resulted in higher photosynthesis rate and vegetative growth rate.

Table 3
Leaf length distribution of vetiver at harvesting time (%)

Leaf length class	Leaf length distribution (%)		
(cm)	M_0	M_1	M_2
L ≤ 10	25,8	36,4	48,3
10 < L ≤ 20	22,2	19,1	20,8
20 < L ≤ 30	17,7	13,0	13,3
30 < L ≤ 40	10,6	15,4	9,2
40 < L ≤ 50	10,6	8,0	7,5
50 < L ≤ 60	6,1	7,4	0,8
60 < L ≤ 70	3,5	0,6	0,0
70 < L ≤ 80	1,5	0,0	0,0
80 < L ≤ 90	0,5	0,0	0,0
≥ 90	1,5	0,0	0,0
Total	100,0	100,0	100,0

Similar findings were reported previously (Nisa & Rashid, 2015). The maximum leaf length of vetiver which was planted in high concentration of palm oil mill effluent (POME) was 42.2 cm whereas in low of POME concentration was 70.2cm . The number of vetiver leaf media in low concentration of POME was 3.18 times higher than in high POME (Darajeh et al., 2014). However, it was reported the vetiver shoot length decreased ± 37% when planted in diesel contaminated soil

In the current study, the initial root number of vetiver was 13-21 with the average of 16.8 pieces, and the root length was in range of 1.5-69.9cm, Table 4 shows root number of vetiver at harvesting time was in the range of 37-45; 27-37; and 23-34 pieces in M_0, M_1, and M_2 respectively. The average root number of vetiver decreased with addition of carwash wastewater in growth media, i.e. from 41.8±3.1 in M_0 to 32.8±3.7 in M_1 and 30.0±2.4 in M_2. The root generation achieved 78% in media with 50% CW (M_1) and 72% in media with 100% CW (M_2). Nevertheless, vetiver has a capability to survive in media of carwash wastewater and generate new organs as well as roots

Roots in vetiver is its most important part. Besides quantitative parameter of root, qualitative characteristics such as nutrient uptake capacity should be considered. This characteristic is strongly related to the root length than the root weight. The root length determines the capacity of plant to obtain water and nutrients for growth (Darajeh et al., 2014; Gupta et al., 2012; Paz-Alberto et al., 2007). In fact, roots of herbaceous species may reach up to 10m in height. Nevertheless, phytoremediation is more effective in 50-100cm depth (Cameselle, Chirakkara, & Reddy, 2013). In this study, the highest total root length was achieved when the vetiver used tap water as growth media at 1,373 cm in average. On the other hand, root length in M_1 and M_2 was 861 and 553 cm respectively.

Table 5 presents the root length distribution of vetiver at harvesting time. The generation of vetiver root with L≤50cm

Table 4
The root number and root length of vetiver at harvesting time

Root growth / Treatment	M_0	M_1	M_2
Root number (per piece) per plant)			
• Range	37-45	27-37	27-34
• Average	41.8±3.1	32.8±3.7	30.0±2.4
Total root length per plant (cm)			
• Range (cm)	1,204-1,670	603-1.019	440-848
• Average (cm)	1,373±187	861±162	553±155
Individual root length			
• Range (cm)	2.6-92.4	1.4-73.0	1.3-64.5
• Average (cm)	32.7±20.5	26.2±19.0	18.7±14,9

Table 5
Root length distribution of vetiver at harvesting time (%)

Root length class	Root length distribution (%)		
(cm)	M_0	M_1	M_2
$L \leq 10$	11,6	24,9	38,3
$10 < L \leq 20$	20,7	17,3	27,8
$20 < L \leq 30$	19,5	19,3	11,7
$30 < L \leq 40$	19,5	16,8	10,0
$40 < L \leq 50$	10,0	9,1	6,7
$50 < L \leq 60$	8,0	5,6	3,9
$60 < L \leq 70$	4,0	4,6	1,7
$70 < L \leq 80$	2,8	2,5	-
$80 < L \leq 90$	3,6	-	-
≥ 90	0,4	-	-
Total	100,0	100,0	100,0

in media 100% CW (M_2) was 94.4%, higher than M_1 and M_0. In other words, root with length of 50-100 cm was only 5.6% in M_2, lower than in M_1 (12.7%) and M_0 (18.7%). There was no root with length ≥70cm was produced by vetiver when using 100% CW media. This data indicates that utilisation of CW as cultivated media would decrease the growth rate of vetiver root. In spite of that, vetiver has a capability to survive and adapt to media CW. Similar study reported that the maximum root length of vetiver in remediation of media with high concentration of palm oil mill effluent (POME) was 47.5 cm. Meanwhile, the maximum root length of vetiver in low concentration of POME was higher, i.e. 70.3 cm with 24 pieces (Darajeh et al., 2014). Another research found that the average root length of vetiver in medium of diesel contaminated soil decreased compared with uncontaminated soil, i.e. 12.6 vs 19.6 cm (Nisa & Rashid, 2015).

Root and shoot ratio is affected by environmental conditions to which the plant is exposed. In this study, there is no significant difference in root-shoot ratio among treatments. Approximately, root-shoot ratio of vetiver was 3:7 before treatment and 2.8:7.2 after treatment in CW. Ash content of vetiver leaf and root in harvesting was 9.3 and 5.8% respectively.

Tensile Strength

There is no significant difference in terms of tensile strength among growth media (Table 6). Tensile strength of Vetiver root with diameter 0.71 mm is higher than roots with diameter 0.81 mm, 33.9 and 16.3 MPa respectively. Tensile strength of root decreased with increase in diameter (Mattia et al., 2005; Teerawattanasuk, Maneecharoen, Bergado, Voottipruex, & Lam, 2015). Tensile strength of vetiver with root diameter 0.74±0.047 mm was 28.007±8.252MPa. This result is consistent

Table 6
Tensile strength of vetiver in different roots

Root diameter class	Diameter (mm)	Sectional area (mm^2)	Tensile strength (MPa)
1	0.810	0.520	16.300
2	0.722	0.416	28.123
3	0.716	0.409	33.688
4	0.710	0.398	33.917
Average	**0.740**	**0.436**	**28.007**
Dev. Std	**0.047**	**0.057**	**8.252**

with earlier research that found tensile strength of vetiver root varied between 22.55-33.83 MPa for diameter of 0.25-2.90 mm (Teerawattanasuk et al., 2015). Tensile strength obtained in the experiment is useful in the phytoremediation with continuous wastewater. Water storm in the system should be calculated not to exceed the tensile strength of the roots.

Pollutant Removal of CW by Vetiver

Plants need essential nutrient for their growth, both macro nutrients such as C, H, O, N, P, K, S, Mg, Ca, and Fe, and micro elements, such as Cu, Zn, B, Mn, and Mo. The root cells have a capacity in selection intake of ions for growth, both of cation and anion, counting of C, H, O, N and P. On the other hand, the root zone of plant is specifically interesting due to its capacity to absorb and store the contaminant. Therefore, high absorbing potential of roots is desirable, mainly in absorption of toxic substances of polluted water.

Generally, the growth rate of vetiver is affected by the pH level of growth media. Plant growth is poor when the active acidity is too high (pH too low). The previous research reported that pH level of domestic effluent changed from 7.26 to 5.98 after treated with vetiver (Truong & Director, 2006). Similar pattern was showed in this study which pH level of CW decreased after treated with vetiver grass for 70 days, i.e. from 7.02 to 6.04 in $M_1$and from 7.86 to 6.33 in M_2. This level complied with the effluent standard in Indonesia, which must be in the range of 6-9. Table 7 presented the pollutant removal in CW. The removal of total N was of 78.5% in $M_1$and 57.9% in M_2. Meanwhile, the elimination of P substances by vetiver was 83.5% for M_1 and 69.0% for M_2. The capacity of vetiver in removal in high concentration of CW was lower than in low concentration and it was reflected also in the growth rate of vetiver in growth medium. The plant growth rate and hydraulic retention time influenced the reduction of contaminants (Gupta et al., 2012).

It was noticed that the objective of biological treatment of wastewater, including of phytoremediation is to remove or reduce the pollutant compounds, including N and P (Jhamaria & Yadav, 2014). It also successfully demonstrated the vetiver

Table 7
Pollutant removal of cw by vetiver

Parameter	Unit	Initial		At harvesting		Removal (%)	
		M_1	M_2	M_1	M_2	M_1	M_2
Nitrite (NO_3)	mg. L^{-1}	0.64	1.27	0.15	0.39	76.4	69.3
Nitrate (NO_2)	mg. L^{-1}	2.05	3.76	0.54	1.53	73.7	59.3
Ammonia (NH_3)	mg. L^{-1}	5.54	11.08	1.08	4.86	80.5	56.1
Total N	mg. L^{-1}	8.23	16.11	1.77	6.78	78.5	57.9
Total P (PO_4)	mg. L^{-1}	6.17	12.10	1.02	3.75	83.5	69.0
C O D	mg. L^{-1}	420	812	101	282	76.0	65.3
B O D	mg. L^{-1}	207	398	65	140	68.6	64.8
Detergent	mg. L^{-1}	6.14	10.29	1.15	4.17	81.3	59.5
Phenol	mg. L^{-1}	0.07	0.12	0.00	0.01	98.6	95.8
Lead (Pb)	mg. L^{-1}	0.08	0.13	0.02	0.05	73.3	61.5
Zink (Zn)	mg. L^{-1}	0.26	0.29	0.03	0.05	88.5	82.8

was capable to remove the N and P from contaminated soil and water, was of 60 and 59-85%, respectively (Truong & Director, 2006). COD removal of carwash wastewater by vetiver achieved 76 and 65.3% in M_1 and M_2, respectively. The BOD removal was not significantly different, between 68.6 and 64.8%. As comparison, the removal efficiency of COD and BOD_5 in natural form of local soil with municipal landfill leachate was 47.7 – 97.9% of COD and 48-99% BOD_5 respectively (Pazoki, Abdoli, Karbassi, Mehrdadi, & Yaghmaeian, 2014). In water passing through the floating wetland system, the median of COD was reduced to 66%, BOD 52%, and total P 65% (Stefani, Tocchetto, Salvato, & Borin, 2011).

In this study, the removal of detergent was 91.3 and 59.5%; phenol was 98.6 and 95.8% in M_1 and for M_2 respectively. Another study found although plant growth was reduced in presence of phenol vetiver adapted to phenol without any decline in potential for phenol remediation. Almost all the phenol was removed at the end of 4 days when concentration was 50-100mg.L^{-1} (Singh, Melo, Eapen, & D'Souza, 2008). Lead (Pb) has been used as an additive in gasoline and resulted in substantial increase in Pb levels in the area adjacent to highways. The present of Pb in fuels also contributes to air pollution, especially in urban areas. In plant, the toxic symptoms of Pb include inhabitation of seed germination and growth rate. The proper system is required to remove or reduce the lead content in the environment. It was reported that vetiver grass can be used to phyto-remediate soil and water in urban areas with various contaminants, such as public parks.

The previous research carried out by Antiochia, Campanella, Ghezzi and Movassaghi (2007) found that vetiver plant could take up large amount of Pb and Zn, both in shoots and roots. The Pb uptake by

shoots is higher than 0.1% (dry weight) from the 4th day and about 3 times by roots. The Zn uptake was even higher, i.e. 0.38% (dry weight) in shoots and 0.61% in roots after 8 days irrigated daily with solutions of 621ppm Pb and 653ppm Zn. Another study investigated that vetiver grass is most tolerant and registered the highest rate of Pb absorption, i.e. 10.15±2.81mg.kg^{-1} (Paz-Alberto et al., 2007). In this study, the removal of Pb by vetiver was 73.3% in M_1 and 61.5% in M_2. This result is higher than the previously found that phytoremediation coefficient of lead by vetiver was 32% (Chantachon et al., 2004). It concluded that vetiver was a good hyper accumulator for Pb and Zn, which was concentrated more in roots than in shoots (Antiochia et al., 2007; Chantachon et al., 2004; Roongtanakiat, Tangruangkiat, & Meesat, 2007).

CONCLUSION

Vetiver has a capability to survive, adapt and generate new organs in carwash wastewater. The first three weeks of cultivation is a sensitive stage in growth, but afterward, it starts to grow normally. Nevertheless, the growth rate of vetiver decreased with the increase in carwash wastewater concentration in growth media. Within 70 days, the pollutant substance in media carwash wastewater 50% and 100% could be removed by vetiver, i.e. 78.5 and 57.9% N, 83.5 and 69.0% P, 76.0 and 65.3% COD, 68.6 and 64.8% BOD, 81.3 and 59.5% detergent, 98.6 and 95.8% phenol, 73.3 and 61.5% Pb and 88.5 and 82.8 % Zn respectively.

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The Response of TLR3 and IL-1β Genes Following Exposure to LPS, Poly (I:C), Zymosan in Culture of Gouramy (*Osphronemus gouramy*) Kidney Cells

Diah Kusumawaty[1]*, Sony Suhandono[2], I Nyoman Pugeg Aryantha[2] and Adi Pancoro[2]

[1]Department of Biology Education, Indonesia University of Education, Jl. Dr. Setiabudi No 229, Bandung 40154, Indonesia
[2]School of Life Sciences and Technology, Bandung Institute of 14 Technology, Jl. Ganesha No. 10, Bandung 40132, Indonesia

ABSTRACT

The aims of the present study are to isolate and characterise structure TLR3 and IL1β gene and evaluate the potential and signaling mechanism following exposure of Polyinosinic: polycytidylic acid (poly (I:C)), Lipopolysaccharide (LPS) and Zymosan as antigens in gouramy (*Osphronemus gouramy*). Gouramy kidney cells were stimulated with LPS, Poly I:C and Zymosan. Following incubation at 28°C, relative expression levels of Toll Like Receptor 3 (TLR3) and interleukin-1β (IL-1β) were examined at one hour and six hour after treatment. A Real Time Polimerase Chain Reaction approach was utilised to search for the effects of Poly I:C, LPS, and Zymosan exposure to gouramy kidney cells between one hour to six hours after treatment, LPS in kidney cell increased expression of interleukin-1β and downregulated expression of TLR3. Poly (I:C) which is an antigen that responds to antiviral, induces an increase in the transcription both of Toll like receptor 3 and IL-1β. Zymosan in kidney cells increased expression of TLR3 but downregulated expression of IL1β. This study shows that TLR3 was activated not only by Poly I: C but also LPS and Zymosan. However, antigen Poly IC-induced labor IL-1β and TLR3 expressed higher than the antigen LPS and Zymosan, so, this research showed a TLR3 response to Poly I:C is more dominant than Zymosan and LPS. TLR3 in gouramy kidney cell

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E-mail addresses:
diah.kusumawaty@upi.edu (Diah Kusumawaty)
sony@sith.ac.id (Sony Suhandono)
nyoman@sith.ac.id (I Nyoman Pugeg Aryantha)
adi@sith.ac.ids (Adi Pancoro)
* Corresponding author

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transcript was identified in response to poly I:C, Zymosan, and LPS. These results indicate that TLR3 in gouramy also plays a role for defense against bacterial infection and virus.

Keywords: IL1β, Innate immune, LPS, Poly(IC), *Osphronemus gouramy,* TLR3, Zymosan

INTRODUCTION

Interleukin 1-beta (IL1-β) is a pro-inflammatory cytokine that is important and has a major role in the immune response specific system or non-specific activation (Oppenheim, Kovacs, Matsushima, & Durum, 1986). IL-1β is also known as major of cytokine because IL-1β may mediate some immune responses and may be synthesised as an inactive precursor, which is processed into biologically active IL-1β as a response to various proinflammatory stimuli. This is why IL-1β is an inflammatory cytokine that is most widely studied in fish (Rauta, Nayak, & Das, 2012). Studies on IL-1β have been done in many fish such as rainbow trout (*Oncorhynchus mykiss*), sea bass (*Dicentrarchus labrax*), carp (*Cyprinus carpio*), orange spotted grouper (*Epinephelus coioides*) and Japanese flounder (*Paralichthys olivaceus*) (Engelsma, Stet, Schipper, & Verburg-van Kemenade, 2001; Lu et al., 2008; Scapigliati et al., 2001; Taechavasonyoo, Kondo, Nozaki, Suzuki, & Hirono, 2013; Zou, Grabowski, Cunningham, & Secombes, 1999).

Other valuable information showed that stimulation of Toll Like Receptor 3 (TLR3) and Toll Like Receptor 4 (TLR4) are one of the several responses that can secrete IL-1β (Maelfait et al., 2008). Previous studies revealed that many Toll-Like Receptors (TLRs) immune signal pathway factors play a major role in immune responses in many teleost fish. One of the relevant TLRs in teleost is TLR3. TLR3 has been known to play a role in response to virus and bacteria. Zebrafish that is injected with *Streptococcus agalactiae* shows induce expression of TLR3 (Hsieh, Pan, & Chen, 2010), whereas in mammalian Toll Like Receptor 3 (TLR3) play role response to virus (Matsumoto, Oshiumi, & Seya, 2011). Gouramy is one of the largest freshwater cultured species in Indonesia, and is one of the important freshwater fish commodities in various regions in Indonesia. In addition, gouramy is also included in the 15 main commodities that can increase the production and income of farmers Gouramy has suffered serious diseases caused by the viral, bacterial and parasitic infections in recent years, which result in enormous economic losses.

However, information on gouramy immune gene is scarce, as the mechanism and regulation of their immune response is still not well understood. Research on gouramy fish associated with bacterial infections has been widely practised, such as to asses the endurance level or mortality rate against infection of *Aeromonas hydrophila* after giving extracts of natural ingredients such as *Phaleria macrocarpa* (Christien, Djayus, & Ezraneti, 2014). Research has also been carried out to determine the immunostimulatory potential of *Spirulina platensis* extract for histopathologic

changes in gouramy fish due to *Aeromonas hydrophila* infection. (Simanjuntak, Wibowo, & Indarmawan, 2016). However, in gouramy fish, molecular innate immune mechanisms has not been widely studied. Kusumawaty, Suhandono, Pancoro, and Aryantha (2017) have also conducted a study to see changes in the expression of TLR2, Myd88 and TRAF6 genes in liver, kidney and spleen of post-infection *Aeromonas hydrophila*.

Gene response information on stimulation of analogue compounds such as LPS (Gram negative), Zymosan (fungi and Gram positive bacteria) and Poly IC (DNA virus) by in vitro method can be used to see early descriptions of unknown gene functions, so it can be used as a model for initial pathway of gene response to microbes. In fish, in vitro stimulation research is generally performed on kidney head cells, because the kidneys in the fish function in settings and central organs for the interaction of the endocrine immune system. (Fierro-Castro et al., 2012; Taechavasonyoo et al., 2013). In addition, fish kidney is the main organ that functions in the setting and is the central organ for interaction of the endocrine immune system (Tort, Balasch, & Mackenzie, 2003). Although in vitro stimulation studies of head kidney cells with analogue compounds have been widely practised, studies on the gouramy have not been carried out as yet.

This study aims to isolate and characterise structure TLR3 and IL1β gene and evaluate the potential and the signaling mechanism following exposure of Polyinosinic: polycytidylic acid (poly (I:C)), Lipopolysaccharide (LPS) and Zymosan as antigens in gouramy (*Osphronemus gouramy*). Phylogenetic tree, BLAST and structure predictive analysis is characterised OgIL-1β and OgTLR3 gene. The temporal expression profiles of OgTLR3 and OgIL-1β genes after stimulation with polyI:C, LPS, and Zymosan were compared to better understand their potential role in gouramy immune responses using real time PCR. In this study, the partial cDNA and threatment kidney cell respon to LPS, poly IC and Zymosan of *Osphronemus gouramy* TLR3 (OgTLR3) and *Osphronemus gouramy* IL1β (OgIL1β) were first described in gouramy. Identified OgTLR3 and OgIL-1β are derived from kidney which is believed to be the core organ for immune-endocrine interaction (Tort et al., 2003).

MATERIALS AND METHODS

Partial cDNA Sequencing of IL-1β and TLR3

Partial cDNA OgIL-1β and OgTLR3 gene were isolated from the kidney of gouramy using miTotalTM RNA Column (Viogen) according to the instructions in the manual protocol. The primer design was performed using amino acid sequence of several species from the Perciformes or Teleostei order. Furthermore, the DNA sequence of the target cds is aligned using the Bioedit programme. The alignment results were then used to design the primers using the Primaclade programme (Gadberry, Malcomber, Doust, & Kellogg, 2004) to obtain the degenerate primer sequence. The subsequently obtained

degenerate primer was used to amplify the partial genes of TLR 3 and IL1B from the carp cDNA. The primers used to isolate IL-1β and TLR3 from gouramy are listed in Table 1. Amplification of gene using Dreamtaq enzyme mix (Thermoscientific) were according to the instructions of the manual protocol.

Synthesis of cDNA using Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) (Thermoscientific) used one µg RNA from kidney cell following the handbook's protocol. The amplicon was sequenced at Macrogen Korea. The sequencing result of OgIL-1β and OgTLR3 genes was checked for identification

Table 1
Primer Listed to Isolated IL-1β and TLR3 from Gouramy

Gene	Sequence 5'-----3'	Size Nt	Ta °C
TLR3_F1	GACCACAGTGCCAGGCCTCA	680	55
TLR3_R1	AAGGCTGGCACCCTCTCCCT		
TLR3_nestedF	GACTGCACRTGYGAGAGCAT	549	60
TLR3_nestedR	TTTRAATCGTCTACACCAGGG		
IL1β_F	TAACACTGAGAGGACAACTG	700	55
IL1β_R	GAAGAGAAACCGCACCAT		

using Basic Local Alignment Search Tool (BLAST) method, aligned and compared with the data of DNA sequences of other OgIL-1β and OgTLR3 genes available at the GenBank database NCBI (National Center for Biotechnology Information). The DNA sequences from species with clear names were put into one group. The group of similar DNA sequences was analyzed using multiple sequence alignment method. The phylogenetic tree was developed using the tool program of Clustal X software and MEGA 5 software (Tamura et al., 2011). The target DNA outcome is also used to locate the target amino acid by predicting it using the OFR finder program on the NCBI as well as estimating the protein domain structure with the SMART program (http://smart.embl-heidelberg.de, 24 August 2015).

Identification of these genes was necessary to design and synthesise the specific primer pair of gouramy that were used to analyse gene expression by using real time PCR. The primers used for gene expression analysis had analysed the quality of the amplification results through electrophoresis and looked at the pattern of amplified graphs using real time PCR

Expression Analysis of IL-1β and TLR3 Genes

The isolated kidney cell from 50gr of gouramy was re-suspended in primary culture medium (RPMI1640 supplemented 100 mg/ml of streptomycin and 100 IU/ml of penicillin). Kidney cell approximately 10^7 cells was treated with 250 µg/ml LPS (from *Escherichia coli* 0127:B8, SigmaeAldrich,

USA), 50 µg/ml polyI:C (Sigma-Aldrich, USA), 10 µg/ml Zymosan, and PBS for control at 28°C. The cells were harvested at one and six hours after treatment. Total RNA was extracted from kidney cell using miTotalTM RNA Column (Viogen) according to instructions in the manual protocol. cDNA synthesis using MMLV reverse transcriptase (Thermoscientific) used one µg RNA from kidney cell following the manual protocol. The cDNA samples were diluted 5-times with deionized water. The changes of mRNA level of the IL-1βand TLR3 were determined by real-time PCR which was performed with SYBR green PCR master mix (First Base) using Biorad CFX 96 Real-time PCR system following the manufacturer's instructions. The expression levels of target genes were normalised to the expression level of elongation factor-1 (EF-1) and Gapdh (glyceraldehyde-3-phosphate dehydrogenase) as an internal control gene and were expressed relative to the average level in the groups at one hour and six hours post treatment.

RESULTS AND DISCUSSION

Structural Characterisation Predicted Partial cds of TLR3 Genes and IL-1β Genes

Sequence of DNA and amino acid of IL1-β and TLR3 gene of gouramy has been submitted to NCBI GenBank. Accession numbers of NCBI GenBank are KT884611.1 (IL1-β) and KT884606 (TLR3). Figure 1A shows the analysis of phylogenetic tree based on partial ORF region of TLR3 gene gouramy (178 amino acids) known to most homologous to part TIR2 domain superfamily of ORF TLR3 large yellow croaker fish (*Larimichthys crocea*). Based on BLAST method in NCBI database, the identity index is 82%. Figure 1B shows ORF partial IL1-β gene (204 amino acids) gouramy most homologous with domain area IL1 superfamily part of ORF IL1-β fish Japanese flounder (*Paralichthys olivaceus*). Based on BLAST method in NCBI database the identity index is 79%. Based on Figure 1A and Figure 1B gouramy lowest homology based on partial both of ORF TLR3 and partial ORF IL1-β is with zebrafish (*Danio rerio*). This shows that gouramy and *D. rerio* are not closely related based on amino acid sequence TLR3 and IL1-β.

Based on Figure 2A, analysis of prediction protein structure using SMART programme is known that 2-47 amino acid regions are part of areas LRRCT (Leucine Rich Repeat C-terminal domain), a trans membrane helix region 51-73 and 106-178 part of areas TIR domain (Figure 2A), in comparison to analysis of protein structure prediction using TLR3 gene sequence of references fishes from GenBank are *L. crocea* [KKF15845.1], *E. coioides* [AEX01718.1], *T. rubripes*. [AAW69373.1] and *D. rerio* [AAT37633.1]. Based on analysis of protein structure prediction using SMART, it is known that the partial cds of gouramy TLR3 homologous to part TIR2 domain superfamily of fish *L. crocea*, *E. coioides* and *D. rerio*. *Takifugu rubripes*, is a fish that does not have a transmembrane region in the TLR3 gene. In Figure 2, it can be seen that there are differences in the

results of the phylohenetic tree using the TLR3 and IL1β genes. This difference is due to the use of partial gene, rather than the complete gene. Although different, the gouramy is still one group with *T. rubripes, L. crocea*, *E. coioides*.

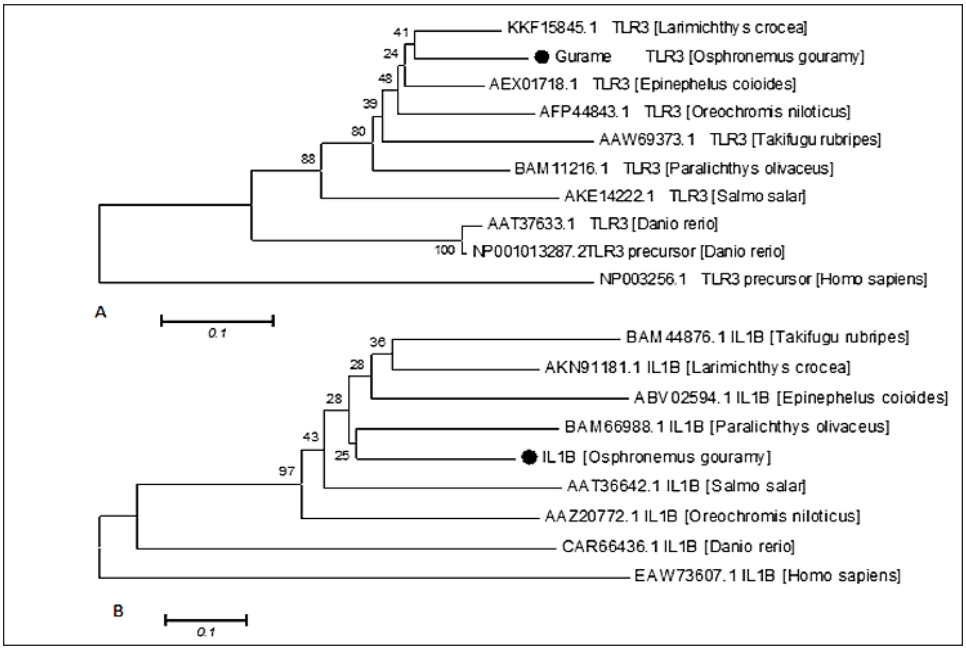


Figure 1. Phylogenetic tree of *O. Gouramy* partial TLR3 (A) and IL-1β (B)

The tree was constructed by the Neighbor-joining method using the CLUSTALW in MEGA 6 and was bootstrapped 5000 times.

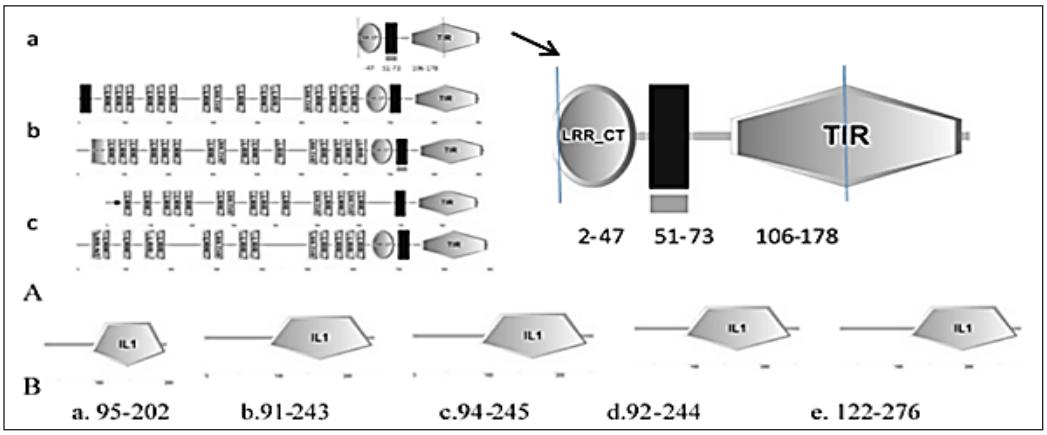


Figure 2. Structure prediction of *O. Gouramy* partial ORF TLR3 Partial (A) and partial ORF IL-1β (B). TLR3: a. *O. gouramy*, b. *L.crocea* [KKF15845.1], c. *E.coioides* [AEX01718.1], d. *T.rubripes*. [AAW69373.1], e. *D.rerio* [AAT37633.1] IL1β: a. *O. gourami,* b. *P.olivaceus* [BAM66988.1], c. *E.coioides* [ABV02594.1], d. *T. rubripes* [BAM44876.1], e. *D. rerio* [CAR66436.1]

Analysis of protein structure prediction OgIL-1β partial gene using the SMART programme found that amino acids 95-202 region is part of the area IL1 superfamily (Figure 2B). For comparison amino acid sequence of the fish *P. olivaceus* [BAM66988.1], *E. coioides* [ABV02594.1], *T. rubripes* [BAM44876.1] and *D.rerio* [CAR66436.1] were analysed using SMART. The analysis of protein structure using SMART showed that all species of fish reference derived from the NCBI GenBank had a similar structure which had a domain IL1 at 91-245 amino acid regions except the *D. rerio* domain IL1 area on 122-276.

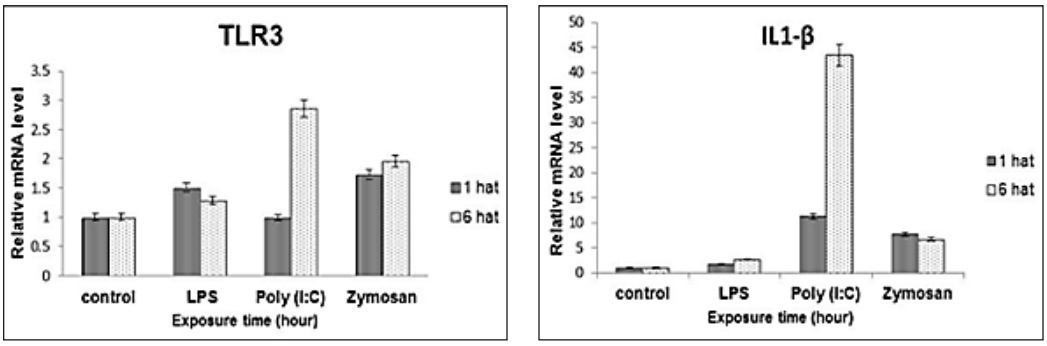
Expression Analysis of IL-1β and TLR3 Genes

The results of TLR3 and IL1-β gene expression analysis on gouramy kidney cell post stimulation (LPS, Zymosan and Poly I: C) can be seen in Figure 3. The relative expression of the genes TLR3 and IL1- β is normalised by the expression of house keeping genes Ef1α and gapdh. One hour after treatment (hat) to six hours after treatment (hat) kidney cell post treated with LPS shows to increase expression of interleukin-1β and downregulate expression of TLR3. Poly (I:C) as an antigen that responds to antiviral induces an increase in the transcription both of Toll-like receptor 3 and IL1β. At the same time, Zymosan in kidney cells increased expression of TLR3, but downregulated expression of IL-1β. This shows that TLR3 was activated not only by Poly I:C but also by LPS and Zymosan.

Expression of OgTLR3 showed up-regulation after treatment with lipopolysaccharide (LPS) in gouramy. This shows that OgTLR3 in gouramy also plays a role in defense against gram negative-bacteria**.** This research is in line with in mice (Alexopoulou, Holt, Medzhitov, & Flavell, 2001; Kadowaki et al., 2001), after infection with the Gram-negative *Edwardsiella ictulari* in a channel and *Edwardsiella tarda* in zebrafish (Phelan, Mellon, & Kim, 2005) and after infection large yellow croaker, (*Pseudosciaena crocea*) with *Vibrio parahemolyticus* (Huang, Wang, & Yao, 2011).

PBS was used as a control expression levels which are normalised to the expression level of EF-1α and Gapdh (hat: hour after treatment). However, the IL-1β and TLR3 have significantly increased transcript in the kidney cell six hours after treatment with Poly I:C. Poly I:C induced labor IL-1β and TLR3 expressed higher than LPS and Zymosan. Therefore, this research has showed the TLR3 response to Poly I:C is more dominant than Zymosan and LPS. This research contrasts with other research projects which have shown LPS to be more dominant than poly I:C to induce expression of IL-1β in Japanese flounder (Taechavasonyoo et al., 2013). The results of this study indicate that gouramy potency against viral infection is higher than gram negative bacteria like *Aeromonas hydrophila*.

Genetic information pertaining to the innate defense system in gouramy is required to understand the level of gene



(a) (b)

Figure 3. Expressions of: (a) OgTLR3; and (b) OgIL-1β in Gouramy after Stimulation LPS, Poly IC and Zymosan.

expression as a molecular response in the innate defense system in gouramy infected with pathogenic microbes. Such information is required to anticipate management of disease control in gouramy in a more targeted future at the molecular level whose application may include (i) development of bacteriosides aimed at the intervention of pathogen virulence, (ii) the development of supplements, feeds or drugs that can be ascertained molecules increase the immune response in fish, especially in gouramy and (iii) the development of gouramy strains of certain pathogens through conventional crosses or through the development of transgenic fish.

CONCLUSION

Phylogenetic tree, BLAST and structure predictive analysis show that OgIL-1β and OgTLR3 like those found in other fish such as *L. crocea, E. coioides, T. rubripes, P. olivaceus* and *D. rerio*. OgTLR3 in kidney cell transcript was identified in response to poly I:C, Zymosan, and LPS. These results indicate that OgTLR3 in gouramy also plays a role in defense against bacterial fungi and viral infection. However, the highest expression of IL-1β and TLR3 post-treatment with poly IC shows the main functions of OgTLR3 against viral infection.

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Isolation of Metyhl- Piperate from n-hexane Extract of Fruit of Cabe Jawa (*Piper retrofractum* Vahl.)

Iqbal Musthapa[1*], Gun Gun Gumilar[1] and Fitri Dara[2]

[1]Biological Chemistry Research Group, Chemistry Department, Faculty of Education of Mathematical and Natural Sciences, Universitas Pendidikan Indonesia, Jl. Setiabudhi 229, Bandung 40154, Indonesia
[2]Research Unit for Clean Technology, Indonesian Institute of sciences, Jl. Cititu Sangkuriang, Bandung 40135, Indonesia

ABSTRACT

Cabe Jawa (*Piper retrofractum* Vahl.) is categorised in Piper's genus under family piperaceae. It is commonly reported to function as antimicrobial, antifungal and anti-inflammatory. The aim of this study is to obtain information on the isolation of secondary metabolites from n-hexane extract of Cabe Jawa fruits. The isolation stage began with the maceration method with n-hexane solvent, then continued with purification stage of fractination compounds which was conducted by using several chromatography techniques including thin layer chromatography (TLC), vacuum liquid chromatography (VLC), and radial chromatography. Methyl piperate compound was isolated from this extract. The structure of this compound was determined using spectroscopic ^{1}H NMR. Based on the results of spectroscopic, analysis of ^{1}H NMR was present at a chemical shift of 3.80 ppm singlet peak with integrity of 3H was the typical peak of a methoxy. At 6.12 ppm chemical shift indicated a signal peak for protons bound to C sp^2. Chemical shift at 6.96 ppm to 7.13 ppm for proton groups was attached to benzene ring. From the results of ^{1}H NMR spectrum assisted by biogenesis approach, it is concluded that the purified compound successfully isolated was a methyl piperate compound.

Keywords : Chromatography, methyl piperate, n-hexane extract, piper retrofractum

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E-mail addresses:
iqbalm@upi.edu (Iqbal Musthapa)
gumilarchemi@upi.edu (Gun Gun Gumilar)
fitrimusthapa74@gmail.com (Fitri Dara)
* Corresponding author

INTRODUCTION

Indonesia is a tropical country which has various types of plants, empirically known to be potential phytopharmaca drug. Thus, these can be utilised as raw materials for the pharmaceutical industry, cosmetics and traditional medicine.

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Plant parts can be utilised as a source for obtaining secondary metabolite compounds. Secondary metabolite compounds are from groups of alkaloids, flavanoids, steroids and terpenoids. Plants are able to change a wide range of chemical compounds that have a variety of interesting bioactivity and this capability is also defined as a mechanism of self-defense against environmental threats. In this case the plant can produce chemical compounds that are pesticides, insecticides, antifungal or cytotoxic (Hernawan & Setyawan, 2003).

There are more than 700 species of genus *piper* of worldwide distribution. Species in this genus have high commercial and medicinal importance (Parmar et al., 1997). Phytochemical investigations of piperine show active physiologic compounds, including alkaloids, flavones, dihydrocaine, kawapyron, lignans, neolignan, profenilphenol, and terpenoids (Kubo et al., 2013). Plants of the genus *Piper*, such as *Piper nigrum, Piper methysticum, Piper auritum* and *Piper betle* have been known for a long time as agricultural commodities for herbs and medicines with high economic value. One of the plants belonging to the genus *piper* is *Piper retrofractum* Vahl. which is known as Cabe Jawa, used as one of Indonesia's traditional medicine ingredients in a mixture of herbs. Several studies have reported traditional herbs using Cabe Jawa as one of their formulations to have have very low bacterial contamination levels. This is due to the antibacterial and antifungal properties of Cabe Jawa. Traditionally, this plant is believed especially by Indonesians, to be able to treat asthma, bronchitis, hemorrhoids, fever, and abdominal pain as well as has stimulant effects on nerve cells that can increase body stamina. A literature survey informed that a number of biological studies have been carried out on this plant extract such as its antioxidant, anti-fungal, cytotoxic and α-glucosidase inhibitory activity (Banerji, et al., 1985; Banerji, Sarkar, Datta, Sengupta, & Abraham, 2002; Chansang, et al., 2005; Jong-Woong, et al.,1992; Luyen, et al., 2014; Muharini, Liu, Lin, & Proksch, 2015; Muharini, Liu, Wenhan, & Proksch, 2015).

Each plant has its own character in the growing environment where the plant is located. Thus, the differences in environmental conditions allow the emergence of certain characteristics, such as differences in plant morphology or different components of the compounds contained in the plant.

This paper focuses on isolation of methyl-piperate from n-hexane extract of fruit of *P. retrofractum*, and describes the isolation and structure elucidation of methyl-piperate. The discovery of these compounds is actually the first time for these species.

MATERIALS AND METHODS

The method of research conducted included several stages such as extraction, separation and purification, as well as the characterisation of compounds by ^{1}H NMR spectroscopy method.

General

^{1}H NMR spectra were recorded with a AGILENT 500 MHz operating at 500 (1H) MHz, using residual (δH 7.26) and deuterated solvent (δC 77.1) peaks of chloroform-*d* as reference standards. VLC (vacuum liquid chromatography) was carried out using Merck silica gel 60 GF254; for TLC analysis, pre-coated silica gel plates (Merck Kieselgel 60 GF_{254}, 0.25 mm thickness) were used. Solvents used for extraction and preparative chromatography were of technical grade and distilled before use.

Plant Material

Fruit samples of Cabe Jawa were collected from Lembang, West Java, Indonesia in January, 2017. The plant was identified by the staff members at Herbarium Bagoriense, LIPI- Bogor Plantation Cansevation Center, and the voucher specimen deposited at the herbarium.

Extraction and Isolation

The dried fruit of Cabe Jawa (500 g) was macerated using n-hexane solvent. The maceration extract was filtered using a buchner funnel and the filtrate was concentrated using a vacum rotatory evaporator.

Separation and Purification

The separation and purification steps of compounds in this study were conducted through two stages of liquid vacuum chromatography (VLC) and radial chromatography. About 15 g of total hexane extract in VLC (n-hexane-ethyl acetate = 9:1 until 0:10) obtained the combined fractions, then separated by chromatotron.

Characterisation

The process of characterising compounds with NMR 1H spectroscopy was performed at the Bandung Institute of Technology, Indonesia.

RESULTS AND DISCUSSION

The extraction process of Cabe Jawa with n-hexane solvent obtained 21.5 g of n-hexane extract. Separation and purification was done by vacuum liquid chromatography (VLC) method. Firstly, it analysed using thin layer chromatography (TLC). This analysis aimed to determine the solvent to be used at the time of separation with VLC. The chromatogram pattern in TLC shows the separation pattern that occurs in VLC.

The first separation was carried out using VLC, the solvent used was an organic solvent which increased its gradient polarity. In this separation, n-hexane and ethyl acetate solvents were used. Based on chromatogram analysis of TLC, eluent hexane and ethyl acetate were used with several compositions of 100% n-hexane (3 elution), n-hexane and ethyl acetate 9: 1 (3 elution), 8: 2 (4 elution), 7: 3 (2 elution), 1: 1 (2 elution), 100% ethyl acetate (1 elution) and methanol 1 elution with 100 ml volume at each elution. From the VLC results, 15 fractions were obtained.

Fractions having the same chromatogram patterns are combined to achieve four combined fractions. The mass of each

fraction is fraction A (1-5) obtained as much as 9.8 g, fraction B (6-9) as much as 3.4 g, fraction of C (10-11) as much as 0.4 g, and fraction D (12-15) as much as 2.7 g. The targeted compound is a nonpolar compound with good separation, so further separation is taken from Fractions A and B. The fractions in VLC return to separate the compounds contained in the fraction.

The second process VLC was done the same way as the previous one, so that 15 fractions were obtained. Based on chromatogram analysis of merging VLC fractions based on similar pattern of stain according to polarity level, three main fractions were obtained among the fractions of A2, as much as 4.6 g, B2 fraction as much as 1.9 g and C2 fraction as much as 1.4 g. The combined fractions were analysed by TLC using 100% dichloromethane eluent. The mass and chromatogram patterns of each fraction were taken into consideration to determine which fractions would be further separated against the C2 fraction which was further purified by using radial chromatography (chromatotron). The result of chromatotron to fraction C2 was obtained by pure compound whose purity with TLC obtained as much as 25.8 mg. The isolated compounds were then analysed using 1H NMR spectroscopy.

Determination of the structure of isolated compounds was analysed using 1H (proton) NMR spectroscopy. Spectroscopic analysis was performed to obtain an idea of the various types of hydrogen atoms present in the isolated compounds. The proton NMR spectrum provides information on the chemical environment of the H atom, the number of H atoms in each environment and the cluster structure adjacent to each H atom. The 1H NMR spectra of isolated compound is shown in Figure 1.

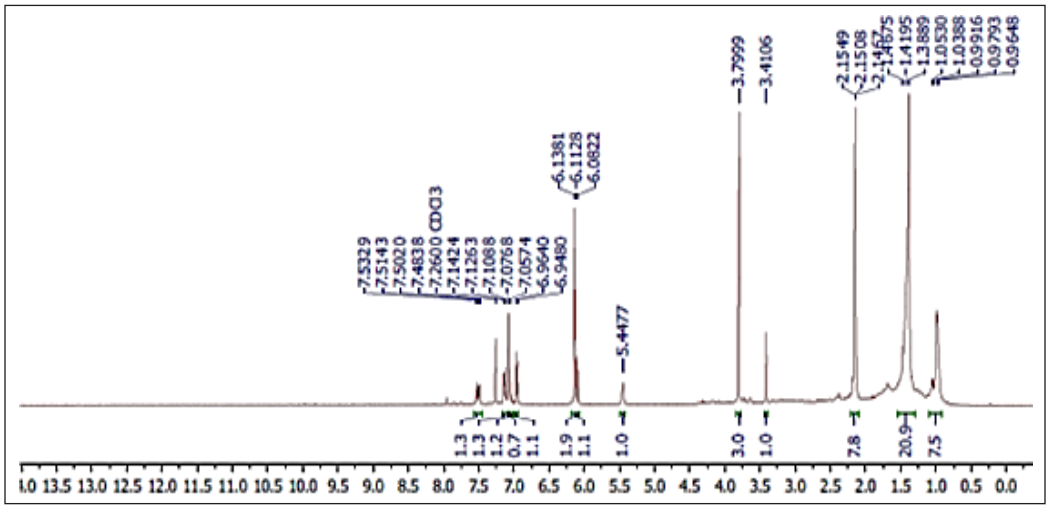

Figure 1. The ^{1}H NMR spectra of isolated compound

Based on the proton NMR spectrum, the number of protons identified was 12 protons. Chemical shift of 3.80 ppm singlet peak with 3H integrity is the typical peak of a methoxy. At 6.12 ppm chemical shift indicates a signal peak for protons bound to C sp^2. The chemical shift at 6.96 ppm to 7.13 ppm is a chemical shift for proton groups attached to the benzene ring. All ^{1}H NMR data of isolated compounds and comparison with the standard of methyls piperate can be found in Table 1.

Table 1
Data ^{1}H NMR spectra methyl piperate

No C	δ_H isolated compound (Multiplisitas, J, Integrasi) (ppm)	δ_H metil-piperat (Multiplisitas, J, Integrasi) (ppm)
1	-	-
2	6,08(d, J=15,3Hz;1H)	5,95 (d,J=15,2 Hz;1H)
3	7,51 (dd, J=6,15,J=4,9;1H)	7,36 (dd, J=15,25, J=11Hz, 1 H)
4	7,02 (dd, J=16; J=11 Hz, 1H)	6,67 (dd, J=15,3, J=10,95Hz; 1H)
5	7,07 (d, J=9,7;1H)	6,76 (d, J=15,4 Hz;1H)
6	-	-
7	7,13 (d, J=9,7;1H)	6,87 (d, J=8,05Hz;1H)
8	6,96 (d, J=8,1H)	6,74 (d,J=8 Hz; 1H)
9	-	-
10	-	-
11	7,11 (s, 1H)	6,95 (s,1H)
$-OCH_3$	3,80 (s, 3H)	3,72 (s, 3H)
$-OCH_2-O$	6,13 (d, J= 12,65 Hz,2H)	5,94 (d, J=1,8;2H)

In the proton NMR spectrum analysis, there is a chemical shift similar to the methyl piperate compound (compound contained in the genus piper), so further analysis compares the value of NMR proton chemistry of the isolated compound with standard NMR proton methyl piperate. Based on the results of the proton NMR spectrum comparison, there is a similar shear peak to indicate the isolated compound is a methyl piperate compound. The integrity of the number of protons is equal to the number of standard methyl piperate protons of 12 protons. Compared to the conclusion that the compound successfully isolated was a methyl piperate compound shown in Figure 2.

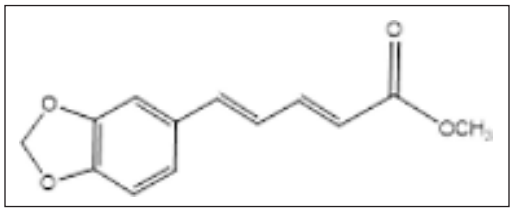

Figure 2. Structure of methyl piperate compound

Characterisation of compounds other than using NMR analysis showed there is also traced biogenesis approach. The biogenesis approach aims to analyse structures that become precursors in the main framework of methyl piperate compounds.

The formation of methyl piperate starts from the precursor L-tyrosine which produces 4-cumaric acid through the enzyme tyrosine ammonia liase (TAL). The reduction process is catalysed by enzymes with high substrate specificity and NADPH. The substitution pattern with meta-methoxy and para-hydroxyl cyclisation on the sinamoil backbone by extension of acetyl-CoA or malonyl CoA chain via Claisen reaction yields keto-ester. The keto-ester subsequently was reduced by NADPH, followed by dehydration for piperoil-CoA and subsequently methylated to give a methyl piperate compound (Dewick, 2002).

CONCLUSION

Methyl piperate was successfully isolated and identified from n-hexane fraction of *Piper retrofractum*. Spectroscopic analysis of 1H NMR showed a chemical shift of 3.80 ppm singlet peak with integrity of 3H confirming the typical peak of a methoxy. At 6.12 ppm, chemical shift indicates a signal peak for protons bound to C sp^2. The chemical shift at 6.96 ppm to 7.13 ppm is a chemical shift for proton groups attached to the benzene ring. From the results of NMR 1H spectrum and assisted by biogenesis approach, it is concluded that the purified compound successfully isolated was a methyl piperate compound.

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Subchronic Toxicity of Ethanolic Extract Velvet Bean (*Mucuna pruriens*) from Indonesia

Ratnaningsih Eko Sardjono[1]*, Iqbal Musthapa[1], Sholihin[1], Fitri Khoerunnisa[1], Atun Qowiyah[2] and Rahmi Rachmawati[1]

[1]Department of Chemistry Education, Universitas Pendidikan Indonesia, Jl. Dr. Setiabudi No. 229, Bandung 40154, Indonesia
[2]Department of Pharmacy, Universitas Garut, Garut, Indonesia, Jl. Raya Samarang No. 52A, Garut 44151, Indonesia

ABSTRACT

The present study is aimed at investigating the subchronic toxicity of ethanol extract of *Mucuna pruriens* seeds from Indonesia. A total of 120 rats Wistar strain, both sexes, were used in this study. Both were divided into one control group, three treated groups, and two satellite groups. The extract in different doses was administered orally for 90 days for the treated groups, while 120 days for the satellite groups. The subchronic toxicity was evaluated using various parameters including death, motoric activity, body weight, haematology parameters, biochemical parameters, and organ weight along with the histopathological study. There was no mortality and no significant change in motoric activity, body weight, several haematological parameters, and glucose levels. Significant differences were found in levels of cholesterol, triglyceride, AST and ALT, BUN, and creatinine. Degeneration of some tissues was seen in the kidneys and the liver. The ethanol extract of *Mucuna pruriens* seeds from Indonesia has a reversible subchronic toxicity to the liver and kidneys.

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E-mail addresses:
ratnaeko@upi.edu (Ratnaningsih Eko Sardjono)
iqbalmust@yahoo.com (Iqbal Musthapa)
Hsholihin@upi.edu (Sholihin)
fitri.khoerunnisa@gmail.com (Fitri Khoerunnisa)
qowiyyah@live.com (Atun Qowiyah)
rahmi.rachmawati@windowslive.com (Rahmi Rachmawati)
* Corresponding author

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INTRODUCTION

Mucuna pruriens or velvet bean is native to tropical Africa and Asia and has been widely used in many countries as a medicinal plant (Deokar, Deore, & Kshirsagar, 2016; Natarajan, Narayanan,

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& Ravichandran, 2012). The seed has been used as a stimulant and nerve tonic to treat impotence and infertility, as well as in the treatment of Parkinson's disease (Kumar & Saha, 2013). The pods are reported as anthelmintic, and the roots are used in the treatment of neurological disorders and blood purification. Another report also mentioned this plant to have antioxidant activity (Agbafor & Nwachukwu, 2011) and can be used in the treatment of cholesterol (Enechi & Ozougwu, 2014), diabetes (Majekodunmi, Oyagbemi, Umukoro, & Odeku, 2011), cough (Sultana, Khan, & Azhar, 2013), rheumatism, gout, cancer, tumors, tuberculosis, asthma, cholera, diarrhea, dysentery, irritation, muscle pain (Deokar et al., 2016; Kumar & Saha, 2013; Natarajan et al., 2012) and to treat snake bites (Fung, Tan, Sim, & Aguiyi, 2012).

Various studies have reported the activity of *Mucuna pruriens* seeds as a promising antiparkinson due to the content of L-DOPA (Cassani et al., 2016; Kasture et al., 2009). L-DOPA is the precursor to the neurotransmitters dopamine and is able to cross the blood-brain barrier. Since Parkinson implies a decrease of dopamine, the presence of L-DOPA can help to increase the dopamine (Hefti & Melamed, 1980; Misra & Wagner, 2007). The highest content of L-DOPA was found in the seeds (Deokar et al., 2016). The seed of *Mucuna pruriens* from Indonesia has been reported to contain about 7.56 to 13.9% L-DOPA (Sardjono, Musthapa, Sholihin, & Ramdhani, 2012). It is also known that *Mucuna pruriens* contains alkaloids, saponins, flavonoids, steroids, triterpenoids, glycosides, reducing sugar, cardiac glycosides and tannins (Akindele & Busayo 2011; Eze, Mohammed, Musa, & Tanko, 2012; Hadimani et al., 2015; Kumar, Rajput, Dhatwalia, & Srivastav, 2009; Pandey & Pandey, 2016).

Toxicity studies have reported that the extract of *Mucuna pruriens* seeds at a dose of 2000 mg/kg body weight did not cause signs of toxicity, did not lead to weight loss and did not cause death in animals (Hadimani et al., 2015; Krishna, Manikyam, Sharma, & Sharma, 2016). The leaf extracts administered orally showed the LD_{50} value of more than 2000 mg/kg (Akindele & Busayo, 2011; Eze et al., 2012). However, the leaf extracts administered by intraperitoneal injection has LD_{50} value of 1509.46 mg/kg (Akindele & Busayo, 2011).

With regard to the use of the plant as an alternative medicine for Parkinson's treatment, toxicological screening is very important for the development of new drugs and for the extension of the therapeutic potential of existing molecules. Previously the authors reported acute toxicity testing and revealed that the *Mucuna pruriens* seed ethanol extract from Indonesia administered orally is safe or non-toxic with $LD_{50} > 5000$ mg/kg (Sardjono, Musthapa, Sholihin, Qowiyah, & Rachmawati, 2017). In the interest of safety in the use of *Mucuna pruriens* as medicinal plants, and considering that humans more often consume the plant extract at lower dose but in longer time, thus it is necessary to assess the subchronic

toxicity that will provide information on health hazards that may result from repeated exposures to the extract. Unfortunately, there is no any report related to the subchronic toxicity of *Mucuna pruriens* seed extract from Indonesia. Therefore, this research aims to evaluate the subchronic toxicity of ethanol extract of *Mucuna pruriens* seed from Indonesia.

MATERIALS AND METHODS

Plant Material

Seeds of *Mucuna pruriens* were obtained from Bantul, Yogyakarta (07°44′04″S 110°12′34″E). The plant was authenticated in School of Life Sciences, Bandung Institute of Technology, Indonesia with 468/11.CO2.2/PL/2017 voucher specimen number.

Extraction of *M. pruriens* Seeds

The seeds were sun-dried and ground into powder. The powder was macerated with 70% ethanol for 3 × 24 hours at room temperature with daily solvent replacement. The liquid extract was evaporated at 40 °C under low pressure in a rotary vacuum evaporator. The dry extract was served as a suspension in 1% tragacanth, with several doses of extract.

Chemicals

Tragacanth, ethanol, distilled water, Turk solution, sodium citrate, formalin buffer solution and reagents for biochemistry test were obtained from local suppliers with technical and pro analyst quality.

Equipment

The equipment used were rotary evaporator, analytical balance, scale, mortar and stamper, needle oral for rat, syringes 3 cc, cage metabolism, platform for behavioural test, surgical equipment, Eppendorf tube, centrifuge Eppendorf, capillary tube for haematocrit, micropipette, hemocytometer, microscopes, counters, Sahli tube, UV-visible spectrophotometer, magnifying glass, microscope slides, glass cover, and commonly laboratory glassware.

Subchronic Toxicity Study

Animals. The rats of Wistar strain, 60 males and 60 females, aged six to eight weeks were obtained from the Bandung Institute of Technology. Variations of weight did not exceed 20% of the average weight.

Experimental Design. Both male and female rats were divided into six groups (six male groups and six female groups) with 10 rats in each group. The control group received tragacanth (1%) orally for 90 days. The three tested groups, received extract with dose of 50, 400, and 1000 mg/kg body weight respectively for 90 days. The satellite of control group received tragacanth (1%) for 90 days, then stopped and nourished until 120 days, and the satellite of high-dose group received extract with dose of 1000 mg/kg for 90 days, then stopped and nourished until 120 days. The extract was administered orally as much as 1 mL per 200 g of rat body weight (Jaijoy et al., 2011; Parasuraman, 2011; Yuet Ping, Darah, Chen, Sreeramanan, & Sasidharan,

2013). Behavioural and motoric activity was observed before and an hour after the administration of the extract on the first day until the 91st day for control and treated groups, and on the 121st day for satellite groups. Body weight was checked daily for 91 days for the control and tested groups and 121 days for the satellite groups. At the end of the study, the experimental rats were sacrificed. For haematological examination, blood samples collected from jugular vein were mixed with EDTA and for examination of clinical biochemistry, blood samples were centrifuged at 6000 rpm for 15 minutes. Haematological examination included examining the levels of hemoglobin, leukocytes, erythrocytes, platelets, haematocrit, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). Examination of clinical biochemistry included the level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, glucose, total cholesterol, and triglycerides. Quantitative determination was done by using UV-visible spectrophotometer with enzymatic reagent.

Macroscopic Observation. Liver, spleen, kidneys, heart, lungs, pancreas, brain, stomach, testis (males), and ovaries (females) were washed and dried with paper absorbed. The organs were observed macroscopically and weighed. Organ weights in comparison to body weights were taken to obtain relative organ weights. The condition of the gastric mucosa was also observed macroscopically.

Histopathology Determination. Liver fixed in 10% formalin buffer was dehydrated in graded alcohol, embedded in paraffin, and cut into 4 μm thick sections. Haematoxylin-eosin was used to stain it (Yuet Ping et al., 2013).

Statistical Analysis

Values are expressed as mean±SD (n = 10). Statistical analysis was done using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD). All statistical analyses were performed using SPSS 22.0 software. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Subchronic Toxicity

Orally administered ethanol extract of *Mucuna pruriens* seeds for 90 days did not cause any mortality and did not cause a significant change in body weight (Figure 1 and Figure 2). However, there was 20% to 30% decrease in motoric activity and muscle strength in the treated groups of both sexes. A decrease in motoric activity and muscle strength is possible due to increase in body weight. The weight loss observed was possibly caused by stress during the experiment. Both male and female groups showed normal gastric mucosa condition and asserted that the ethanol extract of *Mucuna pruriens* is safe for the stomach. This finding is in line with previous report by Golbabapour et al. (2013).

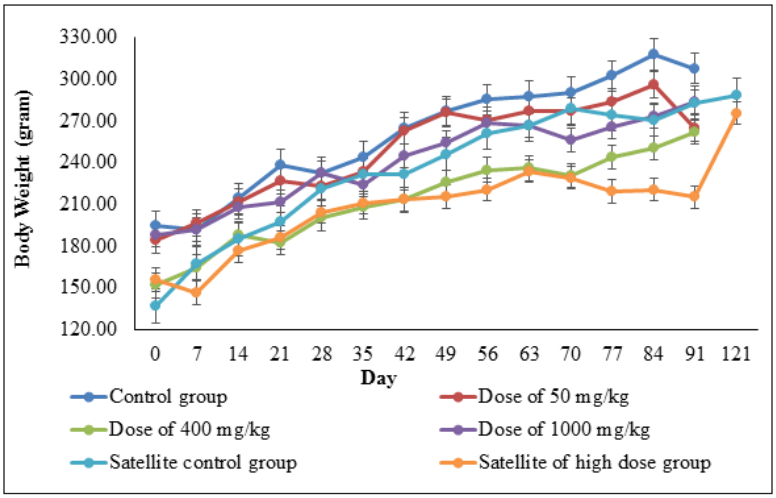

Figure 1. Profile of body weight gains in male rats during experiment

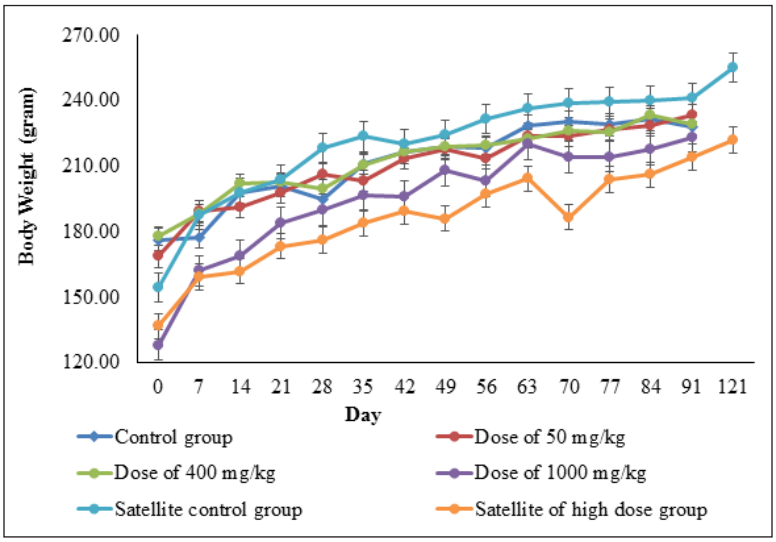

Figure 2. Profile of body weight gains in female rats during experiment

Haematology Parameters

Extract of *Mucuna pruriens* seeds did not affect most of the haematological parameters. Several parameters such as hemoglobin, erythrocytes, leukocytes, platelets, and haematocrit increased significantly, while the number of MCHC and MCH showed a decrease. Haematological examination results can be seen in Table 1. Increase in hemoglobin and erythrocyte is possibly caused by the minerals contained in *Mucuna pruriens* seeds such as Fe, Mg, Zn, Cu and ascorbic acid, which can help the formation of blood cell (Akindele & Busayo, 2011;

Table 1
Haematology parameters of male and female rats orally administered ethanol extract of Mucuna pruriens *seeds for 90 days*

Parameters	Control	Extract of Mucuna pruriens seeds (mg/kg)			Satellite groups	
		50	400	1000	Control	High-dose
Male						
Hemoglobin (gr/dL)	15,84±3.87	**18,44±2,31 (*P*=0,026[a])**	14,71±1,55 (*P*=0,330)	**12,12±2,64 (*P*=0,002[a])**	**12,09±1,00 (*P*=0,002[a])**	**16,38±1,75 (*P*=0,000[b])**
Leukocytes ($\times10^3\times mm^3$)	3,38±0.94	3,86±1,02 (*P*=0,471)	2,72±0,96 (*P*=0,322)	3,94±2,08 (*P*=0,401)	2,72±1,22 (*P*=0,322)	4,54±1,71 (*P*=0,368)
Erythrocytes ($\times10^6/mm^3$)	1,87±0.55	1,85±0,31 (*P*=0,965)	1,23±0,25 (*P*=0,167)	**4,12±2,97 (*P*=0,000[a])**	1,28±0,33 (*P*=0,203)	**2,3±0,53 (*P*=0,000[b])**
Platelets ($\times10^5/mm^3$)	2,52±0,69	2,00±0,20 (*P*=0,234)	2,90±0,42 (*P*=0,379)	**3,57±1,11 (*P*=0,016[a])**	**3,57±1,11 (*P*=0,016[a])**	2,93±0,83 (*P*=0,343)
Haematocrit (%)	42,85±5,37	**50,45±4,73 (*P*=0,035[a])**	43,68±4,48 (*P*=0,814)	**63,32±8,31 (*P*=0,000[a])**	44,61±10,52 (*P*=0,620)	**50,51±8,25 (*P*=0,000[ab])**
MCV (μ^3/sel)	2,52±0,95	2,78±0,42 (*P*=0,675)	3,69±0,86 (*P*=0,063)	2,43±1,43 (*P*=0,885)	3,72±1,32 (*P*=0,057)	2,30±0,60 (*P*=0,828)
MCHC (pg/sel)	37,43±9,93	36,60±3,44 (*P*=0,790)	33,89±4,14 (*P*=0,257)	**19,47±4,99 (*P*=0,000[a])**	**28,90±8,93 (*P*=0,007[a])**	**33,41±7,30 (*P*=0,000[b])**
MCH (g/dL)	92,54±36,31	101,04±11,99 (*P*=0,634)	122,99±21,26 (*P*=0,900)	**48,97±33,82 (*P*=0,016[a])**	100,89±29,71 (*P*=0,640)	74,82±19,18 (*P*=0,149)
Female						
Hemoglobin (gr/dL)	14,98±2,18	15,70±4,25 (*P*=0,53)	15,94±2,39 (*P*=0,408)	13,16±3,50 (*P*=0,118)	14,01±1,83 (*P*=0,403)	**10,56±1,49 (*P*=0,026[ab])**
Leukocytes ($\times10^3\times mm^3$)	3,52±1,48	2,54±0,60 (*P*=0,143)	**2,02±0,95 (*P*=0,026[a])**	**5,40±1,96 (*P*=0,006[a])**	3,90±2,04 (*P*=0,568)	4,18±1,86 (*P*=0,069)
Erythrocytes ($\times10^6/mm^3$)	1,43±0,41	1,17±0,38 (*P*=0,570)	1,33±0,56 (*P*=0,836)	1,96±1,33 (*P*=0,248)	1,32±0,45 (*P*=0,810)	1,45±0,49 (*P*=0,266)
Platelets ($\times10^5/mm^3$)	1,69±0,39	2,35±0,77 (*P*=0,125)	**2,69±0,70 (*P*=0,022[a])**	**3,10±2,58 (*P*=0,011[a])**	2,44±0,47 (*P*=0,084)	2,5±0,48 (*P*=0,169)
Haematocrit (%)	48,06±10,92	**40,85±6,74 (*P*=0,045[a])**	43,26±8,35 (*P*=0,179)	**61,81±4,03 (*P*=0,000[a])**	49,06±13,51 (*P*=0,778)	**45,44±1,91 (*P*=0,000[b])**
MCV (μ^3/sel)	3,66±1,39	3,99±1,95 (*P*=0,597)	3,82±2,05 (*P*=0,802)	3,92±1,38 (*P*=0,677)	4,26±2,09 (*P*=0,338)	3,46±1,10 (*P*=0,463)
MCHC (pg/sel)	32,45±8,17	38,46±9,26 (*P*=0,056)	37,83±7,76 (*P*=0,087)	**21,21±4,86 (*P*=0,000[a])**	29,88±6,83 (*P*=0,410)	**23,29±3,45 (*P*=0,004[a])**
MCH (g/dL)	111,04±35,95	145,87±55,88 (*P*=0,053)	144,58±80,29 (*P*=0,062)	82,47±31,24 (*P*=0,111)	119,20±45,07 (*P*=0,648)	80,27±28,93 (*P*=0,087)

Note: Values are expressed as mean±SD (n=10). $P < 0.05$ was considered significantly different using one-way ANOVA followed by least significant difference (LSD). [a]significant significantly different compared to control group. [b]significant significantly different compared to high-dose group

Table 2
Biochemistry parameters of male and female rats orally administered the ethanol extract of Mucuna pruriens *seeds for 90 days*

Parameters	Contol	Extract of Mucuna pruriens seeds (mg/kg)			Satellite groups	
		50	400	1000	Control	High-dose
Male						
Glucose (mg/dL)	120,80±15,59	133,9±18,42 (*P*=0,089)	135,70±20,11 (*P*=0,053)	110±24,64 (*P*=0,160)	116,90±16,26 (*P*=0,610)	111,8±18,20 (*P*=0,814)
Total Cholesterol (mg/dL)	58,23±33,45	100,90±38,41 (*P*=0,078)	**122,46±47,94 (*P*=0,008[a])**	**254,20±49,74 (*P*=0,000[a])**	**138,53±53,30 (*P*=0,001[a])**	**132,29±65,49 (*P*=0,000[ab])**
Triglyceride (mg/dL)	103,702±59,05	96,35±93,51 (*P*=0,775)	59,03±27,58 (*P*=0,085)	95,29±73,83 (*P*=0,744)	100,70±79,02 (*P*=0,907)	**46,19±23,13 (*P*=0,027[a])**
BUN (mg/dL)	25,02±3,61	**19,26±5,79 (*P*=0,007[a])**	27,71±2,71 (*P*=0,202)	**13,55±3,46 (*P*=0,000[a])**	25,73±4,48 (*P*=0,733)	**21,48±4,65 (*P*=0,000[b])**
Creatinine (mg/dL)	1,08±0,31	1,49±0,98 (*P*=0,148)	1,49±0,98 (*P*=0,148)	**2,00±0,89 (*P*=0,001[a])**	0,91±0,42 (*P*=0,845)	**1,02±0,39 (*P*=0,001[b])**
AST (U/L)	153,40±29,57	160,50±25,81 (*P*=0,564)	177,40±21,25 (*P*=0,053)	153,90±30,20 (*P*=0,968)	152,80±19,27 (*P*=0,961)	**180,60±34,26 (*P*=0,032[ab])**
ALT (U/L)	54,30±12,06	53,80±9,32 (*P*=0,925)	**74,30±10,21 (*P*=0,000[a])**	48,70±10,10 (*P*=0,291)	64,3±10,39 (*P*=0,061)	62,00±18,55 (*P*=0,148)
Female						
Glucose (mg/dL)	99,30 ±15,48	104,10±12,02 (*P*=0,530)	107,50±14,79 (*P*=0,285)	107,90±17,96 (*P*=0,262)	97,10±15,77 (*P*=0,774)	111,10±11,24 (*P*=0,676)
Total Cholesterol (mg/dL)	131,49±61,90	124,64±61,73 (*P*=0,776)	142,52±59,74 (*P*=0,646)	**276,40±52,72 (*P*=0,000[a])**	128,92±55,73 (*P*=0,915)	**80,35±53,42 (*P*=0,000[ab])**
Triglyceride (mg/dL)	119,52±80,20	96,93±66,56 (*P*=0,381)	**59,49±42,27 (*P*=0,021[a])**	**54,89±24,85 (*P*=0,013[a])**	83,05±24,78 (*P*=0,159)	**48,11±21,48 (*P*=0,006[a])**
BUN (mg/dL)	19,26±7,17	20,68±6,38 (*P*=0,499)	**25,27±3,83 (*P*=0,005[a])**	**23,61±2,60 (*P*=0,041[a])**	**23,96±4,61 (*P*=0,027[a])**	21,14±4,44 (*P*=0,241)
Creatinine (mg/dL)	1,18±0,46	1,95±0,45 (*P*=0,007)	1,30±0,50 (*P*=0,683)	**2,12±0,72 (*P*=0,001[a])**	1,15±0,55 (*P*=0,907)	**0,65±0,40 (*P*=0,000[b])**
AST (U/L)	143,1±28,42	112,60±25,92 (*P*=0,014)	117,80±18,71 (*P*=0,042)	107,40±15,32 (*P*=0,004)	**173,50± 47,04 (*P*=0,015[a])**	107,90±17,48 (*P*=0,005)
ALT (U/L)	40,8±8,65	**53,1±17,74 (*P*=0,022[a])**	48,4±9,67 (*P*=0,153)	47,2±14,88 (*P*=0,228)	49,80±6,48 (*P*=0,091)	44,2±6,02 (*P*=0,521)

Note: Values are expressed as mean±SD (n=10). P<0.05 was considered significantly different using one-way ANOVA followed by least significant difference (LSD). [a]significant significantly different compared to control group. [b]significant significantly different compared to high-dose group

Chukwudi, Simeon, & Chinyere, 2011; Ifemeje, 2016; Obioma, Emmanuel, Okechukwu, & Ifemeje, 2014; Tavares, Silva, Campos, Schuler, & Aquino, 2015). Increase in leukocyte is possibly triggered by a metabolic assault from alkaloid and/or phenolic contained in *Mucuna pruriens* (Chukwudi et al., 2011). However, an increase in leukocyte was still below normal levels (10,000/mm^3), which means it was not due to an infection.

Biochemical Parameters

The effect of ethanol extract of *Mucuna pruriens* seeds on biochemical parameters is presented in Table 2. There were no significant differences in glucose levels in male and female groups. The result in glucose levels supports the findings of the ability of *Mucuna pruriens* to maintain the levels of glucose (Bhaskar, Vidhya, & Ramya, 2008; Bhutkar & Bhise, 2013; Eze et al., 2012; Majekodunmi et al., 2011).

However, there were significant changes in total cholesterol and triglyceride levels. Many studies have reported that *Mucuna pruriens* has a powerful anti-cholesterol effect, which can be attributed to its high antioxidant activity. The hypocholesterolemic effects of *Mucuna puriens* may be due to several mechanisms, including inhibition of the cholesterol biosynthesis, prevention of low-density lipoprotein (LDL) oxidation by flavonoids, conversion of cholesterol to bile acids, and inhibition of cholesterol absorption from the intestine by the formation of complexes with compounds such as glycosides and saponins (Dhanasekaran, Tharakan, & Manyam, 2008; Enechi & Ozougwu, 2014). Besides helping to reduce LDL or bad cholesterol, triglyceride and total cholesterol levels, *Mucuna pruriens* also helps in increasing the level of high-density lipoprotein (HDL) or good cholesterol. Therefore, an increase in total cholesterol levels in this study is possibly due to the increase of HDL.

The level of BUN and creatinine in the treated groups showed significant changes too. These changes were reversible, indicated by the change of BUN and creatinine levels close to normal when the administration of the extract was stopped as shown in the satellite of high-dose groups. It indicated that the extract of *Mucuna pruriens* seeds did not cause any significant damage to the kidneys. This finding was also supported by the result of histopathologic assessment.

Further, the extract of *Mucuna pruriens* seeds induced significant changes of ALT. The level of ALT significantly increased, indicating hepatotoxic that causes liver damage (Giannini, Testa, & Savarino, 2005). However, an increase in ALT levels of the rats is relatively safe because the value is slightly higher than normal. This finding is in line with previous reports (Ezeja & Omeh, 2010; Obioma et al., 2014) that showed a hepatotoxicity of *Mucuna pruriens*. However, other reports showed hepatoprotective effects of *Mucuna pruriens* (Chukwudi et al., 2011; Obogwu, Akindele, & Adeyemi, 2014).

Macroscopic Observations (The Relative Organ Weight)

Based on the relative organ weight measurement, the female group showed significant differences both in kidneys and liver, while the male group only showed a significant difference in liver (Table 3). Compared to the results of its biochemical parameters, the significant difference in relative organ weight of the kidneys did not affect the function of the organ. This was also reinforced by the results of relative organ weight measurements of high-dose satellite group which did not show any significant difference with the normal group. It also indicated that toxicity effects of the extract are reversible. However, the significant differences in relative organ weight of liver in both male and female groups indicate toxicity of the extract to the liver.

Table 3
Relative organ weight for all groups after administration of extract of Mucuna pruriens *seeds for 90 days*

Organ	Contol	Extract of Mucuna pruriens seeds (mg/kg)			Satellite groups	
		50	400	1000	Control	High-dose
Male						
Liver	2,341 ± 0,262	2,430 ± 0,202 (P=0,489)	**2,998 ± 0,208 (P=0,000[a])**	**2,624 ± 0,267 (P=0,029[a])**	**2,815 ± 0,214 (P=0,000[a])**	**2,827 ± 0,144 (P=0,000[a])**
Heart	0,257 ± 0,027	0,319 ± 0,033 (P=0,272)	0,282 ± 0,014 (P=0,665)	0,299 ± 0,032 (P=0,458)	0,299 ± 0,032 (P=0,459)	0,311 ± 0,039 (P=0,837)
Lungs	0,637 ± 0,120	**0,835 ± 0,266 (P=0,012[a])**	0,587 ± 0,119 (P=0,522)	**0,842 ± 0,239 (P=0,009[a])**	0,657 ± 0,122 (P=0,797)	**0,618 ± 0,116 (P=0,004[b])**
Kidneys	0,566 ± 0,067	0,640 ± 0,059 (P=0,063)	0,612 ± 0,038 (P=0,250)	0,611 ± 0,068 (P=0,259)	0,614 ± 0,054 (P=0,226)	0,567 ± 0,052 (P=0,267)
Spleen	0,182 ± 0,046	0,161 ± 0,027 (P=0,407)	0,170 ± 0,061 (P=0,633)	0,181 ± 0,068 (P=0,965)	0,184 ± 0,073 (P=0,937)	0,228 ± 0,059 (P=0,066)
Stomach	0,447 ± 0,071	0,443 ± 0,052 (P=0,937)	0,443 ± 0,078 (P=0,930)	0,402 ± 0,141 (P=0,305)	0,469 ± 0,081 (P=0,605)	0,443 ± 0,051 (P=0,346)
Brain	0,624 ± 0,080	**0,730 ± 0,103 (P=0,034[a])**	0,711 ± 0,065 (P=0,081)	0,684 ± 0,104 (P=0,229)	0,669 ± 0,091 (P=0,366)	0,686 ± 0,064 (P=0,956)
Testes	0,478 ± 0,059	0,227 ± 0,084 (P=0,163)	0,535 ± 0,096 (P=0,320)	0,527 ± 0,035 (P=0,394)	0,532 ± 0,085 (P=0,345)	0,505 ± 0,061 (P=0,703)
Female						
Liver	2,571 ± 0,193	**3,292 ± 0,477 (P=0,000[a])**	**2,956 ± 0,173 (P=0,003[a])**	**3,139 ± 0,161 (P=0,000[a])**	**3,360 ± 0,489 (P=0,000[a])**	**3,154 ± 0,373 (P=0,000[a])**
Heart	0,322 ± 0,036	0,324 ± 0,056 (P=0,970)	0,313 ± 0,025 (P=0,869)	0,310 ± 0,034 (P=0,827)	0,463 ± 0,422 (P=0,014)	0,357 ± 0,373 (P=0,409)
Lungs	0,620 ± 0,177	0,653 ± 0,205 (P=0,666)	0,613 ± 0,131 (0,921)	0,628 ± 0,111 (P=0,919)	0,902 ± 0,228 (P=0,000)	0,680 ± 0,133 (P=0,503)
Kidneys	0,667 ± 0,095	**0,530 ± 0,177 (P=0,001[a])**	**0,565 ± 0,028 (P=0,011[a])**	**0,564 ± 0,097 (P=0,010[a])**	0,620 ± 0,142 (P=0,234)	0,624 ± 0,064 (P=0,130)

Table 3 *(continue)*

Organ	Contol	Extract of Mucuna pruriens seeds (mg/kg)			Satellite groups	
		50	400	1000	Control	High-dose
Female						
Spleen	0,375 ± 0,058	**0,232 ± 0,045 (P=0,000[a])**	**0,308 ± 0,040 (P=0,009[a])**	**0,305 ± 0,027 (P=0,006[a])**	**0,240 ± 0,102 (P=0,000[a])**	0,340 ± 0,062 (P=0,156)
Stomach	0,493 ± 0,072	0,580 ± 0,182 (P=0,050)	0,452 ± 0,154 (P=0,339)	0,465 ± 0,068 (P=0,511)	0,464 ± 0,053 (P=0,499)	0,508 ± 0,040 (P=0,320)
Brain	0,780 ± 0,149	**0,673 ± 0,240 (P=0,034[a])**	0,811 ± 0,055 (P=0,526)	0,806 ± 0,052 (P=0,601)	0,735 ± 0,115 (P=0,367)	0,847 ± 0,063 (P=0,408)
Ovaries	0,380 ± 0,117	0,458 ± 0,176 (P=0,172)	0,382 ± 0,183 (P=0,976)	0,431 ± 0,119 (P=0,369)	0,276 ± 0,069 (P=0,942)	**0,562 ± 0,247 (P=0,022[ab])**

Note: Values are expressed as mean±SD (n=10). P<0.05 was considered significantly different using one-way ANOVA followed by least significant difference (LSD). [a]significant significantly different compared to control group. [b]significant significantly different compared to high-dose group

Histopathologic Assessment

A mild glycogen depletion showed in the tissue of the kidneys of the treated groups. Figure 3 shows the tissue of the liver in the treated groups that had moderate glycogen depletion and enlargement of nuclei in some cells of the liver. Although this indicates toxicity effects of the extract, significant pathological changes in the kidneys and liver did not show the phase of necrosis (cell death), thus the kidneys and liver were still quite functional. The satellite of high-dose groups of both genders also showed recovery of the tissue, either in the kidneys and the liver asserted reversible toxic effects of the extract on the kidneys and liver.

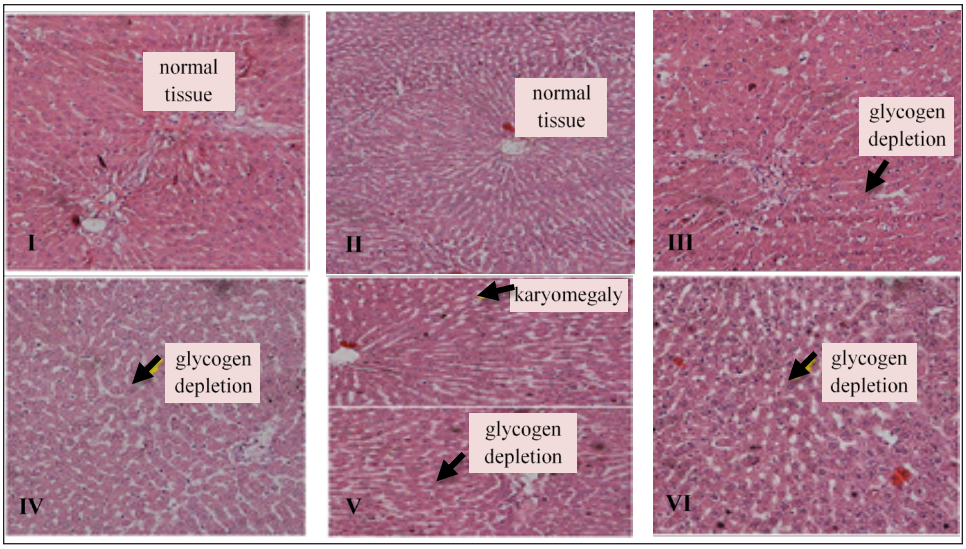

Figure 3. Histopathology of liver tissues

The source of liver are: (I) liver from the control group, (II) liver from satellite control group, (III) liver from a rat treated with 50 mg/kg extract of *Mucuna pruriens* seeds, (IV) liver from a rat treated with 400 mg/kg extract of *Mucuna pruriens* seeds, (V) liver from a rat treated with 1000 mg/kg extract of *Mucuna pruriens* seeds, and (VI) liver from the satellite of high-dose group.

CONCLUSION

Repeated oral consumption of ethanolic extract of *Mucuna pruriens* seed for 90 days did not cause death. The extract did not cause any change in food intake, water consumption, and body weight change. It also did not cause any significant change to the haematology and biochemical parameters. However, the extract caused glycogen depletion and enlargement of nuclei in some cells of the kidneys and liver but did not cause cell death. The toxic effect is reversible characterised by recovery of the kidney and liver tissues in the satellite of high-dose groups after the extract was no longer administered.

CONFLICT OF INTEREST STATEMENT

The authors declare that they did not face any conflict of interest while conducting this study.

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Short Communication

Study of the Tolerance of Black Sea Cucumber *Holothuria leucospilota* to Hypoxia Stress

Neviaty P. Zamani, Khoirunnisa Assidqi* and Hawis H. Madduppa

Department of Marine Science and Technology, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University (IPB), Bogor, Indonesia

ABSTRACT

The aim of this study was to investigate the survival rates and the behavioural responses of *Holothuria leucospilota* under hypoxia stress condition. A total of 15 *H. leucospilota* were collected from Rambut Island, in Thousand Islands, Jakarta, Indonesia. They were exposed to three different dissolved oxygen concentrations (i.e. 2 mg O_2 L^{-1}, 3 mg O_2 L^{-1}, >6 mg O_2 L^{-1}). Mortality was measured at the 4th and 6th day by adding 2 mg O_2 L^{-1} and 3 mg O_2 L^{-1} of dissolved oxygen concentrations respectively. The survival rates of *H. leucospilota* significantly decreased by 80% at 2 mg O_2 L^{-1} and 40% at 3 mg O_2 L^{-1} compared to > 6 mg O_2 L^{-1} in which total survival was 100% at the end of 22 days of exposure to hypoxia. The behavioural responses of *H. leucospilota* to hypoxia stress were shown by decreased metabolic activity by releasing the contents of their stomach described as cuvierian tubules. The lower dissolved oxygen concentrations at 2 mg O_2 L^{-1} and 3 mg O_2 L^{-1} showed a higher metabolic rate in 20% and 50 % of individual organisms during 18 days hypoxia exposure. If their metabolic rate is high, they will need more energy reserves to compensate it. The result of this study revealed that the lowest dissolved oxygen at 2 mg O_2 L^{-1} can exert a stress on *H. leucospilota* which can lead to a high mortality and an impairment of behavioural responses.

Keywords: Behavioural responses, cuvierian tubules, hypoxia, invertebrate, *Holothuria leucospilota*

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E-mail addresses:
npzamani@gmail.com (Neviaty P. Zamani)
nisaassidqi@gmail.com (Khoirunnisa Assidqi)
madduppa@gmail.com (Hawis H. Madduppa)
* Corresponding author

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INTRODUCTION

Black sea cucumber *Holothuria leucospilota* (Brandt, 1835) is a tropical shallow reef holothurian species. *H. leucospilota* as well as other benthic organisms have important roles in the benthic ecosystem. At least seven species, including *H. leucospilota*, are found in Thousand Islands, Jakarta, Indonesia (Madduppa et al., 2017). Holothurian can be used as biotubators, the number of bacterial abundance and the exchange of nutrients and dissolved oxygen in water or marine coastal sediments (MacTavish, 2012). Some bacteria communities in holothurian guts are observed as having the potential to keep the balance in nutrient cycling such as microbes (Amaro et al., 2009; Zhang et al., 2012). Schneider et al. (2011) recorded holothurian that has a role in the coral reef ecosystem to balance $CaCo_3$. Holothurians are currently used as food supplement, food sources and medicine. Mohammadizadeh et al. (2013) found bioactive compounds from *H. leucospilota*, as having antibacterial and antifungal properties. Hence, holothurian is not only commercially valuable, but also has an ecological function for sustaining marine life.

Currently, the major problem affecting the marine ecosystem is related to threats from anthropogenic activities. Nutrients and pollutants enter the marine environment, not only as dissolved solids, but some of them are suspended solids which will be deposited in the sediment. This situation will potentially lead to sedimentation leading to oxygen depletion. Sedimentation of suspended solids may have an effect on holothurian who may not be able to adapt to high levels of sedimentation. Gray et al. (2002) reported that marine life cannot survive when concentration of dissolved oxygen in the water is below 2 mg O_2 L^{-1} to 0.5 mg O_2 L^{-1}. The primary effect on the marine environment is eutrophication, as a result of dissolved oxygen concentration, due to over-enrichment by nutrients. Hypoxia also can be built up under this condition. Consequently, it will lead to the loss biodiversity in the benthic ecosystem services, since the marine organisms will not survive in heavy sedimentation (Worm et al., 2006).

There are internal and external disturbance factors which cannot be predicted in the marine environment (Vilnäss et al., 2012). The internal factor is related to global warming whereby the increasing temperature can lead to reduce oxygen levels in the ocean. The external factor relates to anthropogenic activities which can lead to a drastic decrease in oxygen level. Thus, it would impair the performance of holothurian. Hypoxia phenomenon affects the survival of marine organisms in the ecosystems. Some studies have been reported that hypoxia has become a stressor and has a negative impact on the sustainability of marine organisms leading to reduction in growth and reproduction (Huang et al., 2012; Loddington, 2011; Vaquer-Sunyer & Duarte, 2008).

There is a connection between the physical and biological processes in the marine coastal environment. Holothurian is a deposit feeder which respires with all

tentacles around their skin and consumes a lot of water and absorbs dissolved oxygen. Therefore, this study was conducted to investigate the survival rates and the behavioural of *H. leucospilota* exposed to hypoxia stress tolerance with *ex situ* observations in different dissolved oxygen concentrations. Studies have investigated the cause of hypoxia can lead to negative effect which reverses the loss of biomass on benthic organisms and changes behavioural and physiological responses as a feedback mechanism (Diaz & Rosenberg 1995; Diaz & Rosenberg, 2008; Huo et al., 2018). Since hypoxia condition can constitute a stress for sea cucumbers, we hypothesise the survival rates and behavioural responses to decrease with decreasing concentration of dissolved oxygen.

MATERIAL AND METHODS

Study Site and Sea Cucumber Collection

Individuals of the black sea cucumber *H. leucospilota* were collected in Rambut Island, Kepulauan Seribu, Jakarta, Indonesia (5°58'30.9"S 106°41'42.9"E) during low tide (Figure 1). *H. leucospilota* were found fine-grained sediments just below the low tide line. The length range of sea cucumbers used for the experiment were between 10 cm and 15 cm. The sea cucumbers were kept in cooled insulation box with seawater. During transportation into the laboratory, half of the seawater in cooled insulation box was exchanged every two hours with new stock of the seawater supply. This is to provide an oxygen circulation inside the box. A total of 15 individuals was used in

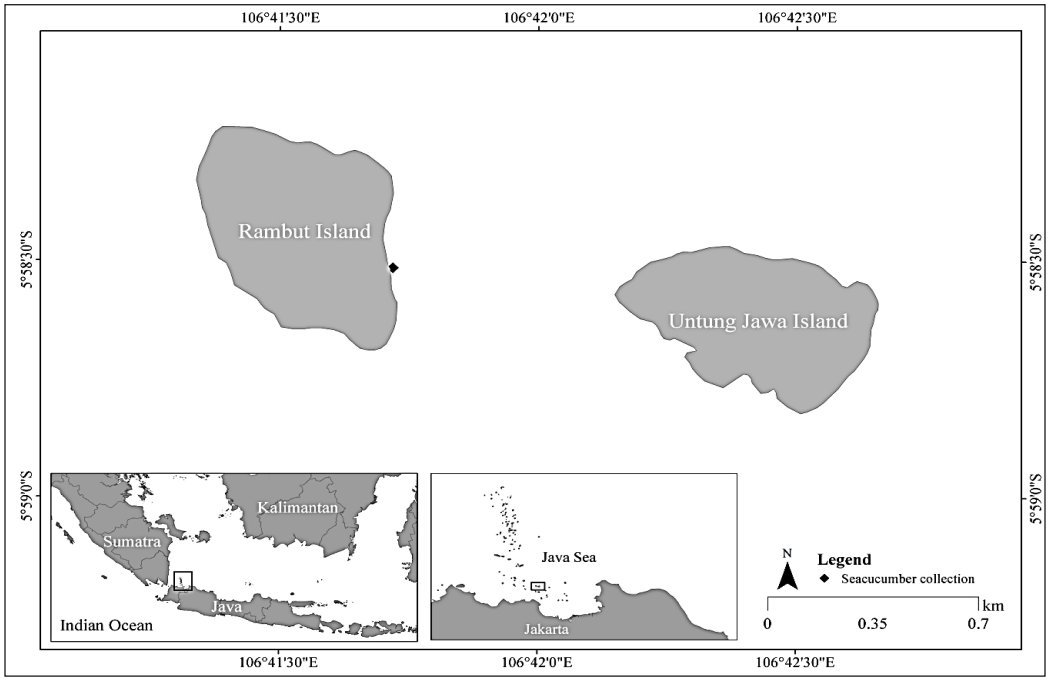

Figure 1. Map of Rambut Island, Jakarta, Indonesia showing the position of sampling *H. leucospilota* with black diamond bullet

the experiment, where each concentration levels used 5 individuals; there were three groups of 5 sea cucumbers each, all having similar length size. The low number of individuals was due to limited availability of sea cucumbers in the field. The sea cucumbers were acclimatised to laboratory conditions, every 5 sea cucumbers were transferred into a glass aquarium with 20 litres of seawater with constant aeration. During post acclimatisation in laboratory conditions for 10 days, 20% of sediment was added from the total volume. The seawater was changed daily to prevent accumulation of metabolic waste.

Experimental Setup

The hypoxia circulation system comprised three different dissolved oxygen (DO) concentrations (2 mg O_2 L^{-1}, 3 mg O_2 L^{-1}, >6 mg O_2 L^{-1}1) (Table 1). Each treatment group consisted of 5 individuals kept in individual containers with 2 l of seawater. The DO concentrations were measured using a digital oxygen meter, the sensor type is CellOx 325 for WTW Oxical 3205, Weilheim, Germany. The hypoxic water collected in the header tank were placed in containers. The inflow was connected to the seawater header tank which had one hole on the top nitrogen inflow. The concentration of dissolved oxygen in the seawater header tank was reduced by pumping bubbling nitrogen gas into it (Cheung et al., 2008; Long et al., 2008; Seitz et al., 2003). According to Vaquer-Sunyer & Duarte (2008), the threshold for hypoxia for echinoderms is ≥ 2 mg O_2 L^{-1}. The target DO concentrations were reached at 2 mg O_2 L^{-1}, 3 mg O_2 L^{-1}, >6 mg O_2 L^{-1}. The DO concentrations of >6 mg O_2 L^{-1} was chosen as the normal condition. The outflow distributed the seawater to fill in the experimental containers (Figure 2).

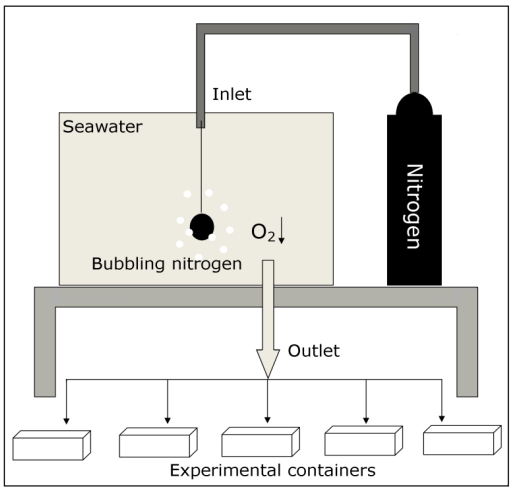

Figure 2. Design of hypoxia circulation system with one bottle of nitrogen immersed in the tank and later stored in the container

Table 1
Treatment groups for hypoxia stress tolerance

Treatment groups	Concentration levels of DO	Number of individuals
X	2 O_2 mg L^{-1}	5 individuals
Y	3 O_2 mg L^{-1}	5 individuals
Z	>6 O_2 mg L^{-1}	5 individuals

Each animal was placed in a tight closely to prevent re-oxygenation of the water. The seawater in the containers was exchanged daily. When changing the water, the containers and the top of the header

tank had to be opened until the water body inside the containers was entirely replaced with hypoxic water from the header tank. The normoxia condition was applied >6 mg O_2 L^{-1} as control and aerated. However, presumably for all DO concentrations did not feed the sea cucumbers during the hypoxia test (Cheung et al., 2008).

In the laboratory study, the lighting condition was simulated like in the marine environment which had a day light on and off (12:12 h). During the experiment, the room temperature at 25 - 27°C and the salinity at 31 - 32‰ was controlled. 3 levels of dissolved oxygen concentration were applied (Table 1). Their behavioural responses under hypoxia condition and the surviving individual was observed twice a day. The saline and the ammonia concentration was measured at the beginning and at the end (death revealed) to ensure it was not caused by increasing salinity and ammonia concentrations (Table 2).

Table 2
Water quality checking data during hypoxia stress tolerance

Treatment groups	Water quality (mean ± SD)	
	Salinity (‰)	Ammonium ($NH4^+$) (mg L^{-1})
X	31.10 ± 0.22	< 0.05 ± 0
Y	31.10 ± 0.22	< 0.05 ± 0
Z	31.10 ± 0.22	< 0.05 ± 0

Statistical Analysis

The survival rates and the behavioural responses of *H. leucospilota* data were analysed using Cox proportional hazard test (Bewick et al., 2004; Goel et al., 2010). Data was analysed using as Kaplan Meier curves which showed the probability for the number of individuals to median time. All statistical tests and curves were calculated and produced using the free R-statistic software (version 3.1.2 (2014-10-31) "Pumpkin Helmet"). The significance result was assumed if p-value was lower than 0.05.

RESULTS AND DISCUSSION

H. leucospilota was exposed to hypoxia stress for 22 days, and it was found that behavioural responses as well as survival rates were low percentages due to oxygen depletion (Figures 3 and 4). Diaz and Rosenberg (1995) had studied three types of oxygen concentrations, the normoxia condition, the moderate hypoxia and severe hypoxia. As shown in Figure 3, 3 mg O_2 L^{-1} affected behavior of the specimens by 50% in 18 days compared with 2 mg O_2 L^{-1}. The different oxygen concentrations had a significant influence on the behavior of *H. leucospilota* which ejected their cuvierian tubules. 20% of the individual organisms showed the behavior by ejecting their cuvierian tubules upon exposure to 2 mg O_2 L^{-1} during 18 days (Figure 3). It was proven by Cox-ph test: Chisq = 8.1249, df = 2, p = 0.01721*. Therefore, that holothurian is more susceptible to 2 mg O_2 L^{-1} concentration.

The line drop of the survival rates in all oxygen concentrations indicated the death phase of holothurians. The survival rates of *H. leucospilota* even showed a decrease of 18% on day 4 at 2 mg O_2

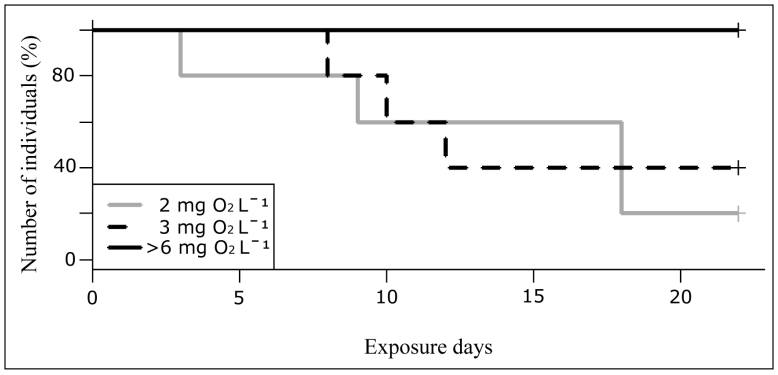

Figure 3. The behavioural responses of *H. leucospilota* showed by ejection of cuvierian tubules during hypoxia stress tolerance with different oxygen concentrations for 22 days (n=5)

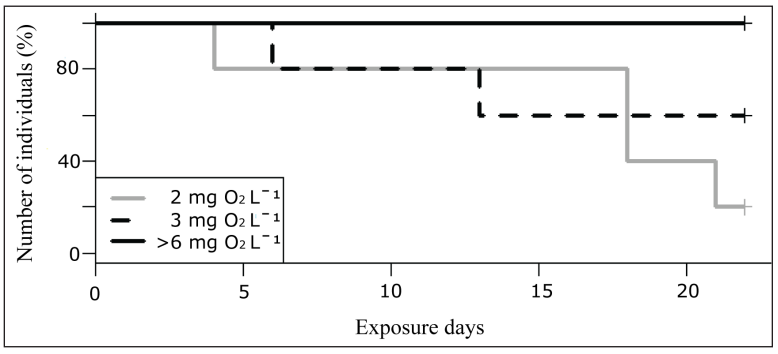

Figure 4. The survival rates of *H. leucospilota* showed by declining curves during hypoxia stress tolerance with different oxygen concentrations for 22 days (n=5)

L^{-1}. The lowest concentration (2 mg O_2 L^{-1}) had only 20% of initial individual organisms (Figure 4). This study revealed the median survival, individual organism in hypoxia concentration decreased with the lowest hypoxia concentration with a strong significant trend (Figure 4, Coxph-test: Chisq = 7.1477, df = 2, p = 0.02805*). The lowest concentration (2 mg O_2 L^{-1}) has a faster death rate compared with the higher concentration (3 mg O_2 L^{-1}) and normal concentration (>6 mg O_2 L^{-1}). At the end of this study, metabolic rate was not measured as it was not available due to the damaged body which was dissolved in the seawater.

H. leucospilota are deposit feeders and under normal conditions, they will use their peltate tentacles to push sediment into their mouths. The behavior responses of Holothurians were examined under hypoxia condition. These behavioural responses were very slow movement with a big open mouth (Figure 5A), and the colour of their tentacles changed from black to white (Figure 5B). This is shown when they eject their cuvierian tubules (Figure 5C). This confirms that the behavioural responses of *H. leucospilota* were strongly affect concentration. Oxygen concentration. Oxygen consumption indicates that holothurian can be very

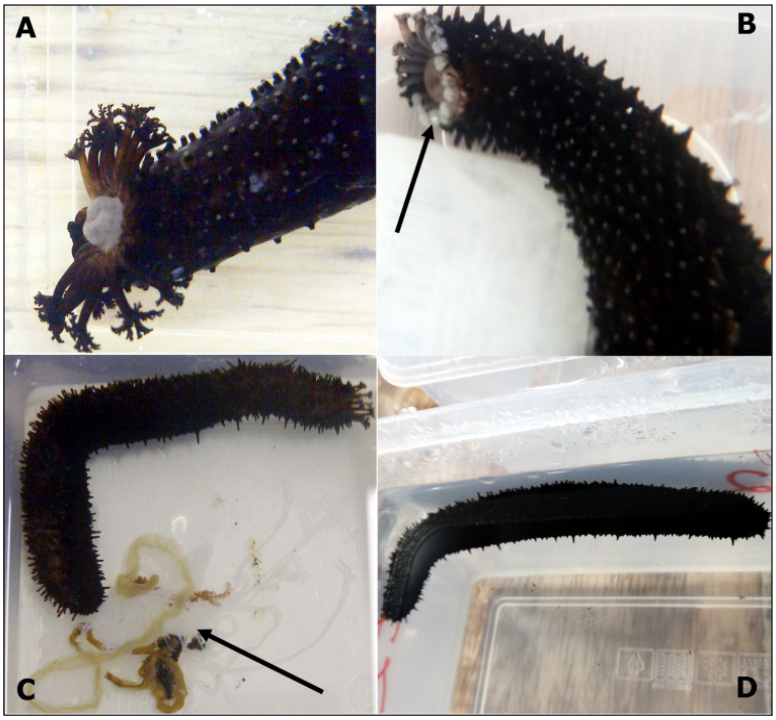

Figure 5. The different appearances under hypoxia (2 mg O_2 L^{-1}; 3 mg O_2 L^{-1}) and normoxia condition (>6 mg O_2 L^{-1}) (A) The anterior body hanging on the top and with a big open mouth (B) The colour of the tentacles changed from black to white (C) *H. leucospilota* ejected part of cuvierian tubules (D) *H. leuscopilota* under normoxia condition.

opportunistic and reduce energy expenditure to a minimum when oxygen is in short in supply (Siikavuopio et al., 2008).

The highest percentages were seen under normoxia condition (>6 mg O_2 L^{-1}), as holothurians did not eject their cuvierian tubules. Under normoxia condition, *H. leucospilota* moved around from one part to another part and looked healthier than in hypoxia condition (Figure 5D). This indicates that the holothurians did not receive any stressor under normoxia condition. The relationship between hypoxia and holothurians behavior is strongly affected by oxygen depletion concentrations.

Astall and Jones (1991) said that holothurians reduced their metabolic rate by ejecting the cuvierian tubules under hypoxia condition. It was relevant to our result which showed the lower dissolved oxygen concentrations led to holothurians ejecting their cuvierian tubules during the experiment. It was observed the lower oxygen concentrations showed a higher metabolic rate, if their metabolic rate is high, they needed more energy reserves to compensate it. They saved their energy with aestivation activity and they ejected their cuvierian tubules to keep their energy levels high.

Specifically, Holothurians ejected their cuvierian tubules as a defence mechanism (Becker & Flammang, 2010; Demeuldre et al., 2014). If they cannot produce cuvierian tubules, their predators can spot them easily and thus, increasing the risk of biodiversity loss. According to Cheung et al. (2008) prolonged reduced oxygen level at 1.5 mg O_2 L^{-1} decreased food consumption in marine scavenging gastropods *Nassarius festivus*. Hypoxia reduces growth of marine organisms and it increases their mortality rate (Rabalais et al., 2002).

There is a relationship between decrease in dissolved oxygen concentrations to holothurians behavioural responses and impact on their mortality. Hypoxia has consequences for living resources and an impact on benthic ecosystem services. The holothurians tried to survive during hypoxia, but unfortunately, some of them died, because they failed to aestivate. There were no significant differences in behavioural responses between under 2 mg O_2 L^{-1} and 3 mg O_2 L^{-1}, except for faster ejection of cuvierian tubules and followed by the mortality. 2 individuals survived until the end of the experiment during hypoxia condition in 3 mg O_2 L^{-1}, and therefore it can be assumed that they succeeded to aestivate themselves (Yang et al., 2006). The threshold for the hypoxia of holothurians was estimated at 3 mg O_2 L^{-1}. This study revealed that holothurian is susceptible to reduction of dissolved oxygen. Holothurians always eject parts of their cuvierian tubules when the environmental conditions do not support their lives.

Holothurian as well as *H. leucospilota* is an important species in benthic ecosystem services. They play an important functional role in the ecology of coastal ecosystem and coral reef ecosystem and a marker of eutrophication within the marine environment. The presence of *H. leucospilota* has an ecological role and in the trophic levels. Holothurian has been considered as one of the marine organisms which recycles nutrient in the coral reefs and seagrass beds ecosystem (Uthicke, 2001; Wolkenhauer et al., 2009). Therefore, Timmerman et al. (2012) concluded nutrient loading had a positive support to the biomass of benthic macrofaunal communities, but it also had potential to increase hypoxia which affected benthic biomass. Hypoxia condition has a negative impact on the marine life and the diversity of marine organism. If one of the marine species in an ecosystem cannot be sustained, this could create new problems for the food web in the ecosystem. This was proven by the Long et al. (2008), and Long and Seitz (2008) that the prey species would be easily detected by their predators.

CONCLUSION

The study showed survival rates of *H. leucospilota* due to hypoxia exposure had declined significantly. The behavioural responses of *H. leucospilota* to hypoxia stress were shown by their decreased metabolic activity by releasing the contents of their stomach described as cuvierian tubules. The results revealed that *H. leucospilota* was susceptible to a low dissolved oxygen less than (2 mg O_2 L^{-1}).

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Short Communication

Comparison of *Nannochloropsis oculata* Productions Cultivated in Two Different Systems: Outdoor Red Tilapia (*Oreochromis* sp.) Culture Tank and Indoor Pure Culture

Ching Fui Fui[1], Sri Yuliani Cancerini[1,2], Rossita Shapawi[1] and Shigeharu Senoo[3]

[1]Borneo Marine Research Institute, Universiti Malaysia Sabah (UMS), 88400 Kota Kinabalu, Sabah, Malaysia
[2]Pt Japfa Comfeed Indonesia Wisma Millenia, Lt. 7 Jl. MT. Haryono Kav. 16 Jakarta 12810, Indonesia
[3]Aquaculture Institute, Kindai University, 3153 Shirahama-cho, Wakayama 649-2211, Japan

ABSTRACT

Production of *Nannochloropsis oculata* or a marine eukaryotic unicellular phytoplankton was the focus of this study. The cultivation of outdoor red tilapia (O*reochromis* sp.) in the tank and indoor was compared in producing phytoplankton. Initial density of *N. oculata* for both culture systems was 0.5×10^6 cell/ml. Findings showed the highest density of *N. oculata* was attained from tilapia culture system at 9.6×10^6 cell/ml harvested at day 7 while in pure culture system was 8.5×10^6 cell/ml harvested at day 4. Contamination was dominated by protozoa (*Gymnodinium* sp.), range of $4.80\text{-}36.67 \times 10^3$ individual cells/ml and $0.00\text{-}41.10 \times 10^3$ individual cell/ml at both tilapia culture and pure culture systems respectively. Levels of ammonium, nitrite and nitrate in tilapia culture systems had significantly lower ($P < 0.05$) concentration. In contrast, total bacteria including vibrio yellow colonies showed higher concentration in tilapia culture system but remained insignificant ($P > 0.05$) for vibrio green colonies in both systems. This study concluded Tilapia culture system is as effective as pure culture system to produce *N. oculata* based on production and quality.

Keywords: Nannochloropsis oculata, pure culture system, tilapia culture system

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E-mail addresses:
cfuifui@ums.edu.my (Ching Fui Fui)
ririndayu@gmail.com (Sri Yuliani Cancerini)
rossita@ums.edu.my / rshapawi@gmail.com (Rossita Shapawi)
sesige@mac.com (Shigeharu Senoo)
* Corresponding author

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INTRODUCTION

Nannochloropsis oculata is a marine microalga from class Eustigmatophyceae (Hibberd, 1981) with diameter size of 2-4 µm (Rodolfi, Zittelli, Barsanti, Rosati, & Tredici, 2002). *N. oculata* has been long considered as one of the most important algae species in marine aquaculture industry particularly owing to its numerous nutritional values including eicosapentaenoic acid (EPA) (Sukenik, 1999), high lipid content (Fabregas, Maseda, Domínguez, & Otero, 2004) and some natural pigments (Lubián, Montero, Moreno-Garrido, Huertas, & Sobrino, 2000). Common cultivation of *N. oculata* in most of the marine fish and crustacean hatcheries is by initially culturing the seed in the laboratory through series of seed upscale from agar to flask, and subsequently mass production will be carried out outdoor. However, there are many constraints and challenges in mass production of *N. oculata* at the early stage (indoor laboratory phase) due to the requirement of specific culturing technique and often involving high production cost. Culture medium for *N. oculata* utilises natural or artificial seawater fertilised with macro and micronutrients based on formulation of F2 medium (Guillard, 1975) or Conway medium (Walne, 1979). Till data, an effective yet economical fertiliser medium is yet to be developed (Bae & Hur, 2011; El Nabris, 2012; Fabregas, Toribio, Abalde, Cabezas, & Herrero, 1987). However, undoubtedly pure culture of *N. oculata* is still significant as it serves as the primary source of seed to initiate mass scale production through an outdoor culturing system.

This study proposed an alternative method of culturing *N. oculata* by using green water from red tilapia (*Oreochromis* sp.) culture system as a fertiliser instead of conventional mediums and fertiliser. Red tilapia was chosen because it is cheap, easy to be cultured and it available all year round. It also has been reported that red tilapia can promote the production of phytoplankton in the ponds or lake by recycling nutrient through excretion (Elser, Marzolf, & Goldman, 1990; McQueen, Post, & Mills, 1986). Nitrogenous organic waste comes from uneaten feeds and excretion of fishes are used by microalgae as nutrients for growth. In a previous study, *Chlorella* sp., a freshwater microalga has been successfully produced in red tilapia culture system (Matsubara, 2011). The objective of this study was to compare the production and quality of *N. oculata* cultured under two different systems (pure culture and tilapia culture system).

MATERIALS AND METHODS

This experiment was conducted at Marine fish Hatchery of PT Suri Tani Pemuka in Pemuteran, Bali, Indonesia in 2015. Two different culturing systems of *N. oculata* were prepared and compared, namely: (1) pure culture; and (2) red tilapia culture. *N. oculata* seed was obtained from Gondol Research Station for Coastal Fisheries Bali, Indonesia. In pure culture system, *N. oculata*

seed was initially cultured in laboratory and fertilised using f/2 medium (Guillard, 1975) and later upscaled from 100 ml flask into 18 gallons. Next, the seeds were gradually transferred into a 10 tonne tank made of high-density polyethylene (HDPE) placed at the outdoor area and fertilised with agricultural grade fertiliser. The water quality was maintained as follows: dissolved oxygen, DO (6.5 to 7.5 mgL^{-1}), pH (7.5 to 8.0) and temperature (28 to 32ºC). Tanks were filled with 20 ppt saline water up to 800 litres and aerated evenly. Tanks were left uncovered to allow sunlight penetration for photosynthesis to take place. Light intensity ranged from 10,000 – 12,000 lux (light meter 840020, Sper Scientific, USA). The seeds were further fertilised using local improved formula of agricultural grade fertiliser (PT. Andalan Chemist, Kalimantan Timur, Indonesia). Three of one tonne tank were used as experimental tank and the initial seed of *N. oculata* was introduced at 1.0×10^6 cells/mL. Similar to pure culture system, three 1 tonne HDPE tank were used as experimental tank and the initial seed of *N. oculata* at 1.0×10^6 cells/m were introduced. Red tilapia. (Total length (TL): 10.00 ± 0.61 cm, body weight (BW): 18.0 ± 1.3 g) were stocked at 1.5 kg/tonne and fed commercial pellet at 1% of their body weight twice daily. The water quality was maintained in pure culture method. Analyses of *Ex-situ* parameters were performed on Nitrite (NO_2), Nitrate (NO_3) and Ammonium (NH_4) using Aquamerck water test kits respectively. A total of three samples of 1ml water were sampled for water quality analyses for 14 days. *N. oculata* density and contamination counts were performed by means of digital images obtained through an inverted microscope (40×) (Nikon, Eclipse E600, Japan). To calculate density of *N. oculata*, one ml of sample from both cultures were pipetted on to a Neubauer chamber (haemocytometer) placed under a light microscope, and cells were counted at 10x magnification and calculated by equation of density= count/4 x (dilution) x 10,000. Meanwhile, for contamination analysis, 5 ml of 10% formalin was added into 30 ml water sample taken from both systems in order to minimise protozoa movement for counting and identification purpose. Bacterial count was done using Tryptic Soy Agar (TSA) for total bacterial content and Thiosulfate Citrate Bile Sucrose Agar (TCBS) Water sample from both systems taken and cultured in 37°C for 24 hours in incubator and colonies that visible in the agar plate were counted (Kobayashi, Enomato, Sakazaki, & Kuwahara, 1963). All the quantitative data from this study were analysed using a non-parametric Mann-Whitney Test.

RESULTS

The findings revealed the highest density of *N. oculata* in red tilapia culture system and pure culture system were obtained at day 7 (9.6 × 106 cell/mL) and day 4 (8.5 × 106 cell/mL) respectively. From day 5 onwards, the density of *N. oculata* in red tilapia culture system remained significantly higher

($P < 0.05$) until the end of experiment. Contamination was dominated by protozoa (*Gymnodinium* sp.) in the range of 4.80 – 36.67 × 10^3 cell/mL and 0.02 – 41.10 × 10^3 cell/mL in both red tilapia culture and pure culture systems respectively. Contamination was first detected in red tilapia culture system and pure culture system at day 1 and 3 respectively, and their densities fluctuated until the end of experiment.

There was no significant difference in terms of water quality parameters between both systems. The DO and pH recorded in red tilapia culture system and pure culture system were 6.78 – 10.33 mg/L and 7.80 – 10.37 mg/L, and 7.9 – 8.2 and 8.07 – 8.53 respectively. A good control of water quality is a critical factor for higher yields of *N. oculata* in both systems. In Tilapia culture system, *N. oculata* culture did not crash and this revealed a balanced 'interaction' between nutrients, oxygen and pH that allow its growth. In contracts, *ex-situ* findings revealed the concentrations of ammonium in pure culture system (1 – 10 mg/L) was significantly higher ($P < 0.05$) compared with red tilapia culture system (0.17 – 2 mg/L). Ammonium concentration was extremely low in red tilapia culture system from day 4, but it took 12 days in pure culture system to reach lower concentration. Meanwhile, level of nitrate in pure culture system (1.00 – 4.00 mg/L) was significantly higher ($P < 0.05$) compared with red tilapia culture system (2.67 – 15.00 mg/L). The level of nitrite in pure culture system (0.12 – 0.17 mg/L) was significantly higher ($P < 0.05$) compared with red tilapia culture system (0.01 – 0.33 mg/L).

DISCUSSION

Red tilapia culture system has often been associated with the production of quality 'green water' for microalgae propagation in aquaculture pond (Carmen et al., 2007) and this is consistent with the findings of this study. Commercial feed given to red tilapia in this study was considered the main source of nutrients in the culture system as it provides additional nutrient for phytoplankton growth (Jin, Chang, Ji, & James, 2011). Although pure culture system had provided similar nutrient for *N. oculata* growth, microalga culture, balance abiotic and biotic elements such as water quality, nutrients, a light source, aeration and mixing are critical to ensure a satisfactory yield (Creswell, 2010), which was seen limited in pure culture system. Undoubtedly, nutrients in pure culture system were also considered sufficient in this study owing to the addition of nutrient rich of enrichment media, incorporating trace metals, vitamins and several organic and inorganic salt.

Contamination by various microorganisms, such as bacteria, fungi, algae, and protozoa, can affect the growth and quality, and sometimes leading to rapid collapse of the cultures (Ucko et al., 1989), therefore choosing the most ideal microalgae species that are able to tolerate wide range of contaminants and

environmental changes is vital. Although total bacteria count including vibrio yellow and green colonies was relatively higher in red tilapia culture system, there was no significant difference between both systems. A study conducted by Cremen, Martinez-Goss, Corre and Azanza (2017) showed the use of green-water derived from red tilapia culture system not only encouraged the growth of more favourable groups of algae, particularly *N. oculata*, but it also prevented algal collapses and vibriosis.

In the present study, the parameters that contributed to the stability of the *N. oculata* bloom were highly associated with the low N:P ratios and slightly high salinity. The biotic and abiotic factors in the red tilapia culture system were assumed to have provided optimum conditions that favoured the growth of *N. oculata*. The findings are consistent with those of Buttino (1994), affirming that commercial feeds given to red tilapia are the main source of nutrients where nitrogen and phosphorus provide optimum condition for *N. oculata* growth.

CONCLUSION

This study concluded red tilapia culture system can be an alternative means to produce *N. oculata* and it has a significant advantage over pure culture system.

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APPENDIX

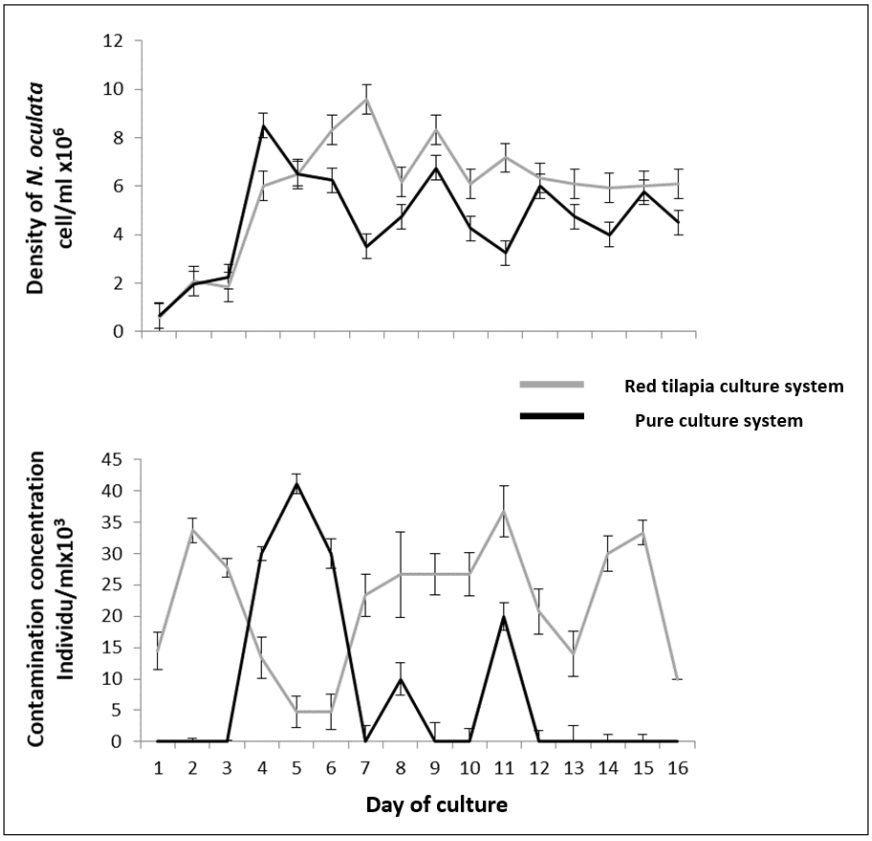

Figure 1. Changes of density and contamination concentration in *N. oculata* cultivated in two different systems. Black and grey lines represent tilapia culture and pure culture systems respectively.

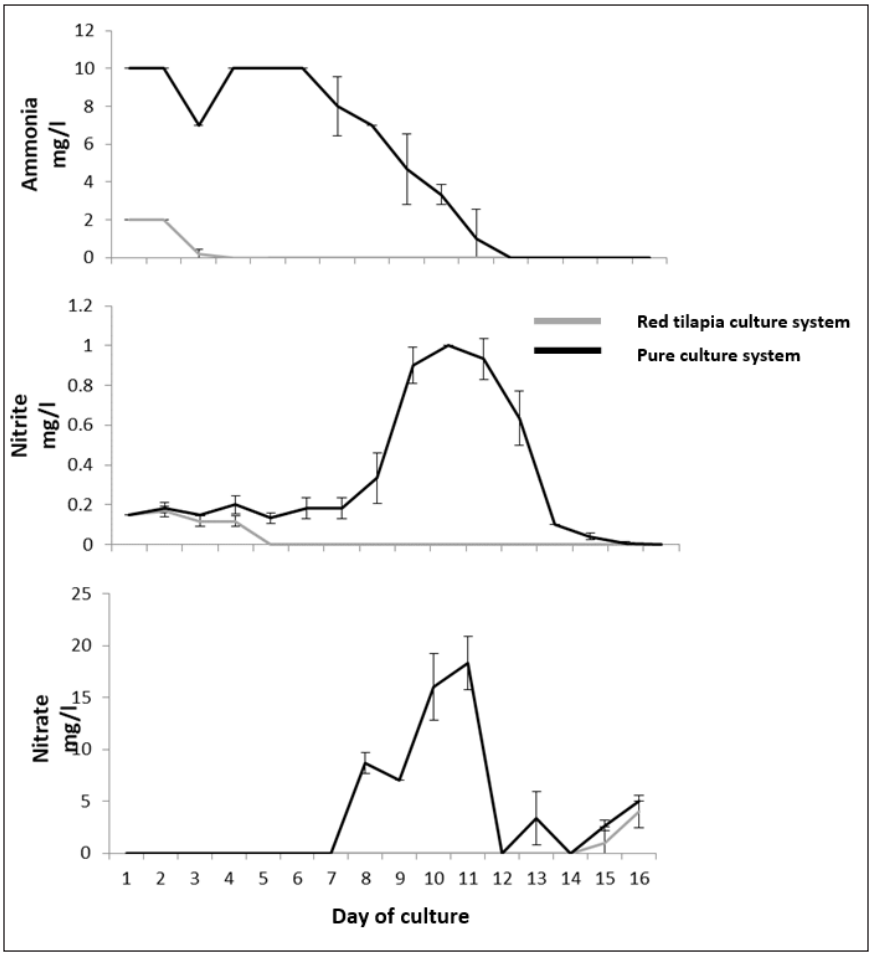

Figure 2 Changes of ammonium, nitrate and nitrite concentration in *N. oculata* cultivated in two different systems. Black and grey lines represent tilapia culture and pure culture systems respectively.

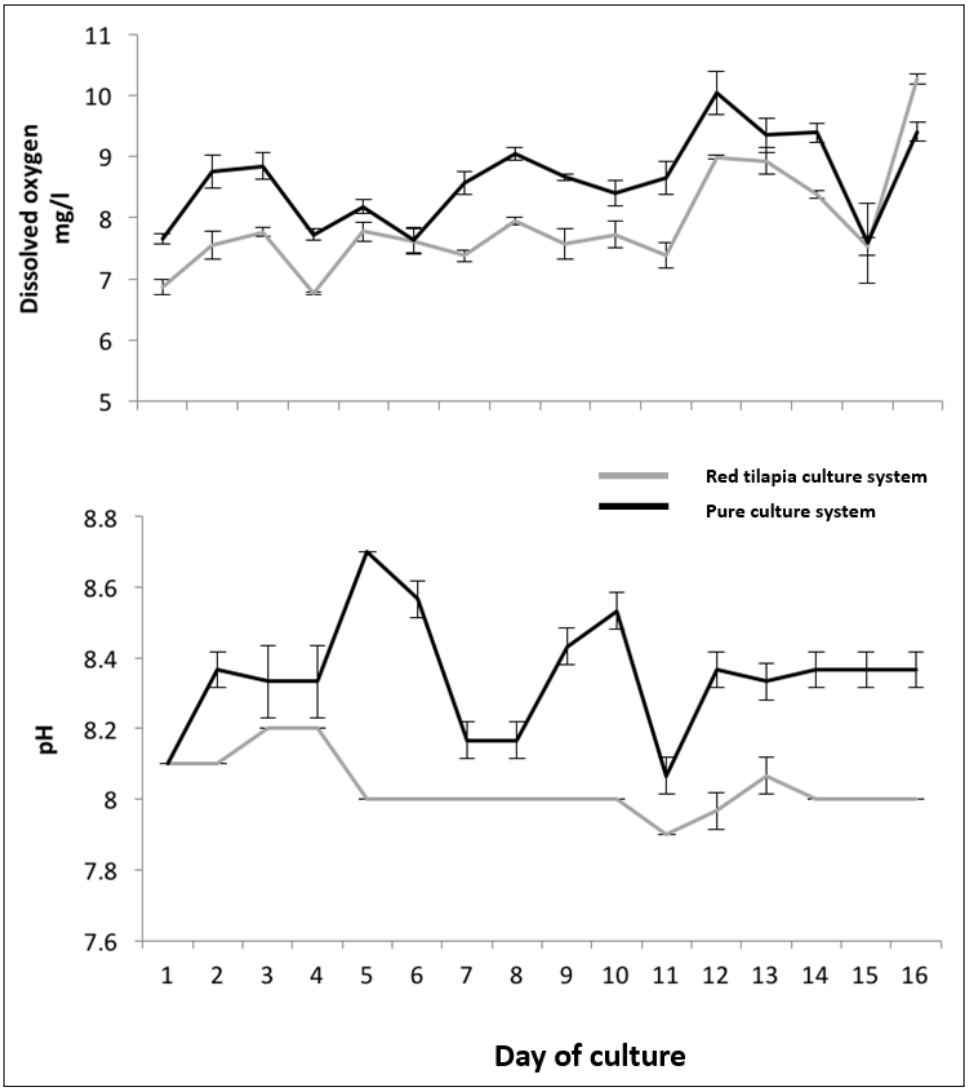

Figure 3 Changes of dissolved oxygen and pH level in *N. oculata* cultivated in two different systems. Black and grey lines represent tilapia culture and pure culture systems respectively.

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Authors' addresses. Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers:

George Swan[1] and Nayan Kanwal[2]
[1]Department of Biology, Faculty of Science, Duke University, Durham, North Carolina, USA.,
[2]Office of the Deputy Vice Chancellor (R&I), Universiti Putra Malaysia, Serdang, Malaysia.

A **list** of number of **black and white / colour figures and tables** should also be indicated on this page**.** Figures submitted in color will be printed in colour. See "*5. Figures & Photographs"* for details.

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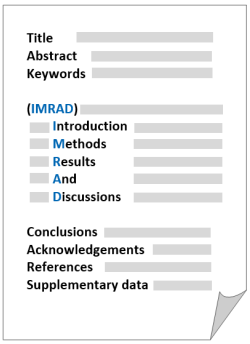

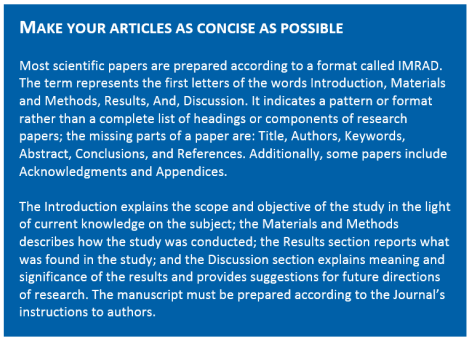

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PERTANIKA JOURNALS

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Office of the Deputy Vice Chancellor (R&I)
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