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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.

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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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Our goal is to bring the highest quality research to the widest possible audience.

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Pertanika is almost 38 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.

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A decision on acceptance or rejection of a manuscript is reached in 3 to 4 months (average 14 weeks). The elapsed time from submission to publication for the articles averages 5-6 months.

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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**.

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

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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.

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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.

JTAS

Journal of Tropical Agricultural Science

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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 | 1619 | 1616
E-mail: executive_editor.pertanika@upm.my
URL: www.journals-jd.upm.edu.my

PUBLISHER

Kamariah Mohd Saidin
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
URL: http://penerbit.upm.edu.my

ABSTRACTING/INDEXING

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Foreword

Welcome to the **Third Issue 2016** of the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open-access journal for the 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 **nine articles**, out of which one is a review article and eight are regular papers. The authors of these articles come from different countries, namely, **Malaysia**, **Iran**, **Bangladesh** and **Pakistan**.

The review paper briefly reports on the effects of superabsorbent polymers (SAPs) on soils and plants. The findings show that the SAPs could store water and nutrients and release them in drought stress conditions in light soils. Therefore, an acceptable biological and grain yield with less irrigation water depth could be achieved (*D. Khodadadi Dehkordi*).

The eight articles cover a wide range of topics. In the first research paper, the effects of flooding and alternate wetting and drying on the yield performance of upland rice are reported by the researchers from Universiti Sultan Zainal Abidin (*Mohd Khairi, Mohd Nozulaidi* and *Md Sarwar Jahan*). The next research paper discusses the impact of different temperature-time profiles during superheated steam roasting on some physical changes of Robusta coffee (*Ooi Ee Shan, Wahidu Zzaman* and *Tajul A. Yang*). The other papers consist of the report of the improved pre-treatment protocol for scanning electron microscopy coupled with energy dispersive X-ray spectroscopy analysis of selected tropical microalgae (*Yau, S. K., Yusoff, F. M., Khong, N. M. H., Foo, S. C.* and *Lai, J. I.*); population fluctuation and dispersion patterns of apple snails, *Pomacea* spp. (Gastropoda: Ampullariidae) in a rice ecosystem (*Arfan, A. G., Muhamad, R., Omar, D., Nor Azwady, A. A.* and *Manjeri, G.*); urine versus pre-mix (sugar:salt): baits for stingless bees (Hymenoptera: Meliponini) (*Kumara, T. K., Farisya, M. S. N., Wan Noor Aida, W. M., Suhaimi Omar, Marcela, P.* and *Aurifullah, M.*); effect of dietary soy lecithin on laying performance, egg quality and meat texture of aged layer hen (*Akit, H., Sazili, A. Q., Ismail, N. A., Atan, N. A., Shazali N.* and *Loh, T. C.*); preliminary studies towards identification of ginger wilt disease in Sabah, Malaysia (*Cosmas, L. L., Atong, M.* and *Poili, E.*); and finally, estimation of grain quality components and their correlation of basmati rice (*Oryza sativa L.) (Mahmuda Ratna, Shahnewaz Begum, Md Abu Kawochar, Shiuli Ahmed* and *Jannatul Ferdous*).

I 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.

I would also like to express my gratitude to all the contributors, namely, the authors, reviewers and editors, who have made this issue possible. Last but not least, the editorial assistance of the journal division staff is fully appreciated.

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
Nayan Deep S. KANWAL, FRSA, ABIM, AMIS, Ph.D.
nayan@upm.my

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Review Article

The Effects of Superabsorbent Polymers on Soils and Plants

D. Khodadadi Dehkordi

Department of Water Engineering and Sciences, Ahvaz Branch, Islamic Azad University, Ahvaz, Iran

ABSTRACT

Current climate change is projected to have significant effects on temperature and precipitation profiles, increasing the incidence and severity of drought. Drought is the single largest abiotic stress factor leading to reduced crop yields. Given the large share of water use in the agriculture sector and very low efficiency in this sector, selection and development of the new strategies to improve and optimise irrigation water use with significant savings is essential. The usage of Superabsorbent polymers (SAPs) is one of the strategies in this regard. This paper briefly mentions to the previous studies about the effects of SAPs on soils and plants, suitable usage rate of SAPs for improvement of soils, raising of WUE and amount of irrigation water saving in this field. The results showed that SAPs could store water and nutrients and release them in drought stress conditions in light soils. Therefore, an acceptable biologic and grain yield with less irrigation water depth could be achieved.

Keywords: Superabsorbent polymer, Irrigation interval, Deficit irrigation

INTRODUCTION

Many countries have inadequate water supplies to meet their current urban, environmental and agricultural needs. During the time of increased water scarcity, population and water demands continue to grow (Postel et al*.,* 1996; Bouwer, 2002). Thus, the challenge is to grow enough food for 2 billion more people over the next 50 years while supplying growing urban and environmental needs for water (Gupta & Deshpande, 2004; Gordon et al*.,* 2005). Some analysts have estimated that 60% of added food requirement will

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E-mail addresses:
davood_kh70@yahoo.com or dkhodadadi@iauahvaz.ac.ir
(D. Khodadadi Dehkordi)

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come from irrigation (Plusquellec, 2002). Raising food production to support the larger world population requires sustaining improved performance of irrigation (Oster & Wichelns, 2003; Rockstorm et al., 2007; Ward & Velazquez, 2008). Drought stress is the most important factor limiting plant growth in arid and semi-arid regions. One of the new methods used for managing water in soil is the use of superabsorbent materials as a storage tank to prevent water waste and increase irrigation efficiency (Khodadadi Dehkordi & Seyyedboveir, 2013d).

SUPERABSORBENT POLYMERS

Superabsorbent polymers (SAPs) have been established as a soil conditioner to reduce soil water loss and increase crop yield. They are hydrophilic networks that can absorb and retain 1000 times more water or aqueous solutions than their original size and weight (Sojka & Entry, 2000). Thus, the application of SAPs to soil may increase water-holding capacities and nutrient utilisation efficiency (Lentz & Sojka, 1994; Lentz et al., 1998) and reduce water loss (Al-Omran & Al-Harbi, 1997). SAPs are used in soil to create a water reserve near the rhizosphere zone (roots) and benefit agriculture (Zohuriaan-Mehr & Kabiri, 2008; Han et al., 2010). Due to water resource crisis, water-saving agriculture is essential for sustainable development of human societies. Furthermore, droughts are predicted to become increasingly severe due to climate change (Gornall et al., 2010). Superabsorbent hydrogels (SAHs) are moderately crosslinked 3-D hydrophilic network polymers that can absorb and conserve considerable amounts of aqueous fluid even under certain heat or pressure. Due to their unique properties that are superior to conventional absorbents, SAHs have found potential application in many fields such as agriculture (Karadağ et al., 2000; Liu et al., 2006; Puoci et al., 2008), hygiene products (Kosemund et al., 2009), wastewater treatment (Kaşgöz & Durmus, 2008; Kaşgöz et al., 2008; Wang et al., 2008), sealing materials (Vogt et al., 2005) and drug delivery system (Sadeghi & Hosseinzadeh, 2008). Currently, further extension of application domains of SAHs was limited because the practically available SAHs are mainly petroleum-based synthetic polymer with high production cost and poor environmental friendly properties (Kiatkamjornwong et al., 2002).

Hence, the development of multi-component Superabsorbents derived from natural polymer and eco-friendly additives becomes the subject of great interest due to their unique commercial and environmental advantages (Kurita 2001), and such materials have also been honoured as the material families of ‘‘in greening the 21st century materials world” (Ray & Bousmina, 2005). Thus far, many natural polymers such as starch (Lanthong et al., 2006; Li et al., 2007), cellulose (Suo et al., 2007), chitosan (Mahdavinia et al., 2004; Zhang et al., 2007b), guar gum (Wang & Wang 2009) and gelatin (Pourjavadi et al., 2007) have been utilised for fabricating multi-component Superabsorbents. It was concluded that the composition and preparation technologies of Superabsorbents

are the dominant factors affecting the properties of SAHs (Wang & Wang, 2010). Many types of material have been used for preparing Superabsorbents. In addition, most traditional water absorbing materials are acrylic acid and acrylamide-based products, which possess poor degradability. About 90% of Superabsorbents are used in disposable products and most of them are disposed of by landfills or by incineration (Kiatkamjornwong et al*.,* 2002). In addition, there will be an environmental problem with SAPs (Zhang et al*.,* 2006; Zhang et al*.,* 2007b). Meanwhile, it has low absorption rate under high concentration of electrolyte, undesirable water-keeping capacity and high cost (Wang & Liu, 2004). In order to solve those problems, considerable attention has been paid to the naturally available resources such as polysaccharides and inorganic clay mineral (Ray & Bousmina 2005; Li et al*.,* 2007). It has good commercial and environmental values with the advantages of low cost, renewable and biodegradable polysaccharides for deriving Superabsorbents (Yoshimura et al*.,* 2005; Pourjavadi & Mahdavinia, 2006). Recently, a series of new Superabsorbents characterised by eco-friendliness and biodegradability made from some natural materials such as starch, cellulose, chitosan (Farag & Al-Afaleq, 2002; Lanthong et al*.,* 2006; Peng et al*.,* 2008; Wu et al*.,* 2008) were used to react through radical graft polymerisation with vinyl monomers and crosslinking agent (Ma et al*.,* 2011). Teimouri and Sharifan (2013) evaluated the effects of two monovalent salts (NaCl and KCl) in different concentrations on hydrate and dehydrate of some SAPs including Aquasorb, Stockosorb, Clophony and A 200. The results showed that A 200 and Clophony had the most hydrate and dehydrate, respectively.

Superabsorbents minimise micronutrients from washing out to water tables and cause more efficient water consumption, reduction in irrigation costs and intervals by 50%, water stress and mechanical damages to transplants during transferring, in addition to providing plants with eventful moisture and nutrients (Abedi Koupai & Mesforoush, 2009) and improving plant viability, seed germination, ventilation and root development. Moreover, Superabsorbents can increase water holding capacity of light soils and keep this capability for about 2 to 4 years (Khoram-Del, 1997). Superabsorbents were introduced to the markets in early 1960s by the American company of Union Carbide (Dexter & Miyamoto 1959). The product absorbed water thirty times as much as its weight but did not last long and was sold to greenhouse retail markets. Soon it was determined that the product was unsuccessful in the market because of its low swell (high cost per unit of water held) and short life (Joao et al*.,* 2007). Materials having the capacity to absorb water 20 times more than their weights are considered as a superabsorbent (Abedi Koupai & Sohrab 2006). Hydrogels are three-dimensional networks of SAPs swelling in aquatic environment. Due to their cross bonds, they tend to hold a part of solvent in their structure instead of dissolving. Their

performance depends on their chemical properties such as molecular weight, formation condition, along with chemical composition of soil's solution or irrigation water (Abedi Koupai & Asadkazemi, 2006; Abedi Koupai et al., 2008). There are three types of hydrophilic polymers including natural (polysaccharide derivatives), semi artificial (cellulosic primitive derivatives) and artificial (Mikkelsen, 1999). Artificial polymers are used more than natural ones because they are more stable against environmental breaking down (Peterson, 2002). Meanwhile, SAPs do not threat human life and environment (Boatright et al., 1997; Shooshtarian et al., 2011). A famous SAP in Iran is in title of Super AB A 200 is made by Rahab Resin, licensed under the Polymer and Petrochemical Institute of Iran. This superabsorbent is tripolymer of acrylamide, acrylic acid and acrylate potassium, as shown in Figure 1 (Khodadadi Dehkordi et al., 2013e).

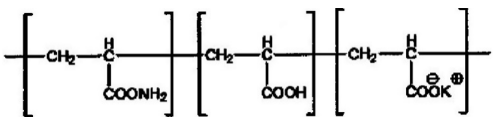

Figure 1. Chemical structure of Super AB A 200 SAP

THE EFFECTS OF SUPERABSORBENT POLYMERS ON SOIL

Since SAP can ease the burden of water shortage, proper use is helpful in arid and semiarid areas (Bakass et al., 2002; Zohuriaan-Mehr & Kabiri, 2008; Han et al., 2010). It has positive effect on water retention on various types of soils that can improve the physical properties of soil; these include increasing their water-holding capacity and nutrient retention of soil, delaying the time to reach permanent wilting point and prolonging plant survival under water stress (Huttermann et al., 1999; Oscroft et al., 2000; Viero et al., 2002; Abedi Koupai & Asadkazemi, 2006; Orikiriza et al., 2009). Yang et al. (2014) used SAPs as for water retention to improve the utilisation of water resources on rocky slopes eco-engineering. This superabsorbent polymer was provided by SIDA Co., Beijing, China. In this study, SAPs were used in three levels of 0.15%, 0.3% and 0.45% and mixed with sandy loam soil. The study was aimed to evaluate the saturated water content, evaporation rate and water holding capability of SAP treated soils, determine seed germination rate and plant survivals in soil with SAP by absorbing and spraying experiments. The addition of SAP to the sandy loam soil resulted in a significant increase of the soil water retention compared to the controls. In addition, seed germination was significantly higher in SAP amended soil than in the soil without SAP, whereas survival times of grass and woody were prolonged under water stress. In specific, 0.30% SAP treatment was the optimum selection for sandy loam soil improvement on steep rocky slopes. These studies indicated that SAP with good water retention properties was very effective in enhancing water uptake and utilisation of water for plants growth, and could be expected to have wide potential applications in rocky slopes eco-engineering.

SAP has hydrophilic groups that are able to absorb and retain fluid and release the fluid later under certain conditions (Zhang et al., 2006). A polymer is categorised as a superabsorbent if its ability to absorb water is more than 100 times its original weight (Zhang et al., 2007a). Most SAPs available in the market have low biodegradability making them environmentally unfriendly in the long run. Therefore, extensive studies have been conducted to use natural based polymers, namely, starch and cellulose, for biodegradable SAPs (Nakason et al., 2010). In general, SAP is synthesised by grafting or grafting crosslinking copolymerisation. The monomers used in grafting copolymerisation include acrylic acid and acrylamide (Li et al., 2007; Teli & Waghmare 2009), while N,N-methylene-bisacrylamide (MBA), trimethyl propane triacrylate and 1,4-butadienol dimethacrylate are used as crosslinkers (Swantomo et al., 2008). Commonly used initiators are persulfate salts, hydrogen peroxide (Moad & Solomon, 2006) and cerium salts (Al et al., 2008). Mas'ud et al. (2013) studied on increasing the benefits of cassava waste pulp by converting it into a superabsorbent. This conversion was carried out by a graft copolymerisation of cassava waste pulp using acrylamide, ammonium persulfate and N,N′-methylene-bisacrylamide as a monomer, an initiator and a crosslinker, respectively. The results showed that cassava waste pulp had a great potential to be used as a superabsorbent, which could give benefit to cassava.

In specific, SAPs improve water penetration rate, structure and texture of soil (Helalia & Letey, 1988; Helalia & Letey, 1989), soil-water retention (Tayel & El-Hady, 1981), soil infiltration and aeration, size and number of aggregates, water tension, available water (Abedi Koupai et al., 2008), soil crispiness (Azzam, 1980) and facilitate water management practices in soil (Shooshtarian et al., 2011). Abilities such as nutrient release and soil nitrification (El-Hady et al., 1981), increase in nutrient absorption, osmotic moisture of soil and decrease in transplanting stresses cause improvement in plant growth reaction (Hadas & Russo, 1974) and increase in yield and reduction in growth and production costs of plant. By absorbing hundred times of its origin weight, superabsorbent can be used as a cultural medium itself or can even be used alone as a rooting medium. Furthermore, it reduces impact pressure in turfs, usage of pesticide (i.e., herbicides, fungicides), absorbs soluble fertilizer and releases it in time, other than improving drainage when used as a soil amendment. In some cases, overuse of hydrogel can cause reverse results because it reduces soil air, followed by filling vacant spaces and gel swelling. There are many reports of no or low effect of gels in overused application in terms of plant growth indices. The main reason, as mentioned, is linked with occupation of numerous vacant spaces of soil leading to severe reduction in soil ventilation (Abedi Koupai & Mesforoush, 2009). In the report, it was illustrated that usage of high levels of superabsorbent in cultural medium caused reductions in soil porosity and air volume and could end up reducing

plant growth rate as well. Thus, there is limitation in term of using SAPs levels (Woodhouse & Johnson, 1991; Shooshtarian et al., 2011). Moradi and Azarpoor (2011) reported that superabsorbent could increase water-holding capacity of soil, infiltration, cation exchange capacity and reduce water consumption. Meanwhile, Tabatabaei and Heidari (2011) evaluated the effects of SAP (Stockosorb) on wetting front dimensions and irrigation intervals. Superabsorbent treatments were found to contain S_0, S_1 and S_2 equal to 0, 5 and 10 gr m^{-2}, respectively, and soil texture was sandy loam. The results showed that increasing superabsorbent amounts could cause the length of wetting front to decrease and its width to increase. In addition, after exceeding 24 hours of irrigation time, soil moisture of S_2 treatment was 46% more than S_0 treatment. Taheri-Sodejani et al. (2015) evaluated the using natural zeolite for contamination reduction of agricultural soil irrigated with treated urban wastewater. In this study, the effects of application method, dosage and particle size of natural zeolite were studied on EC, pH, BOD_5, Na^+, $Ca^{2++}Mg^{2+}$ and nitrate concentration of an urban wastewater by passing it through zeolite-added soil columns. The results showed that by adding zeolite to the soil column, the values of pH, EC and Na^+ of the column outlet were increased, while its $Ca^{2++}Mg^{2+}$, nitrate concentrations, as well as the BOD_5, were decreased. The BOD_5 levels of the column effluent in the control, mixed and layered treated soils with zeolite were lower than the BOD_5 of used fresh wastewater by 38.42, 54.98, and 71.84%, respectively. However, the nitrate concentration of the column effluent in the control, mixed, and layered treated soil with zeolite were lower than the nitrate content of the fresh wastewater by 12.18, 32.19, and 54.90%, respectively. Finally, the results showed that the application of the natural zeolite into the soil in a layered treatment reduced the pollutant transferred to the soil-depth more effectively and this consequently improved the quality of drainage water. Haghighat-Talab and Behbahani (2006) evaluated optimising model of water consumption in hydroponic greenhouses using *PR3005A* SAP. The results showed that the use of SAP could increase water use efficiency to 44 percent per m^3 in hydroponic greenhouses. The polymers are effective in correcting aggregation, prohibiting capillary water soar, decreasing cumulative evaporation and improving growth and efficiency in vast range of plant species (Johnson & Veltkamp, 1985; Choudhary et al., 1995; Al Omran et al., 1997; Sivapalan, 2006). Al-Darby (1996) reported that by increasing the concentration of hydrogel (0, 0.2, 0.4 and 0.8% - Jalma), the amounts of available water and saturated electrical conductivity progressively increased and decreased, respectively. In addition, the results of that experiment also showed reduction in water infiltration and spreading. Finally, he recommended the 0.4% application of Jalma hydrogel and stated that adding this amount of hydrogel led to better improvement in hydraulical properties of sandy soils. This amount of superabsorbent reduced in deep

penetration while simultaneously providing adequate amount of infiltration and water conservation. Dorraji et al. (2010) reported that increasing level of polymer resulted in reduction of soil electrical conductivity. They noticed that after 0.6% polymer application in sandy, loam and clay soil, electrical conductivity declined by 15.3, 20 and 16.9%, respectively, compared to the control. Reduction in electrical conductivity is due to the ability of hydro gels to absorb and conserve a great deal of water and physiological solutions in themselves. Great amount of water causes a decrement in the concentration of salt and it leads to electrical conductivity reduction (Ramezani et al., 2005). It was concluded that in a soil type with loamy clay texture, the application of 0.4% polymer (Stuckosorob) increased survival percentage more than 0.2% with a significant difference compared to the control in *Pinus halepensis* (Huttermann et al., 1999). In the same experiment, when plants were stressed, the evapotranspiration rate was 90%; however, using 0.4% of that material reduced it by 50%. In fact, the polymer could reduce stress in plants. The survival percentage after the last irrigation increased from 49 to 82 days (Huttermann et al., 1999). In this study, the amount of plant growth in the control treatment was 43% less than that of 0.04% treatment. Karimi et al. (2008) stated that applying SAP of Igita caused some changes in the percentages of solid, gas and liquid phases in soil. In the pre-planting stage of their experiment, volume increment was between 10 and 40%, 5 to 32% and 9 to 37% in clay, loamy and sandy soils, respectively.

Montazer (2008) evaluated the effects of SAP (Stockosorb) on flow advance time and soil infiltration parameters in Furrow irrigation. Superabsorbent treatments were containing S_0, S_1, S_2 and S_3 equal to 0, 5, 7 and 9 gr m^{-2}, respectively. The result showed that adding Superabsorbent to Furrows increased flow advance time and soil infiltration. In particular, the soil infiltration in S_3 treatment was more than that of the S_0 treatment about 67%. Banej Shafie et al. (2006) evaluated the effect of SAP (Super AB A 200) on the moisture characteristics of sandy soils. The results showed that when a mixture of sand and SAP was provided in a way that 0.2 to 1.0 percent (w w^{-1}) of the mixture was polymer, the condition of water in the mixture would be similar to clay soil. When the amount of polymer reached 1%, the condition would be tougher than the previous one. In other words, the polymer caused more absorption of water in sand. In blown sand, the stored water was kept in the soil by a suction that was higher the suction in clay. Therefore, in order to increase the capacity of plant available water in blown sand and irrigation interval of the planted seedlings for afforestation in dry areas, adding polymer to the blown sand would result in undesirable conditions. Furthermore, using polymers increases the cost of operation. They are unsustainable materials that may have some other disadvantages. Results of this experiment suggest that usage of clay,

instead of polymer and blown sands, would create better conditions.

Dashtbozorg et al. (2013) evaluated the effects of different sizes of SAP (Taravat A 200) on water retention capacity in two different soils. In this study, there were seven treatments of water absorbent materials including control (without the water absorbent material) and Taravat A 200 SAP in six sizes (0.21-0.25, 0.25-0.5, 0.5-1, 1-2, 2-3.4 and 3.4-4.75 mm), and each of them was used in the form of 2g per kg soil. Then, soil water content was measured for each treatment at the suctions of 0, 0.1, 0.3, 0.5, 1, 3, 5 and 15 bar and the soil moisture curves were plotted separately. The results showed that there was a significant difference between the treatments and two soil textures in various suctions and the interaction of these factors was at the level of 1%. Also, it was observed that SAP with the size of 1-2 mm resulted in an increase in the soil water holding capacity significantly compared with other treatments, especially in the light soil texture. In another experiment, Nadler (1993) observed that using polyacril amid increased water holding capacity in sandy and loamy soils but it had less effect in clay. As for the available moisture, the best results gained from the applications of PR3005A polymer (4 and 8 gKg^{-1}) and in loamy soils. The moisture amount in this situation was increased by 2 to 4 times, respectively (Ghaiour, 2000). Sivapalan (2006) stated that the remaining water in sandy soil was equal to 23% and 95% with the application of polymer 0.03 and 0.07% of its weight, respectively.

It is demonstrated that residual water amount in soil volume becomes more when it blends with Superabsorbent material (Elliot, 1992; Shim et al*.,* 2008). The major factor is related to prohibiting from water subsidence. It is estimated that the additional water causes an increment in the frequency of irrigation in plants (Wang & Gregg, 1990; Mousavinia & Atapoor, 2006). Karimi et al. (2008) reported that utilising the Igita absorbent in soil increased water holding capacity and available water in soil and thereafter, the water intervals also increased. Increases in water intervals in clay soils were about 30 to 130%, 60 to 120% in loamy soil and 150 to 300% in sandy soil. The saved water quantity was 30, 40 and 70% in clay, loam and sandy soils, respectively. Abedi Koupai and Sohrab (2006) conducted an experiment to evaluate water holding capacity and water potential of three kinds of soils; they concluded that on the whole, the application of PR3005A at 6 to 8 gKg^{-1} increased the amount of available moisture by 1.5 to 3 times, respectively. In relation to increment in porosity, the effect of polymer was more outstanding in sandy soil because of more swelling grade, and this caused capillary porosity for about four folds compared to the control samples and decrement in aerial priority. In this experiment, the effects of polymer on irrigation intervals was estimated about 2 to 3 times compared to the control and it emphasised on decreasing costs and efficient water consumption. Ramezanifar et al. (2011) evaluated the effects of SAP (Taravat A 200) on the moisture curve of two different

soils. In this study, two different soil textures including light and medium textures and five levels of SAP [S_0, S_1, S_2, S_3 and S_4, equal to 0 (control), 2, 4, 6 and 8 gr kg^{-1}, respectively] were considered. The results showed that SAP could increase soil moisture content. In every soil, increasing SAP levels contributed to increases in volumetric water content of soils, whereas the most effect was related to the S_4 treatment in light soil texture.

Haghshenas-Gorgabi and Beigi-Harchegani (2010) evaluated the effects of zeolite on water holding capacity in sandy and sandy loam soils. In this study, there were four levels of zeolite (0, 2, 5 and 8 percent) and soil moisture was determined at 0 to -15000 cm. The results showed that the operation of zeolite in sandy soil was better than sandy loam. Besides, adding 8% of zeolite in sandy soil increased some moisture parameters including field capacity from 11% to 13%, available water (1.5 times), residual moisture (2%) and saturated degree (6%). Sharifan et al. (2013) evaluated the effects of SAP (Taravat A 200) on the infiltration equation parameters (Kostiakov-Lewis) through the advance time calculated. In this research, there were four levels of SAP (0, 7, 11, 16 gr m^{-2}). The results indicated that by adding polymers increased advance time and soil cumulative infiltration. Seyed-Doraji et al. (2010) evaluated the effects of different levels of SAP (Taravat A 200) on water holding capacity and the porosity of soils with different salinities and textures. In this research, the polymers were added to different soil textures (sand, loam and clay) at the levels of 0, 0.2, 0.4 and 0.6% w w^{-1} and the salinity of the soils was adjusted at the levels of 0, 4 and 8 dS m^{-1}. The application of 0.6% w w^{-1} polymer at the lowest salinity level increased available water content by 2.20 and 1.20 times greater than the controls in sandy and loamy soils, respectively. Thus, the application of polymers to soil, especially in the sandy soil may increase water-holding capacity and decrease salinity, but it may help improve irrigation projects in arid and semi-arid areas.

Sarafrazi et al. (2011) evaluated the effects of SAP (Acryl amid potassium) on soil volumetric water content and grass water potential. In this study, the experiment was conducted in a randomised completed block design in four levels of polymers including 0 (control), 3, 6, 12 and 24 gr m^{-2} with three replications. The results showed that by increasing the SAP levels, soil volumetric water content also increased. In addition, consumed water was saved up to 75% in the SAP treatments in comparison with the control treatment. Meanwhile, Habibollahi and Hooshmand (2012) evaluated the effects of hydrophilic polymer on wetting dimensions, under drip irrigation. Their study investigated the effects of SAP (Taravat A 200) on vertical wetting depth under drip irrigation, including the four treatments (control (0), 0.1, 0.2, and 0.3 wt%). The investigation showed that the use of drip irrigation with SAP for 4 litres per hour discharge in loam soil, the soil wetting front penetration depth was reduced, while water accumulation in the surface layer (layer modified by the

SAP) increased. Pajuohesh et al. (2008) evaluated the effects of SAP on runoff volume in slopes and various intensities of rainfall. In their study, the main treatment was the three dominant slopes (10, 20, 30 percent), accessory treatments involved five levels of SAP (0, 20, 40, 60, 80 kg ha^{-1}) and three levels of various rainfall intensities were 25, 30, 40 mm hr^{-1}. The results showed that the SAP treatments of various rainfall intensities in comparison with control plate had significant effects in decreasing the output runoff volume to 5 level percent. Dashti et al. (2013) evaluated the effects of synthetic and natural Superabsorbent on nitrate movement in sandy soil. In their study, the treatments consisted of control and superabsorbent. The superabsorbents were synthetic ones (2 gr kg^{-1}) and natural ones (15 gr kg^{-1}). For this purpose, 9 pots were prepared and filled with sandy soil texture. Then, the amount of nitrate was measured by using a spectrophotometer in different values of porous volume (0.1, 0.3, 0.5, 0.7,1, 1.3, 1.7, 1.9, 2 and 2.5). The results showed that at the first stage of leaching, the natural Superabsorbent (manure) was more effective than the synthetic one. However, as the leaching continued, the synthetic superabsorbent absorbed more nitrate compared to natural one. The greatest effect of synthetic superabsorbent was seen at points 0.3 and 0.5 of porous volume and as the leaching was carried on, its effect on absorbing nitrate diminished. The largest effect of manure was obtained at points 2 and 2.5 of porous volume. Han et al. (2010) evaluated the porosity change model for watered SAP (Acrylate sodium co-polymers (ASC) treated soil. The study was aimed to evaluate the bulk density curve of watered SAP-treated soil and construct and test the model for porosity change of watered SAP-treated soil. The results showed that the application of SAP reduced soil bulk density, improved soil permeability and caused soil swelling.

Bai et al. (2010) evaluated the effect of SAPs on soil moisture, bulk density, pH, electrical conductivity (EC) and available P and K after different wetting and drying cycles. Four types of SAPs, labelled as BF, JP, BJ and WT with organic macromolecules, were mixed with sandy soils to give the concentrations of 0%, 0.05%, 0.1%, 0.2% and 0.3%, with the aim to determining water retention and soil properties after amendment with SAPs. Soil moisture increased by 6.2–32.8% with SAP application, while soil bulk density was reduced by 5.5–9.4% relative to the control, especially with a moderate water deficit when the relative soil moisture contents were about 40–50%. The largest increase in soil moisture and the greatest reduction in bulk density resulted from the WT treatment. The effects of SAPs on soil pH and EC were contrary. Soil available P increased slightly while available K significantly decreased, except following the first wetting and drying cycle. Available K increased with drying, but the opposite effect was observed for available P. Particular SAPs (JP and WT) which seemed more suitable under alternating dry and wet conditions. The effects on soil-water retention and other soil

properties varied according to the structure of SAP and soil moisture. Khodadadi Dehkordi et al. (2013a,b,c) performed some research on SAP (Super AB A 200). The results showed that quadratic function was optimum water-yield production function in deficit irrigation situation with the presence of SAP for corn. Besides, with the increase of SAP ratios in sandy soil, the unsaturated hydraulic conductivity decreased. However, with the increase of SAP ratios in sandy soil, the saturated hydraulic conductivity increased. Also, with the increase of SAP ratios in sandy soil, the marginal production index (MPI) and the value of marginal production (VMPI) of corn yield increased. In addition, the results showed that with the increase of SAP ratios, the average matric potential of corn root zone along the growth season, reduced significantly.

In many studies, it is confirmed that reduction or lack of positive effectiveness was due to dissolved salt in water or fertilisers (Taylor & Halfacre 1986; Lamont & O'Connel, 1987). Effect of saline water is reduction in their capability of water absorption and conservation. Akhter et al. (2004), in a comparison, evaluated effects of water type on the amount and rate of absorption, and reported that the maximum time for absorption with distilled water, tap water and saline water were 7, 4 and 12 hrs, respectively. Moreover, the amount of absorption in 1 hr was measured as 505, 212 and 140 gg^{-1}, respectively. Naderi and Farahani (2006) conducted an experiment on three gel types (Yellow, Aquasorb and White) properties, and estimated that using tap water instead of distilled water reduced swelling degree in three SAP from 290, 250 and 218 gg^{-1} to 160, 164 and 150 gg^{-1}, respectively. Reduced impact of polymers in saline is because of the absorption process in polymers occurring based on thermodynamic balance and the osmotic pressure differences between gel network and exterior solution are decreased by increasing the ionic power in saline solution. Accordingly, swelling in solution medium is declined with growing ionic power in saline solutions (Kabiri, 2002). In a study, the application of SAP in loamy-sandy soils of Kuwait was assessed in order to evaluate the establishment of *Conocarpus lancifolius*. Results showed that an increase in water salinity more than 2.5 dSm^{-1} causes reduction in polymer effectiveness, and plants irrigated with 5 dSm^{-1} used 42% less than that of with 1.6 dSm^{-1} (Bhat, 2009). There are large quantities of trace elements in polluted soils, particularly in mining regions, causing an interruption in plant growth and establishment (Walker et al., 2004; Celemente et al., 2006; PerezdeMora et al., 2007). Regarding the fact that installing new green spaces in these regions has been in environmental organisations schedule of many countries, there is a need to find a way to overcome this limitation. One of these ways is treating polluted soils with hydrophilic polymers to have better establishment and growth (DeVarennes & Queda, 2005; Mendez et al., 2007; Guiwei et al., 2008). Naderi and Farahani (2006) estimated that the solute ions in water greatly decreased gel swell and

water absorption, whereas the best amount of pH was reported as neutral. They also suggested that it is better to apply ionic gels as soil pH in Iran is above 7 in most regions, provided that they possess low quantity of bivalent cations. Wallace and Wallace (1986) estimated that, in general, the most favourable results associated with anionic polymers. In other studies, however, the size of particles effects on absorption rate was found between polymer size and growth of *Ardisia pusilla* (Shim et al*.,* 2008; Shooshtarian et al*.,* 2011).

THE EFFECT OF SUPERABSORBENT POLYMERS ON PLANT

The previous studies on SAPs have focused on their effects on particular soil physical and chemical properties (Nadler et al*.,* 1996; Zhang & Miller, 1996) such as pH, electrical conductivity (EC) and soil water content (Bai et al*.,* 2010) for soil erosion control and irrigation management (Sojka et al*.,* 1998) and the effects on plant growth and production (Busscher et al*.,* 2009; Islam et al*.,* 2011). However, a few studies have investigated the effects of SAPs on soil microorganisms and plant available water in the natural environment. Therefore, Li et al. (2014) evaluated two types of SAPs [Jaguar C (JC) and Jaguar S (JS)] applied at 200 kg ha^{-1} by bulk and spraying treatments in a field trial to investigate their effects on winter wheat growth, physical properties of the soils, as well as microbial abundance and activity. It was found that addition of SAPs promoted the formation of macro soil aggregates (particle size >0.25 mm) and soil bacterial abundance under winter wheat cultivation. SAPs also significantly increased soil water content (SWC) and soil maximum hygroscopic moisture (SMHM) in the booting and filling stages but had no effects on the soil available water-holding capacity (AWC) compared with the control in the filling stage. The effects of SAPs were found to depend on the application strategy as only the bulk JC treatment improved the wheat yield, soil microbial biomass carbon (MBC) and soil microbial respiration (SMR). The results showed that the application of SAPs did not lead to detectable adverse effects on the soil microbial community and might even enhance soil microbial activity. Various applications of SAPs and active fields of applied research works on SAPs have been made. It was first applied in the agricultural production of corn and soybean, as well as seedling transplanting. Fanta et al. (1971) found that SAP contributed to water saving and yield enhancement. Later, SAP is also used in many areas such as pharmaceuticals, food packaging, paper production, the agricultural and horticultural industry, oil drilling, etc. (Wang et al*.,* 1998, Wang et al*.,* 2000a; Wang et al*.,* 2000b; Li et al*.,* 2004; Han et al*.,* 2010). In the agriculture and horticultural industry, the application of SAP is in the form of seed additives, seed coatings, root dips and so on (Zohuriaan-Mehr & Kabiri 2008). Many studies, in general, have indicated that SAPs cause improvement in plant growth by increasing water holding capacity in soil (Boatright et

al., 1997; Khalilpour, 2001; De Varennes & Queda, 2005) and delaying duration to wilting point in drought stress (Gehring & Lewis, 1980). Water conserving by gels creates a buffered environment which is effective in short term drought tensions and can reduce losses in the establishment phase of some plant species (Johnson & Leah 1990). Water consumption efficiency and dry matter production respond positively to Superabsorbent existence in soil (Woodhouse & Johnson, 1991; Shooshtarian et al., 2011). Figure 2 shows the SAP of super AB A200 around plant root.

Figure 2. Super AB A200 SAP around plant root

Fazeli-Rostampoor et al. (2011) reported that drought stress and application of SAP (Taravat A 200) had significant effect on corn grain yield and water use efficiency (WUE). In this study, 3 different depths of irrigation were considered as the main treatments I_1, I_2, I_3 as 100, 70 and 40 percent of water requirement of plants respectively, whereas different levels of SAP were used as the secondary treatments (S_0, S_1, S_2 and S_3, equal to 0 (control), 35, 75 and 105 kg ha^{-1} respectively). The most corn grain yield and WUE were related to I_1 and S_3 treatments and the least of them were related to I_3 and S_0 treatments. Zangooei-Nasab et al. (2012a) reported that applying SAP (Stockosorb) had significant effect on plant height, dry weight of aerial organs, root dry weight and root length of Saxaul plant. In this study, three different irrigation intervals including I_1, I_2 and I_3 were considered as daily, three-day and five-day respectively and different levels of SAP including S_0, S_1, S_2 and S_3, equal to 0 (control), 0.1, 0.2, 0.3 and 0.4 weight percent, respectively. The most effect of SAP was related to 0.4% treatment that had not any significant difference with 0.3% treatment and the most effect of irrigation interval was related to the three-day treatment. Abedi Koupai and Mesforoush (2009) evaluated the effects of SAP (Super AB A 200) on the yield performance, growth indices (length of shoot), water use efficiency, and N, K, Fe and Zn uptake of a nursery plant (*Cucumis sativus* var. Gavrish). The greenhouse trial was conducted using factorial experiment with a completely randomised design layout in which the treatments were two soil textures (sandy and clay loam), three irrigation regimes consisting 50%, 75% and 100% ETc and the hydrogel treatments were containing 0, 4, 6 and 8 gr kg^{-1} hydrogel.

The results show that use of 4 g kg^{-1} SAP Super AB A200 in a light texture soil and without stress or 25% deficit irrigation is recommended to achieve the best marketable yield and desired water use efficiency. Banej Shafei (2000) investigated the effect of a SAP (Super AB A200) on increment of soil water accessibility, fertiliser efficiency, growth and establishment of *Panicum capillare*. The results illustrated that 0.3% application of this gel caused higher production of dry matter in three different soil textures (light, medium and heavy) and three irrigation intervals (4, 8 and 12 days) in all the treatments.

Karimi and Naderi (2007) evaluated the effects of different rates of a SAP (Vinyl alcohol acrylic acid) on dry matter yield (Y) and water use efficiency (WUE) of forage corn. A greenhouse experiment was carried out as a factorial complete randomised design with 18 treatments and 3 replicates. Six levels of SAP (0, 0.05, 0.1, 0.2, 0.3 and 0.4 dry basis percentage, S_0 to S_5) and three soils differing textures (clay, loamy and loamy sand, A_1 to A_3) were used. Forage corn was planted in the pots. The pots were irrigated based on 60% depletion of soil available water for the all treatments. Yield (Y), evapotranspiration (ET) and water use efficiency (WUE) were measured. The results indicated that the effects of soil, SAP and their interactions were significant ($P<1\%$) on Y and WUE. Clay soil (A_1) had maximum Y, while WUE and loamy soil (A_2) and loamy sand soil (A_3) had minimum Y and WUE. There were significant differences in WUE between S_1 and the other rates. Meanwhile, S_5 and S_0 had maximum and minimum Y and WUE values, respectively. The results indicated that there was no significant difference between S_4 and S_5 treatments. Moreover, the application of SAP at five levels increased Y and WUE. In summary, the application of 0.05, 0.1 and 0.3 dry basis percentage of SAP, in clay, loamy and loamy sand soils, caused maximum Y and WUE, respectively.

Ahrar et al. (2009) evaluated the effect of hydrogel amendment and *Cucurbita pepo*. Rootstock on hydroponically cultured greenhouse cucumber. Results showed that incorporating hydrogel into media could improve media physical properties and increase its water holding capacity. This condition could also decrease leaching fraction and increase yield and water/ fertiliser use efficiency. Moghimi et al. (2011) evaluated the effects of perlite different amounts on grain yield and water use efficiency in winter wheat (Zarrin cultivar). A field experiment using completely randomised block design with seven treatments (P_0, P_1, P_2, P_3, P_4, P_5 and P_6) including: zero (reference), 75, 150, 300, 600, 1200 and 2400 $kg.ha^{-1}$ with four replicates was conducted. Results of statistical analysis showed that the use of rates in all the treatments significantly increased crop . The results showed that adding perlite to the soil, grain yield and biological yield of wheat increased up to 39.9 and 31.5 percent, respectively, which statistically is significant at one percent level. Also, there was a significant difference between grain proteins of treatments at

five percent level. The results also showed that in the treatment with application of 2400 kg.ha^{-1} perlite, water use efficiency increased by up to 40.12 percent. The results of a study to evaluate the effects of 5 levels of SAP on turf grass in Tehran (Iran) illustrated that the substance caused increases in colour intensity, density and coverage area but a reduction was found in the wilting rate. Furthermore, it is stated that the most efficient amount was 100 gr per 1 m^2 (Khushnevis, 2006). According to the estimated results of Evaporation Pan in part of Tehran (Iran), each 1 m^2 of turf grass requires around 14 to 18 litres of water in warm seasons daily. Providing this amount of water is rather difficult. Using 100 g of SAP in the mentioned area can reduce water consumption by 50% (Ataei & Ghorbani, 2001). Another study on turf grass indicated that 8 g application of a SAP per 1 Kg of soil enhanced the available moisture up to 4.2, 1.8 and 2.2 times in sandy-loamy, clay and loamy soils respectively in a suction range of 0.3 and 15 bar (Mousavinia & Atapoor, 2006). Al Humaid and Moftah (2007) reported that application of K400 Stockosorb polymer in 0.4 to 0.6% of weight caused water potential of Buttonwood (*Conocarpus erectus* L.) seedlings to increase significantly in dry region of Saudi Arabia. These seedlings survived three times more than those controlled under drought stress. They also expressed that root and shoot growths were significantly increased using hydrogels. Abedi Koupai and Asadkazemi (2006) illustrated that applying 4 and 6g Kg^{-1} of SAP (Super AB A 200) decreased one third of Arizona cypress (*Cupressus arizonica* Greene.) water demand in comparison to the controls. Lawrence et al. (2009) claimed that under drought stress in green house, amending soil with SAP (Polyacrylate) (0.2 and 0.4% in weight) caused biomass increment in 9 ornamental tress species. They also announced that adding this material to the soils held their moisture in field capacity range and caused an increase in water consumption efficiency, which is used in photosynthesis. Another experiment was conducted to determine the effects of two kinds of SAPs (Stockosorb and Luquasorb) on *Populus popularis* grown under drought and saline tension. It was observed that 0.5% application of two kinds of polymer in cultural medium could reduce the plant growth and leaf gas exchanges prevention rate induced by mentioned stresses (Shi et al*.,* 2010). Hutterman et al. (1999) reported that SAP (Stockosorb K 400, a highly cross-linked polyacrylamide with about 40% of the amide group hydrolysed to carboxylic groups) caused improvement in the shoot and root performance of *Pinus halepensi*s Mill under dry condition.

Sarvas et al. (2007) in an experiment on *Pinussylvestris* L. seedlings observed that by overusing SAP (Stockosorb) in soil, plants were more likely to be exposed to Fusarium diseases and mostly perished. They suggested that investigations needed to be carried out to find out the most suitable amount of hydrogel to be used in different situations and for plant species. Results of another study showed that adding

polymer up to 0.3% had positive effect; in concentrations over 0.4%, however, the effects were reversed (Al Harbi et al., 1999). Fry and Butler (1989) concluded that in order to reduce drought stress in Tall fescue (*Festuca arundinacea*) in sandy soil, the amount of SAP has to be 80 folds compared to the recommended amount. Khadem et al. (2010) evaluated the effects of animal manure and SAP (Taravat A 200) on leaf relative water content, cell membrane stability and leaf chlorophyll content (SPAD) of corn under drought stress. In their research, water stress was applied by three different irrigation intervals (irrigation after 70, 105 and 140 mm evaporation of basin class A) which were allocated to main plots. A combination of six levels of animal manure and SAP allocated to subplots are as follows: S_1: control, S_2: 100% animal manure (40 t ha^{-1}), S_3: 100% SAP (200 kg ha^{-1}), S_4: 50% animal manure+50% SAP, S_5: 35% animal manure+65% SAP, S_6: 65% animal manure+35% SAP. The results showed that the highest leaf relative water content (RWC) was obtained with 100% SAP (200 kg ha^{-1}), and it was reduced by increasing drought stress. Meanwhile, cell membrane stability (CMS) increased with increasing drought stress and decreased by using animal manure and SAP. Leaf chlorophyll index (SPAD values) increased in response to drought stress and by using different combinations of animal manure and SAP. 1000-seed weight and grain yield was decreased by drought stress. Grain yield decreased by yield components reduction, especially 1000-seed weight due to drought stress. 1000-seed weight and grain and biological yield increased by using animal manure and SAP together as the maximum yield grain was obtained by using 65% animal manure and 35% SAP.

Rahmani et al. (2010) evaluated the effects of different levels of water deficiency stress and SAP (Super AB A 200) on yield, antioxidant enzymes activity and cell membrane stability in mustard. The treatments included five levels of water deficiency stress (irrigation after 80 mm evaporation from class A evaporation container, cut irrigation from booting stage, flowering stage, silicling stage and grain filling stage) and three levels of applying SAP (amount of 0, 5 and 7 weight percentage). Results showed that the effects of water deficiency stress and SAP were significant. The mutual impacts of water deficiency stress and SAP were not significant for any treatments. Normal irrigation and usage of concentration of 7%, level 3, of SAP with mean of 5.803 and 25.78 t h^{-1} showed the most seed and biological performance. Cut irrigation from booting stage without applying SAP with mean of 206.1 and 5231 (mg.protein^{-1}) showed the most activity of catalase and super oxide dismutase and with mean of 2683 Ls cm^{-1} showed the least cell membrane stability. Ghasemi and Khoshkhui (2008) evaluated the effects of SAP (Super AB A 200) on irrigation interval, growth and development of Chrysanthemum. In their study, four different irrigation intervals (I_1, I_2, I_3 and I_4 as two-day, three-day, four-day and five-day, respectively) and different

levels of SAP [S_0, S_1, S_2, S_3, S_4 and S_5, equal to 0 (control), 0.2, 0.4, 0.6, 0.8 and 1 weight percent, respectively] were taken into consideration. The results showed that SAP had significant effects on the number and diameter of flowers, dry and fresh weight of flowers, branches and roots, number of leaves, leaves area, as well as number of branches and plant length in drought stress conditions. The best treatment was I_1S_4. Jalili et al. (2011a) evaluated the effects of SAP (Tarawat A 200) and irrigation period on generative growth of Rosa bushes. In their research, randomised complete block design in natural field for two factors (SAP in 4 levels including 0, 40, 90 and 140 gr and irrigation period in 4 levels [6, 10, 14 and 18 days]) was used with 3 replications. The findings showed significant impacts of SAP in increasing irrigation and various growth parameters in Rose. In qualitative parameters, 40 and 90 gr levels of SAP had a positive effects on number of main branch parameters; however, it had no significant effect on total number of branches and number of flowers. As for irrigation factor, 6 and 10 day levels in number of main branch and total number of branches parameters had a significant difference with that of other levels. Increasing irrigation periods and decreasing water availability for plants led to reduction in flowers.

Jalili et al. (2011b) evaluated the effects of SAP (Tarawat A200) and irrigation period on Almond seedling. Their research used randomised complete block design in natural field for two factors (SAP in 4 levels including 0, 60, 100 and 125 gr in 100 kg soil and irrigation period in 4 levels including 7, 12, 18 and 24 days) with 3 replications. Results showed no significant difference between the treatments in the first year. In the second year, however, a significant difference was observed between the treatments in most of growth indices. Alami et al. (2011) evaluated the effects of SAP (Super AB A 200), Paclobutrazol and irrigation period on *Lolium perenne cv.* Barbal. For this purpose, they used randomised complete block design for three factors (SAP in 4 levels including 0, 3, 6 and 9 gr kg^{-1}, irrigation period in 3 levels including 2, 4 and 6 days and Paclobutrazol in 2 levels including 0 and coated grains by 30 mg l^{-1} Paclobutrazol) with 3 replications. The results showed that the best density achieved in 6 gr kg^{-1} SAP and two-day irrigation period. In an experiment, it was stated that *Conocarpus lancifolius*, in warm and dry climate of Kuwait, needed 50% less irrigation water when treated with Agrihope Superabsorbent in 0.4% of weight concentration. Furthermore, at that concentration, available water capacity increased from 7.29 (in control) to 18.75% (Bhat et al., 2009). Although clay soil holds great deal of water, the available water for roots would be less than half. On the other hand, more than 90% of water absorbing by Superabsorbent is available to plant roots (Joao et al., 2007). Abedi Koupai and Sohrab (2006) estimated that 2 to 8 g of hydrogel (Super AB A 200) per each 1 kg of soil increased the moisture quantity roughly 1 to 2.6 times respectively in comparison with the control. In an experiment to evaluate

the effects of Aquasorb on irrigation of three species seedlings including *Atriplex canescens*, *Pinus Eldarica* and Populus euphratica, it was estimated that using 1% polymer three times higher than the control interval could have the same result as the control irrigation. In general, they reported that it is recommendable to use polymer in planting time for the mentioned species to reduce irrigation rate and interval with proper survival percentage (Poormeidany & Khakdaman, 2006). Sheikhmoradi et al. (2011) evaluated the effects of SAP (Super AB A 200) and irrigation period on some qualitative characteristics of sport grass. In this study, it was considered four different irrigation intervals [I_1, I_2, I_3 and I_4 as one-day, two-day, four-day and six-day, respectively] and four levels of SAP [S_0, S_1, S_2 and S_3, equal to 0 (control), 20, 25 and 30 gr $m^{-2,}$ respectively]. The results showed that applying SAP was significant (at 1% level) in some growth indices including shoot height, total chlorophyll and plant density. The best treatment was I_2S_3. Banej Shafie et al. (2012) evaluated the effects of SAP (Manufactured by Flowergel co., of Netherlands) application and irrigation period on the growth of pistachio seedlings. In their study, the treatments applied were SAP in 3 levels (0, 50 and 100 gr), irrigation amount in 2 levels (5 and 10 lit) and irrigation period in 3 levels (10, 20 and 30 days). The results showed that using 50 gr SAP halved irrigation amount (5 lit) in 10-day period intervals. Using 100 gr SAP with 10 lit water increased period of irrigation from 10 to 20 days. In addition, applying SAP increased the height growth and diameter at collar growth compared to controls. It was concluded that SAP diminished water consumption and irrigation frequency by 50%. Nikoorazm et al. (2009) evaluated the effects of applying SAP (Tarawat A 200), irrigation regimes and polymer usage style on lettuce growth. In their study, four levels of SAP (0, 20, 40 and 60 gr per plant), four irrigation regimes (5, 8, 11 and 14 days) and polymer usage style (layering and mixed whit soil) were performed on growth lettuce under greenhouse conditions. The results showed no significant differences between the irrigation regimes on fresh and dry weight. Moreover, the high levels of SAP (60 gr per plant) increased fresh and dry weight compared to the control (without polymer) and the lowest level of polymer (20 per plant). These results indicated that high amounts of SAP had positive effects on growth lettuce.

Abdi and Hedayat (2009) reported that adding 2 to 5% SAP (Super AB A 200) could increase length and diameter of branches, petiole length and leaf area of Lycopersicom esculentum Mill and Capsicum annuum L. Bordbar et al. (2011) evaluated the effects of SAP (Super AB A 200) and irrigation period on yield and yield components of sunflower. In this study, three levels of irrigation period (12, 16 and 20 days) were used as main factor and four levels of SAP (Tarawat A 200) (0, 30, 60 and 90 kg ha^{-1}) as the sub factor. The results showed that application of 90 kg ha^{-1} of SAP with 12 days irrigation period caused the highest yield, whereas the control treatment with 20 days irrigation

period had the lowest yield. Generally, by increasing the amount of SAP and with shorter irrigation period, the yield and yield components were significantly increased. Zangooei-Nasab et al. (2012b) evaluated the effects of SAP (Stockosorb) and irrigation period on some physical characteristics of soil and growth indices of Atriplex plant. In this study, three different irrigation intervals (I_1, I_2 and I_3 as daily, three-day and five-day, respectively) and five levels of SAP (S_0, S_1, S_2, S_3 and S_4, equal to 0 (control), 0.1, 0.2, 0.3 and 0.4 weight percent, respectively) were considered. The results showed that SAP had significant effect (at 5% level) on some growth indices including plant height, dry and fresh weight of aerial organs, dry and fresh weight of root and root length. The best treatment was I_2S_4. Besides, SAP caused significant increase in soil saturated degree and available water content and decrease in bulk density and EC.

Yadollahi et al. (2012) evaluated the effects of SAP (Stockosorb) and organic matters in retention of water and establishment of Almond gardens in rainfed conditions. The results showed that SAP and organic matters could increase soil relative moisture significantly. This situation could increase growth indices of Almond seedlings. Razban and Pirzad (2011) evaluated the effects of varying amounts of SAP (Super AB A 200) under different irrigation regimes on growth and water deficit tolerance of German Chamomile. In their study, treatments included water deficit stress (irrigation after 50, 100, 150 and 200 mm evaporation from pan class A) and varying amounts of SAP (0, 60, 120, 180, 240 and 300 kg ha^{-1}). The results showed that the effect of SAP was non-significant in Capitulum yield, the numbers of Capitulum per plant, Capitulum diameter, Capitulum length, receptacle height, Capitulum weight and leaf soluble carbohydrates. In contrast, interaction effect between deficit irrigation and polymer was significant on biomass yield, chlorophyll a+b, total chlorophyll and proline. Yazdani et al. (2007) evaluated the effects of SAP (Tarawat A 200) on soybean yield and yield components. In their study, there were four levels of SAP (0, 75, 150 and 225 kg ha^{-1}) and three irrigation intervals (6, 8 and 10 days) on growth, yield and yield components of soybean. The results showed that the highest level of SAP (225 kg ha^{-1}) increased seed yield, 100-grain weight, number of pod per main branch, oil and protein yield and seed protein content in comparison with the control treatment and the lowest level of polymer (75 kg ha^{-1}). These results indicated that high amounts of SAP had positive effects on yield and yield components of soybean even under drought-stress conditions.

Keshavarz and Farahbakhsh (2012) evaluated the effects of Superabsorbent (zeolite) and drought stress on yield components of forage millet. In their study, there were three levels of Superabsorbent (0, 150 and 300 kg ha^{-1}) and four irrigation levels (40, 60, 80 and 100 percent of field capacity). The results showed that adding of Superabsorbent could increase dry and fresh weights, height and diameter of stem, node and claw number of forage millet. The most

amount of growth indices was related to 300 kg ha^{-1} Superabsorbent treatment. Poorpasha et al. (2011) evaluated the effects of SAP (Super AB A 200) and Nitrogen fertilizer on yield and yield component of wheat. In their study, there were three levels of SAP (0, 100 and 200 kg ha^{-1}) and four levels of Nitrogen fertiliser application (0, 50 and 100 percent of fertiliser need). The results showed that by applying SAP, some indices (including fertile claw number, grain yield and harvest index) increased. Besides, the treatment with 200 kg ha^{-1} SAP achieved the most grain yield. By applying of 200 kg ha^{-1} of SAP and 100% of fertiliser need achieved the most fertile claw number and grain yield. Roostai et al. (2012) evaluated the effects of SAP (Super AB A 200) and animal manure ratios on the quantitative and qualitative characteristics of soybean under drought stress. In this study, the main factor was irrigation with 50, 100 and 150 mm (evaporation from class A pan) and sub-factor was (0) control, 40 $t.ha^{-1}$ animal manure, 200 $kg.ha^{-1}$ SAP, 50 percent SAP plus 50 percent animal manure, 65 percent SAP plus 35 percent animal manure and 35 percent SAP plus 65 percent animal manure. The results showed that pod number, grain number per plant, 1000-grain weight, grain, biological, protein and oil yield increased when SAP and animal manure were applied significantly. Finally, the combination of 35 percent SAP and 65 percent animal manure was the best treatment in this experiment. Allahyari et al. (2013) evaluated the effects of SAP (Aquasorb) application on yield and yield components of two chickpea cultivars under rainfed conditions. In this study, the experiment treatments included three levels of SAP (0, 150 and 300 gr m^{-2}) and two chickpea varieties (Jam and ILC482). The results showed that all the studied characteristics were affected by SAP. The highest grain yield (160.541 gr m^{-2}) obtained in Jam cultivar by applying 300 gr m^{-2} polymer and lowest grain yield (71.276 gr m^{-2}) obtained in ILC482 cultivar without polymer application. Finally, SAP application increased pod number in plant, 100-grain weight and biological yield compared to the control.

Islam et al. (2011) evaluated a water-saving SAP (Granular type) for minimising NO_3^- leaching from soil and optimising corn growth and yield. Thirty-six undisturbed soil lysimeters were installed in a field lysimeter facility in drought affected northern China to study the growth and yield characteristics of summer corn (*Zea mays* L.). The amount of NO_3^-leaching losses under different fertiliser (standard, medium or 75% and low, or 50% of conventional fertilization rate) and SAP [control (0); level-1 (15 kg ha^{-1}) and level-2 (30 kg ha^{-1})] treatments. The results showed that corn yield fell by 19.7% under medium and 37.7% under low fertilisation. The application of SAP increased yield significantly by 44.4% on level-1 and 80.3% on level-2, respectively. Similarly, plant height, leaf area, number of grains as well as protein, soluble sugar and starch contents in the grain also increased with SAP treatment. Application of SAP at 30 kg ha^{-1} plus half of conventional fertilisation could reduce maximum (64.1%)

nitrate leaching losses from soil. Rahimian and Hosieni-rad (2007) evaluated the effects of SAPs (Stockosorb and Super AB A 200) on yield and water use of tomato. The treatments were as 1- control (without polymers and irrigation with 7-day interval), 2- ten kg ha^{-1} Stockosorb and irrigation with 7-day interval, 3- thirty kg ha^{-1} Stockosorb and irrigation with 14-day interval, 4- fifty kg ha^{-1} Stockosorb and irrigation with 14-day interval, 5- seventy kg ha^{-1} Stockosorb and irrigation with 21-day interval, 6- ninety kg ha^{-1} Stockosorb and irrigation with 21-day interval, 7- ten kg ha^{-1} Super AB A200 and irrigation with 7-day interval, 8- thirty kg ha^{-1} Super AB A200 and irrigation with 14-day interval, 9- fifty kg ha^{-1} Super AB A200 and irrigation with 14-day interval, 10- seventy kg ha^{-1} Super AB A200 and irrigation with 21-day interval, 11- ninety kg ha^{-1} Super AB A200 and irrigation with 21-day interval. The results showed that there were significant differences (at 1% level) between the treatments. The best treatment was No.7 that yielded 45.3 ton ha^{-1} tomato that was 40% more than control yield. This was followed by treatment No.2 that yielded 40.3 ton ha^{-1} tomatoes, which was 28% more than control yield. Khodadadi Dehkordi and Seyyedboveir (2013d) evaluated the effects of drought stress and different levels of SAP (super AB A 200) and their effects on water use efficiency (WUE) and yield response factor (Ky). In their study, 3 different depths of irrigation were considered as the main treatments I_1, I_2, I_3 as 100, 75 and 50 percent of water requirement of plants respectively and different levels of SAP were used as the secondary treatment (S_0, S_1, S_2 and S_3, equal to 0 (control), 15, 30 and 45 gr m^{-2}, respectively). The results revealed that the effect of Superabsorbent treatments on biologic and grain yield of corn was significant at 1% level. Besides, the independent effect of irrigation and SAP treatments at 1% level and interaction between them at 1% level on WUE of corn were significant. I_1S_3 treatment had the most WUE amount (16.18 kg ha.mm^{-1}) and I_3S_0 treatment had the least WUE amount (6.48 kg ha.mm^{-1}). Figure 3 shows the manner of putting SAP in the ridges. In this manner, SAPs are put on the furrows at first, after that they are hilled up by ridges soil (Khodadadi Dehkordi & Seyyedboveir, 2013d).

Figure 3. Application of SAP in ridge and furrow system

CONCLUSION

The results showed that irrigation water depth was reduced by applying Superabsorbent in water restriction conditions. SAPs could remain in soil for 3 to 5 years and retain their capability. The results showed that the application of Superabsorbent in light soils

with less irrigation water depth achieved more grain yield and biologic yield. This is due to the nutrients and water storage by Superabsorbent in light soils that created favourable conditions for plant growth. In addition, the results also showed that soil water-holding capacity in light soils could be increased with Superabsorbent. From the economic aspect, SAPs are costly in many countries. Thus, subsidy needs to be given to farmers to encourage them to use it. Therefore using SAPs can be regarded as profitable and if farmers use them in growing plants, it can help save water resources. In conclusion, applying SAPs could improve light soils characteristics and help cultivation in these soils, particularly in areas with limited water supplies and restricted nutrient conditions.

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Effects of Flooding and Alternate Wetting and Drying on the Yield Performance of Upland Rice

Mohd Khairi, Mohd Nozulaidi and Md Sarwar Jahan*

Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, 22200 Besut, Terengganu, Malaysia

ABSTRACT

Water-wise rice cultivation is a growing concern in rice production. We justified the effects of different water levels on rice production of upland variety. A completely randomised design was arranged with four treatments (T1: flooding at 5 cm depth, T2: flooding at 1 – 3 cm depth, T3: saturated to 1 cm flooding, and T4: alternative wetting and drying, or AWD) with five replications. Yield, plants and soil parameters were evaluated. Upland rice variety showed improved yields and yield parameters under flooding at 5 cm depth treatment than alternative wetting and drying treatment. Flooding water significantly increased plant height, tiller numbers, panicle numbers, panicle height, grains per panicle and yield compared to AWD treatment. Chlorophyll (Chl) content increased gradually with increasing plant age but flooding at 5 cm treatment increased Chl content after the secondary tillering. Net photosynthesis rate (Pn) and relative water content (RWC) decreased in plants under alternative wetting and drying treatment than control treatment. Saturated to 1 cm flooding treatment saved 42% of water used in the treatment of flooding at 5 cm depth, which showed a similar water use efficiency (WUE) to alternative wetting and drying treatment. However, treatments of flooding at 1 – 3 cm depth and saturated to 1 cm flooding showed a similar effects on rice yield. Meanwhile, saturated or above soil water condition did not affect soil pH, soil electric conductivity (EC) and phytoavailability of nutrients in the soil. These results suggested that saturated to 1 cm flooding irrigation could increase rice yield of upland variety and save fresh water for other purposes at the same time.

Keywords: Water use efficiency, chlorophyll content, photosynthesis, relative water content, flooding effects, rice yield

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E-mail addresses:
khairi0102@gmail.com (Mohd Khairi),
nozulaidinordin@gmail.com (Mohd Nozulaidi),
sarwarjahan@unisza.edu.my (Md Sarwar Jahan)
* Corresponding author

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INTRODUCTION

Rice is a staple food which provides about 32% of total calorie uptake and about 90-91% of total rice produced and consumed in Asia (IRRI, 2012). In Asia, irrigated agriculture accounts for about 90% of the freshwater, while rice farming only consumes about 50% of that fresh water (Cantrell & Hettel, 2005). Between 1955 and 1990, water availability per capita dropped by 40-60%, and further dropping at 15 – 54% is expected in the next few years (Gleick, 1993). Malaysia needs approximately 20 billion m^3 by 2020 to fulfil domestic and industrial demands (Keizrul & Azuhan, 1998). In addition, water rationing during rainless situation shows a critical condition for fresh water in Selangor state (Koon & Pakiam, 2014). Therefore, it is important to produce rice that needs less water to cut down water supply for rice cultivation and ensure sustainable rice production.

Plant growth and grain yield are affected by water deficit in soil (Pirdashti et al., 2004). Less water affects transpirational functions to regulate effectively in irrigated rice variety (Vandeleur et al., 2009), while upland rice may persist under less water condition and sustain production (Dahamarudin & Rivaie, 2013). Soil water status that is below saturated condition affects the production of rice (Sariam et al., 2004). Under less water condition, irrigated rice variety shows low tissue water potential (Kato et al., 2004) which may affect net photosynthesis rate. Water and soil stresses affect net photosynthesis rate, photosynthetically active radiation (Inani et al., 2015; Munirah et al., 2015a; Syuhada et al., 2016). Chlorophyll function, on the other hand, is important to regulate light reaction in photosystem II to produce light energy, which might affect crop yield (Jahan et al., 2016). In addition, flooded soil-system reduced oxygen in soil may cause a decline of redox reaction but less water ensures higher redox value in the soil (Sarwar, 2004) that might indicate oxygen availability for root growth and absorb nutrients from soil effectively. Therefore, water in rice cultivation should be applied in a logical way to reduce water use in rice cultivation without affecting yield and plant properties.

Rice production system requires approximately 1900 to 5000 litres of water to produce 1 kg of grain (Haefele et al., 2009). By 2025, about 10% of irrigated rice will face water scarcity (Bouman et al., 2007). Therefore, we need to focus on upland rice production to minimise water use and increase rice production. To date, a lot of research has been done on reducing water use in lowland rice cultivation (Sarwar, 2004; Bouman et al., 2007; Jahan et al., 2013a) but less attention was given to study the effects of different water regimes on upland rice cultivation. Therefore, this study was conducted to find the effects of less water use on rice yield and physiological parameters of upland rice. In this study, we provide information that shows upland rice variety provides higher production and better physiological performance under 1 cm flooding than alternative wetting drying condition.

METHODOLOGY

Plant Materials and Experiment Setup

Four-day old pre-sprouted rice seeds of WAB 96-1-1 were grown in a pot measuring of 25 cm × 25 cm x 35 cm. All pots were filled with soil leaving 5 cm spaces from the top of the pot and two holes were made at 0 cm and 1 cm above the soil level to maintain water levels. There were four treatments [T1: flooding at 5 cm depth (control; irrigate when the water level dropped at 3 cm), T2: flooding at 3 cm depth (irrigate when the water level dropped at 1 cm), T3: flooding at 1 cm depth (irrigate when the soil water level dropped at saturated level) and T4: alternative wetting and drying (AWD; wetting at 5 cm flooding when the water level dropped at drying level of -33 Kpa)] arranged according to the completely randomised design (CRD) with five replications. Soil water potential values were determined by using ECHO soil moisture sensors. The experiment was conducted under a rain shelter. Insect, disease and weeds were controlled according to Sarwar et al. (2004). Fertilisers were applied according to the previous studies (Sarwar & Khanif, 2005; Khairi et al., 2015a).

Measurement of the Parameters

At harvest, yield and yield related parameters were measured according to Jahan et al. (2012, 2013a). A portable SPAD-502 chlorophyll meter (Minolta Technologies, Japan) was used to measure chlorophyll content in leaves (Abdulkadir et al., 2015; Khairi et al., 2015b; Nozulaidi et al., 2015). The second uppermost collared-leaf was used to determine Chl content at different weeks after planting. A CI-340 portable photosynthesis meter (CID Biosciences, Inc., USA) was used to determine net photosynthesis rate (Munirah et al., 2015b). The leaf relative water content was measured according to Chelah et al. (2011). It is important to note that water needed for land preparation was not considered in this experiment. This experiment was conducted under rain shelter, therefore, rainwater was considered as zero (0). Water saving was calculated against the control treatment. Water use efficiency (WUE) was calculated from the grain yield divided by the amount of irrigation water applied in the treatments. Meanwhile, soil pH and soil EC were measured using portable Mettler Toledo MP120 pH meter and soil EC meter, respectively. Soil extracts were collected using soil sampler model SPS200, whereas nitrogen (N), phosphorus (P) and potassium (K) were measured according to Sarwar (2004) and Jahan et al. (2013a).

Statistical Analysis

The data were analysed for the analysis of variance (ANOVA). The means were compared by using Duncan's Multiple Range Test (DMRT) at 5% level by utilising the SPSS software (Version 17), MS Excel and Minitab 16.

RESULTS

Effects of Flooding Water on Plant Height, Yield and Yield Parameters

Results indicated that different water levels did not affect plant height in the first few weeks but it was affected thereafter (Figure 1A). Plant height gradually increased with increasing plant age regardless of the effects of treatments (Figure 1A). Application of water increased tiller and panicle numbers/hill and panicle height of rice plants compared to alternative wetting and drying treatment (Figure 1B). Seed size did not affect by water stress (data not shown). Flooding water (Treatments T1 to T3) significantly increased total grains and filled grains per panicle (Figure 1C). Furthermore, alternative wetting and drying treatment significantly affected rice yield (Figure 1C) which made the lowest yield, although continuous flooding at 5 cm made the highest yield.

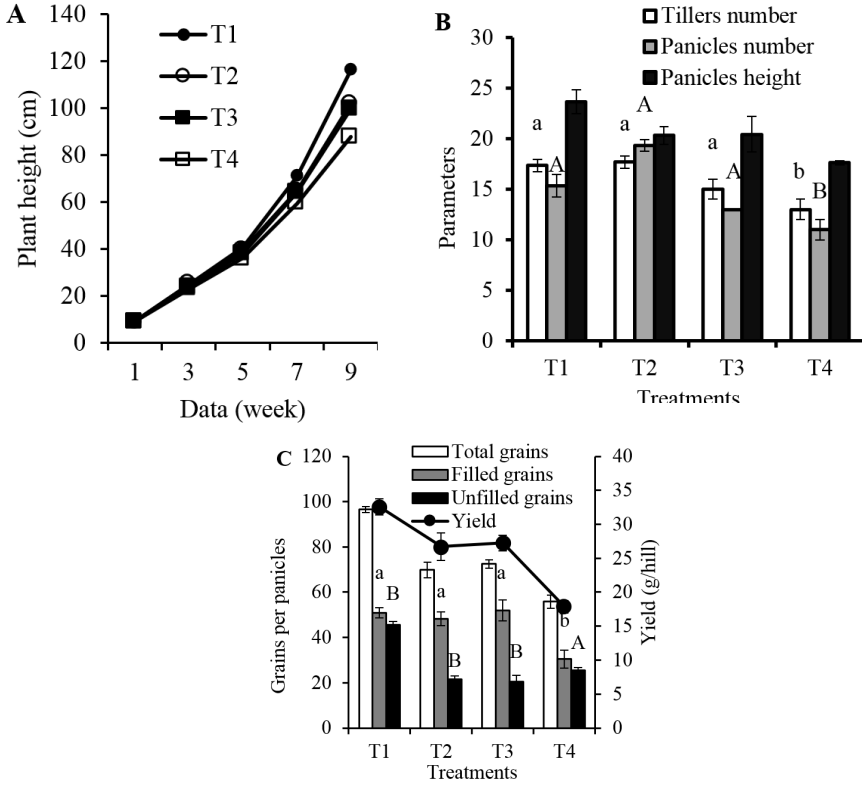

Figure 1. Effects of different water levels on yield and yield parameters of upland rice variety.

A. Plant height was measured at different weeks under different water levels; T1 (closed round), T2 (open round), T3 (closed square) and T4 (open square). B. Plant produced tillers number (open bars), panicles number (gray bars) and panicle height (black bars) under different water regimes. C. Left panel shows total grains per panicle (open bars), filled grains per panicle (gray bars), unfilled grains per panicle (black bars) and right panel shows the yield of rice (line graph). Here, T1 stands for flooding at 5 cm depth, T2 stands for flooding at 1 – 3 cm depth, T3 stands for saturated to 1 cm flooding and T4 stands for alternative wetting and drying.

Effects of Flooding Water on Chlorophyll Content and Net Photosynthesis Rate (Pn) in Leaves

Chlorophyll content did not affect in all treatments for the first five weeks but significantly increased chlorophyll contents afterward (Figure 2). Figure 3 showed that Pn rate decreased with decreasing water input in soil but the decrement was not significant until T3 treatment. Pn rate was significantly higher in plants under different treatments (T1 to T3) than that of plants under T4 treatment.

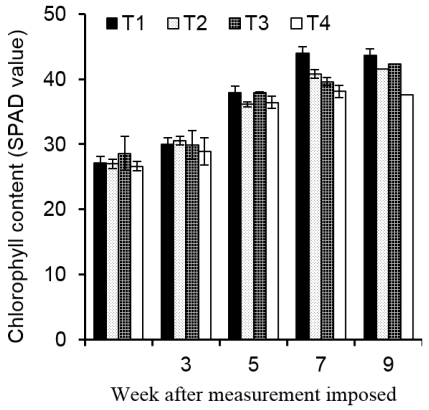

Figure 2. Effects of different water regimes on chlorophyll content in leaves of rice plants.

T1 (closed bars), T2 (dotted bars), T3 (grid bars) and T4 (open bars). Vertical bars indicate standard deviation. Here, T1 is for flooding at 5 cm depth, T2 is for flooding at 1 – 3 cm depth, T3 is for saturated to 1 cm flooding, and T4 is for alternative wetting and drying.

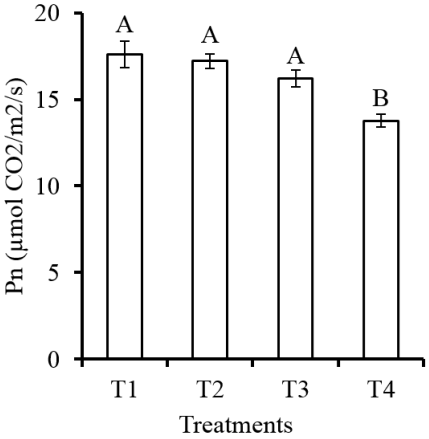

Figure 3. Effects of different water regimes on net photosynthesis rate in leaves of rice plants. Here, T1 is for flooding at 5 cm depth, T2 is for flooding at 1 – 3 cm depth, T3 is for saturated to 1 cm flooding, and T4 is for alternative wetting and drying.

Effects of Flooding Water on Leaf RWC, Water use and WUE

The highest RWC was recorded in T2 treatment and non-significant difference with T1 and T3 (Figure 4A). In contrast, the lowest RWC was recorded in T4 treatment. Water volume used in T3 and T4 treatments was almost similar (Figure 4B) but it was significantly lower than T1 and T2 treatments. Water volume used in rice cultivation increased with increasing depth and duration of irrigation water used (Figure 4B). The potency of water use was found to be the sequence of T1 > T2 > T4 > T3. The treatments T4, T3 and T2 saved water about 43, 42 and 29%, respectively, over the control (data not shown). Figure 4B also shows that water use efficiency in T3 treatment was significantly higher than other treatments, while T2 and T4 treatments showed similar WUE whereas control (T1) showed significantly lower (Figure 4B, line graph).

Effects of Flooding Water on Soil pH and Soil EC Value in Soil

Soil pH and EC readings were determined to justify the effects of flooding water on soil chemical properties (Sarwar, 2004). Flooding water had no significant influence on soil pH until T3 treatment and soil pH significantly decreased in T4 treatment as compared to the control (Figure 5A). On the other hand, flooding water reduced soil EC value compared to AWD treatment, which indicates solute accumulation in soil (Figure 5B).

Effects of Flooding Water on Phytoavailability of Nitrogen, Phosphorus and Potassium in Soil Solution

Figure 6A shows that the concentrations of N and K decreased with time under water stress and even decreased with decreasing water levels (Figs. 6A & 6C). These results are consistent with some previous results by

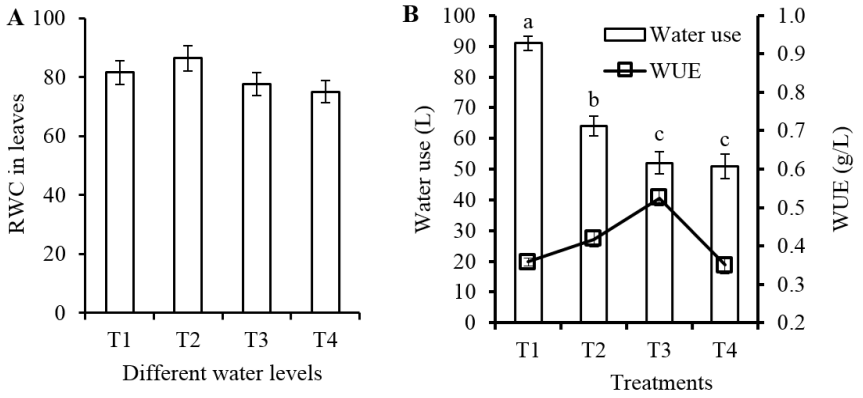

Figure 4. Effects of different water regimes on relative water content and water productivity.

A. Relative water content in leaves of rice plants grown on different water treatments. B. Water use (open bars) and WUE (line graph) under different water treatments. Here, T1 is for flooding at 5 cm depth, T2 is for flooding at 1 – 3 cm depth, T3 is for saturated to 1 cm flooding, and T4 is for alternative wetting and drying.

Jahan et al. (2012, 2013b). Flooding water did not affect P concentration in soil solution except for T4 treatment, which significantly decreased P availability in soil solution. This finding is also consistent with the previous results by Sarwar et al. (2004).

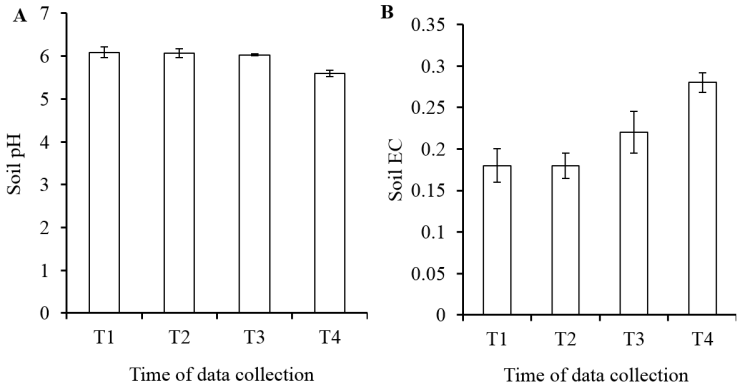

Figure 5. Effects of different water regimes on soil pH (A) and soil electric conductivity (B). Here, T1 is for flooding at 5 cm depth, T2 is for flooding at 1 – 3 cm depth, T3 is for saturated to 1 cm flooding and T4 is for alternative wetting and drying.

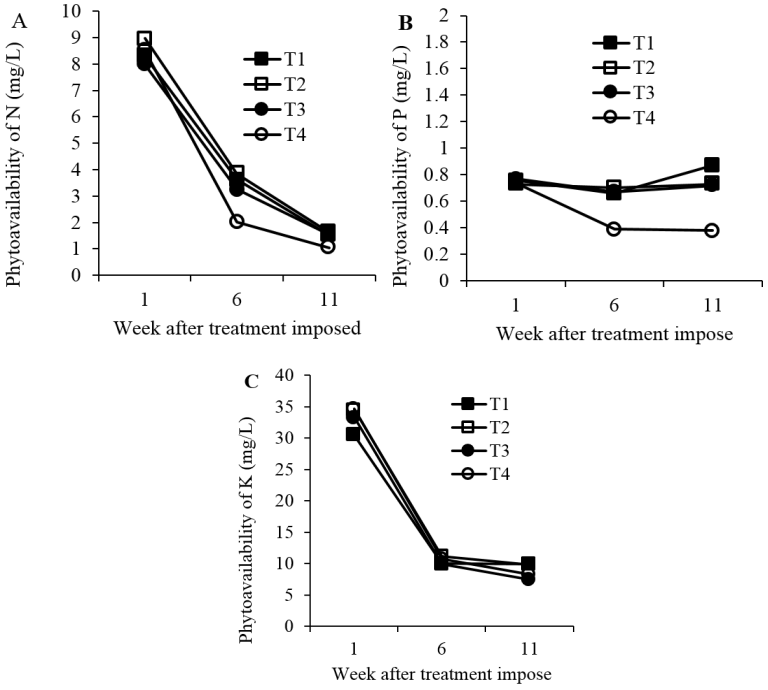

Figure 6. Effects of different water regimes on phytoavailability of nutrients.

Phytoavailability of nitrogen (A), phosphorus (B) and potassium (C) in soil solution different water treatments, T1 (closed square), T2 (open square), T3 (close round) and T4 (open round). Here, T1 is for flooding at 5 cm depth, T2 is for flooding at 1 – 3 cm depth, T3 is for saturated to 1 cm flooding, and T4 is for alternative wetting and drying.

DISCUSSION

Malaysia still practices conventional flooded rice cultivation system, which leads to use higher amounts of fresh water compared to the less water rice production system (Sarwar et al*.,* 2004). This study showed that uses of flooding water increased production of upland rice (Figure 1). Upland plants might enhance physiological function under flooding condition than AWD condition. These results are supported by the effects of AWD treatment on grain filling stage that might lead to a reduction of grain filling per panicle (Figure 1C). In contrast, saturated and above water condition did not affect filled grains per panicle, the finding which is consistent with that of Sarwar et al. (2004). Therefore, these results suggested that upland rice might increase yield and yield parameter when grown under saturated or above soil water condition than alternative wetting and drying condition.

Chlorophyll content in leaves controls crop productivity by modulating physiological function in plants (Jahan et al*.,* 2016). The finding of this study stated that flooding water treatment induced the accumulation of chlorophyll content (Figure 2), which might increase photosynthesis rate in plants (Figure 3). These results suggested that application of flooding water in upland rice variety might affect Chl-functioned plant growth and development (Khairi et al*.,* 2015b). Furthermore, higher Chl content in wild type plants indicates higher GSH content in plants compared to Chl-deficient mutant (Jahan et al*.,* 2011). This also supports that flooding treatments (T1, T2 and T3) might affect GSH content that enhanced physiological function in the plant. In addition, Kura-Hotta et al. (1987) stated that photosynthesis is affected by water stress, which supports this study that saturated or above water condition did not affect Pn rate in upland rice plants (Figure 3).

Drought reduces relative water content in leaves under T4 treatment (Figure 4A). Water use efficiency increased in T3 treatments compared to T4 and T1 treatments (Figure 4B) indicating that less water might ensure sustainable rice production and reduce water use over the control. In contrast, soil pH decreased (Figure 5A) but EC value increased in the soil of T4 treatment (Figure 5B). Under anaerobic condition, bio-chemical reaction might not affect soil pH; this indicates unaffected phytoavailability of nutrients in the soil (Figure 6). Nitrogen decreased in the soil of T4 treatment; this might due to different transformation processes of nitrogen in the soil, e.g. nitrification. P is less deficient in flooded soil than in upland soil due to the highly available forms of P in flooded soils (Thiyagarajan & Selvaraju, 2001), while Olk et al. (1995) suggested that plant-available K decreases after flooding of dry soil due to fixation. These results confirmed that saturated or above water condition did not affect nutrients phytoavailability in the soil.

CONCLUSION

Soil water condition at saturated or above it did not affect water use, Chl content, soil

chemical properties and rice production. In addition, T3 treatment reduced water use but increased WUE and rice yield. Farmers could implement irrigation water at saturated to 1 cm flooding to increase rice production by using upland variety without affecting soil and plant parameters but saving a larger amount of fresh water instead.

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Impact of Different Temperature-Time Profiles during Superheated Steam Roasting on Some Physical Changes of Robusta Coffee

Ooi Ee Shan[1], Wahidu Zzaman[1,2] and Tajul A. Yang[1]*

[1]Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia
[2]Food Engineering and Tea Technology, Shahjalal University of Science and Technology, Sylhet-3114, Bangladesh

ABSTRACT

Roasting is the most important step in the coffee processing. The impact of superheated steam roasting temperature (150, 200, 250°C) and time (10-50 min) on the color (L*, a*, b*, browning index), moisture and hardness attributes of *Coffea canephora* (Robusta coffee) beans were studied. Increases in roasting temperature and time caused a decrease in all the responses except for browning index, a* and b* values of roasted coffee beans. A decrease in the hardness of coffee bean during roasting has been correlated to the loss of moisture content. Coffee beans exhibit greater bean volume, pore volume and larger micropores during roasting process, which indirectly lead to the loss of moisture content. With regards to the prolonged superheated steam roasting, it significantly affects colour attributes and moisture content. As the roasting temperature increased up to 250ºC, colour attributes such as browning index, a* and b* values decreased significantly whereas moisture content increased slightly.

Keywords: Superheated steam, roasting, coffee bean, moisture, color and hardness

INTRODUCTION

Coffee plants belong to the botanical genus *Coffea* in the family of flowering plants called *Rubiaceae*, which contains around 600 genera and 13,500 species. Although the genus *Coffea* L. have more than 100 species, only two, *Coffea arabica* (Arabica coffee) and *Coffea canephora* (Robusta coffee), are commercially cultivated (Mishra

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E-mail addresses:
eshanooi@hotmail.com (Ooi Ee Shan),
wahidanft@yahoo.com (Wahidu Zzaman),
taris@usm.my (Tajul A. Yang)
* Corresponding author

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& Slater, 2012). Coffee is one of the most widely consumed beverages around the world (Ding et al*.,* 2014). Coffee gains its popularity around the world for its aroma and flavour. Besides, its popularity is also attributable to the stimulating effects of its caffeine content. Recent studies suggest that coffee consumption may have beneficial effects on human health and coffee has been identified as major contributor to the *in vitro* antioxidant capacity of the diet (Van Dam, 2008).

Coffee roasting is an important unit operation to develop the specific organoleptic properties (aroma, flavour and colour) in which the process has a great degree of influence on coffee final quality (Hernández et al*.,* 2008; Mussatto et al*.,* 2011). Roasting is a highly complex process as it involves various chemical reactions and considerable physical changes, which all depend on the temperature-time profiles used during roasting process (Wang, 2012). Heat and mass transfer occur simultaneously inside coffee beans. The roasting process consists of two stages, first the drying stage where the moisture is rapidly driven out of the bean. Moisture content significantly decreases during the first 5 to 10 minutes of roasting (Kristin, 2011). Colour development has been reported to occur rapidly after the initial drying stage.

Colour development is one of the parameters that is used to measure the degree of roast and acts as an indicator of final quality product (Manzocco et al*.,* 2000; Baggenstoss et al*.,* 2008). Clarke and Vitzthum (2008) reported that Maillard reaction and oxidative polymerisation or degradation of phenolics compounds are the major reactions responsible for colour formation during roasting process. The chemistry underlying the Maillard reaction is very complex as it involves not only one reaction pathway but a whole network of various reactions (Martins, 2003).

In addition, changes in the textural characteristics undergo during roasting plays an important control parameter for coffee bean roasting. During the roasting, beans become more crumbly and brittle as they lose their strength and toughness, which are typical characteristics of roasted products. Besides, a decrease in moisture content affects the texture properties, which causes the beans to become more fragile and brittle upon roasting (Kita & Figiel, 2007). Reaching of a certain degree of brittleness is very important for the grinding process, which is carried out before the extraction of coffee brew (Jokanovic et al*.,* 2012). Meanwhile, the fineness of grind affects the extraction of soluble solids, which relates directly to the brewing extraction (Pittia et al*.,* 2001).

Roasting is a heating process that drives off the free and bound moisture of a food sample. The coffee roasting process is carried out in many ways; these is done by conduction using hot metal surfaces (conventional drum roasters), convection using air as the heating medium (fluid bed roasters) and radiation methods (infrared roasters). A widely used method of roasting coffee beans is convection roasting which is based on subjecting the raw beans to a

flow of hot air in the temperature range between 200 to 230ºC for 12 to 20 minutes (Jokanovic et al*.,* 2012). However, it has a number of drawbacks such as difficulty in handling particulate solids and lowering the product quality (Panda, 2013). The processing method used on coffee is usually limited to the traditional roasting method; thus, superheated steam roasting may offer an alternative method. Superheated steam is a type of unsaturated steam generated when additional heat is applied to raise the steam temperature above the saturated dry steam (Zzaman et al*.,* 2013). When drying with superheated steam, the water removed from the product during the drying process becomes part of the drying medium, whereas in hot-air drying method, the moist air must be replaced by fresh air (Moreira, 2001; Borquez et al*.,* 2008). Superheated steam is cleaner and it provides higher evaporation rate, yields better colour and less oxidation in food, thus reducing the loss in nutritional value during the drying process (Moreira, 2001; Wang et al*.,* 2012).

Numerous studies have been conducted on *Coffea arabica* (Arabica coffee) varieties using the conventional roasting method but no studies have been done on superheated steam roasting of *Coffea canephora* (Robusta coffee). Therefore, the purpose of this work is to study the changes in moisture, colour and hardness of *Coffea canephora* using superheated steam roasting in order to predict their effect with different time-temperature profiles.

MATERIALS AND METHODS

Sampling

Green coffee (*Coffea canephora*) was obtained from the local Hang Tuah Coffee factory located in Tasek Gelugor Seberang Prai, Malaysia. The beans were stored in a dry place, which is also known as "out-gassing". The purpose is to excrete and separate moisture from the beans. The beans were then subjected to sorting and selection processes manually at the laboratory of Food Technology Department. The defective beans (black, partly black, broken, infested) were discarded and the non-defective beans were packed in hermetically sealed containers. The moisture content of green coffee was 10.06 %.

Superheated Steam Roasting

The roasting process was carried out by placing the Robusta coffee beans in the superheated steam drying oven and exposed them to the roasting temperatures of 150, 200, 250ºC at different time intervals (10-50 min). Sharp AX-1500 K superheated steam oven with a pressure of approximately 1 bar, steam generation capacity of 16 cm^3/ min, and steam engine heater of 900 W was used during the roasting process. Roasted beans were stored in an air tight container for further analysis.

Moisture Content Determination

Moisture content was determined using a halogen moisture analyser (HB43-S model, Mettler Toledo, UK). The results were the mean of three measurements.

Colour Measurement

Colour measurement was done using CIE Lab scale (CM-3500D Minolta spectrophotometer, Minolta, Japan). Three measurements were done for each sample. The reflectance used was d/8 (diffuse illumination/ 8 ° viewing angle) geometry, pulsed xenon arc lamp as the light source and 2.5 s for the measurement time. The instrument was calibrated with zero calibration (CM-A100) and followed by white calibration plate (CM-A120) before measuring the sample. The colour values are expressed as L* (whiteness or darkness), a* (redness/ greenness), and b* (blueness/ yellowness). The L*, a*, and b* values are the three dimensions of the measured colour, which gives specific colour value of the material. The browning index (BI) (Eq. (1)) was also used to estimate total colour changes during roasting (Maskan, 2001).

$$BI = \frac{[100\ (x-0.31)]}{0.17} \quad [1]$$

where

$$x = \frac{(a+1.75\ L)}{(5.645\ L+a-3.012\ b)} \quad [2]$$

Texture Measurements

The texture analysis of the Robusta coffee beans were performed using a Universal Texture Analyser (CNS, Farnell, UK) equipped with the Texture Pro™ texture analysis software. A bean was placed horizontally on the platform and double compression was applied using a 36 mm cylindrical probe P/36 R at a test speed of 5.0 mm/s. The instrument was calibrated with a 30 kg load cell, 15 mm of return distance, 10 mm/s of return speed and 5 g of the contact force. The maximum peak of the first compression (N) in the force-time curves indicates the hardness value (Kahyaoghu & Kaya, 2006; Shakerardekani et al., 2011).

Statistical Analysis

The moisture contents, colour values, and textural parameters of roasted coffee beans were analysed using the two-way analysis of variance method (ANOVA) to determine the effects of temperature and time on these responses. The ANOVA tests were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Moisture Content

Moisture content of green and roasted coffee beans typically varied within the ranges of 9.06 – 9.24 and 1.11-3.12 wt. (%), respectively (Bicho et al., 2012). The moisture content of coffee bean has a strong influence on the degree of brittleness and it is important during the grinding process as it influences the coffee extraction (Jokanovic et al., 2012). As shown in Figure 3, increasing the roasting temperature from 150 to 250°C causes the moisture content to decrease significantly ($P < 0.05$).

During the early phase of roasting, progressive decrease in moisture content was observed in Robusta coffee bean due to water evaporation as the bean core

temperature is increased above the boiling point of water (Wang, 2012). According to Correa et al. (2010), increased roasting temperature activated the water molecules to higher energy levels, allowing them to break away from their sorption sites, and thus reducing the moisture content of roasted beans.

From Figure 3, moisture content increased slightly from 30 to 50 min for 250°C. This might be attributed to the hygroscopic nature of coffee beans (Ismail et al*.,* 2013; Coradi et al*.,* 2014). Superheated steam is sprayed onto the beans during roasting process and since coffee beans are hygroscopic in nature, the water molecules which condense on to the bean surface will rapidly be absorbed (Clarke & Macrae, 1988).

Colour Changes

Colour is an important quality indicator in the roasting process which is typically used as an indicator of the degree of roast (Chu, 2012). The changes in the CIE L*, a*, b* and BI values of roasted Robusta coffee at different temperatures are shown in Figure 1. The two-way ANOVA indicated that temperature and time significantly ($P<0.05$) affected the colour values of ground Robusta coffee during roasting.

The L-value indicates the whiteness of a sample. Results showed that the L-value was significantly ($P < 0.05$) influenced by the roasting temperature and time. In general, the L-value of coffee beans tends to decrease during the roasting process which may be due to the brown pigment produced by non-enzymatic browning (Sacchetti et al., 2009; Wang, 2012). As an exception, the L- value of the coffee beans increased slightly up to 10 min of roasting time and decreased thereafter for the entire roasting temperature from 150 to 250ºC. The initial increase in the L-value during the early periods of roasting was similar to the observation for hazelnut (Özdemir & Devres, 2000), corn kernels (Chung et al*.,* 2014) and sesame seeds (Kahyaoglu & Kaya, 2006). Özdemir and Devres (2000) reported that the reason of the initial lightening in roasted hazelnuts may be due to the denaturation of soluble protein. Meanwhile, according to Kahyaoglu and Kaya (2006), denaturation of proteins, concentrated amount of oil particles embedded in the protein matrix and low moisture content were the factors contributing to the initial lightening in sesame seeds.

The a-value shows redness for roasted products. The a-value of Robusta coffee roasted at temperature below 250ºC tended to increase over the entire roasting period; however, the beans roasted at 250ºC increased up to 20 min of roasting and then decreased over the remaining period. Gökmen and Şenyuva (2006) reported that changes in CIE a* colour value are correlated with the acrylamide content in coffee. The amount of acrylamide increased rapidly at the onset of roasting then decreased exponentially as the rate of degradation exceeds the rate of formation. The CIE a* profile followed a similar trend. It is also reported by other researchers (Taeymans et al*.,* 2004) that darker coloured coffee

contains lower acrylamide content than the light coloured coffee. Hence, an initial increase in a-value to an apparent maximum as there is an increased rate of brown pigments formation through the Maillard reaction. However, a-value decreased at 250 ºC after 20 min was possibility due to the acrylamide degradation.

The b-value shows the degree of yellowness (Figure 1). The b-value of roasted coffee increased at the lower roasting intensity but decreased significantly with a higher roasting intensity. The b-value of the coffee beans roasted at 150, 200 and 250ºC decreased gradually after 40, 30 and 20 min of roasting, respectively. According to Afoakwa et al. (2014), an increase in the b-value with the increasing roasting time is due to thermal oxidation of polyphenols and Maillard products formation.

Browning index (BI) represents the purity of brown color develops during the roasting processes, where enzymatic and non-enzymatic browning takes place (Maskan, 2001). Generally, it is an indirect way to measure the contents of pigment compounds produced from browning reactions (Chung et al*.,* 2013). Changes in the browning index of coffee beans during roasting are shown in Figure 1. Browning index change was significantly affected ($p < 0.05$) by roasting temperature and time. The browning index of the beans roasted at 150ºC increased throughout the roasting process, especially the beans roasted at 200ºC, which sharply increased after 10 min of roasting. The increase in the browning index was probably due to the brown pigment formation from Maillard reactions, thermal oxidation, Strecker degradation

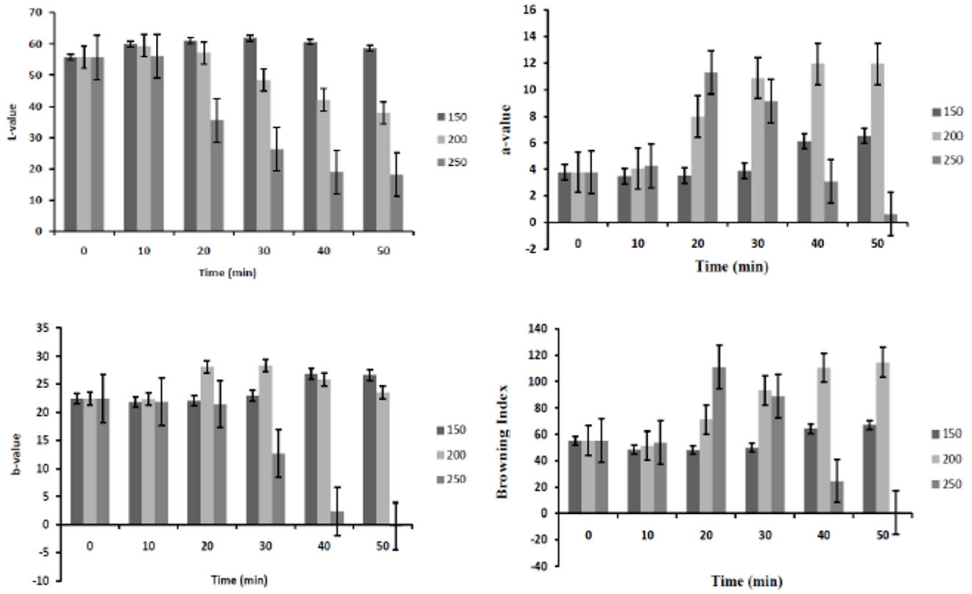

Figure 1. Changes in the colour attributes (L*, a*, b* and Browning index) of Robusta coffee bean during superheated steam roasting at different temperatures and time

and polymerisation of polyphenols to form tannins during the roasting process. However, the browning index of the beans roasted at 250ºC decreased after 20 min of roasting. These results showed that Robusta coffee roasted at the temperature below 200ºC has higher content of Maillard reaction products compared with the coffee beans that roasted at 250ºC.

Changes in Hardness

Heat treatment determines the textural characteristics of the roasted coffee. Green coffee beans have thick cell walls and lack intracellular spaces and exhibit exceptional hardness. In the coffee industry, grinding is a process which is carried out on roasted coffee beans before coffee brew extraction. Therefore, the degree of brittleness is very important in the grinding process and it influences the coffee brew extraction efficiency (Pittia et al., 2001; Nedomová et al*.,* 2013).

The hardness of roasted Robusta coffee beans showed significant decrease ($p < 0.05$) as roasting temperature and time increased, indicating a progressive increase in the brittleness of the bean. As shown in Figure 2, the hardness significantly decreased ($p < 0.05$) from 150°C to 250°C for all roasting time (10-50 min). However, there was a slight increase in the hardness at the roasting temperature of 150°C for 30 min. Based on Wilson et al. (1997), polysaccharide cytoplasmic matrix of coffee bean starts to denature during the early stages of roasting, and this is followed by moisture loss, increase in bean volume and larger cell wall micropores as the roasting process continue. Loss of moisture and disruption of parenchyma is the principal cause for the increase of brittleness, crispness and crunchiness in the roasted beans. Hence, reduction of the hardness indicates a progressive decrease in moisture content in the bean and increase in the brittleness of the coffee beans as a more porous structure formed during the roasting process (Li et al*.,* 2005).

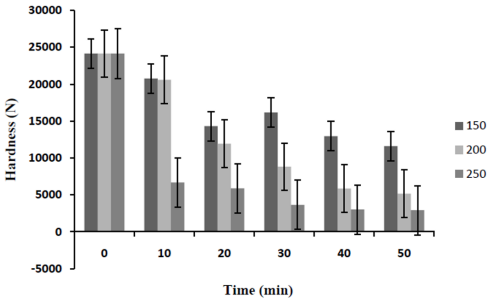

Figure 2. The effects of different roasting temperatures (150 to 250°C) and time (0 to 50 min) on hardness of coffee beans

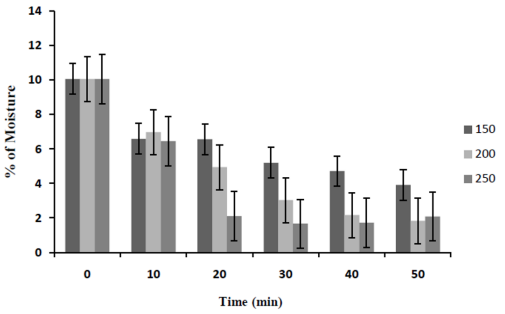

Figure 3. The effects of different time-temperature profiles during superheated steam roasting on moisture content of Robusta coffee beans

CONCLUSION

Roasting process is an important step in coffee industry, where the desired aroma, flavour and colour of coffee are developed (Noor Aliah et al., 2015). Thus, roasting process has a crucial impact on the final quality of coffee.

Colour of the roasted beans used to define the end of the roasting operation and is often correlated to the degree of roasting (light, medium, or dark roast); the higher the roasting temperature, the darker the colour of the bean (Buffo & Cardelli-Freire, 2004). Moisture content and toughness of the coffee beans have been used to study mechanical properties such as brittleness of coffee beans. Meanwhile, degree of brittleness is very important for the grinding process and it affects the extraction of soluble solids to obtain the good quality coffee brew (Nedomova et al., 2013).

In this study, Robusta coffee was subjected to superheated steam roasting within an appropriate temperature range (150 – 250°C) and time (10 – 50 min) exhibited desired colour, hardness, as well as moisture content. As the roasting temperature and time increased, the colour of bean became darker, more brittle texture and thus reduced the moisture content. The results of this study showed that the effects of both superheated steam roasting temperature and time have significant influence ($p < 0.05$) on moisture content, hardness, and colour changes of Robusta coffee beans. Therefore, the new method of superheated steam can be employed to produce desired coffee and coffee products because it takes shorter time to achieve satisfactory results although slightly high temperature is required. Further studies on chemical properties will be conducted to evaluate the final coffee quality after roasting using superheated steam.

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Improved Pre-treatment Protocol for Scanning Electron Microscopy Coupled with Energy Dispersive X-ray Spectroscopy Analysis of Selected Tropical Microalgae

Yau, S. K.[1], Yusoff, F. M.[1,2]*, Khong, N. M. H.[1], Foo, S. C.[1] and Lai, J. I.[1]

[1]*Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

[2]*Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Suitable protocol for identification and classification of microalgae using scanning electron microscopy, coupled with energy dispersive X-ray spectroscopy (SEM-EDX), is important to obtain accurate information of their ultrastructure description. The objective of this study was to modify microalgae pre-treatments for reliable SEM-EDX analysis. Sixteen cultured tropical microalgae were subjected to two-step chemical fixation of glutaraldehyde and osmium tetroxide, sample washing in sodium cacodylate, ethanol and acetone dehydration, critical point-drying, mounting and gold sputter-coating prior to SEM visualisation and elemental characterisation. In this study, short period of chemical fixation and optimum separation forces, at 3213 x *g* for 3 min during every chemical solution change, were successfully established with high quality SEM images. Ultrastructure, particularly clear and useful images of cell wall ornamentation in *Scenedesmus* spp. and *Desmodesmus* sp.; areola patterns in *Biddulphia sinensis* and *Thalassiosira* sp. and morphological appearances such as interconnecting structures in *Coelastrum* sp. and *Crucigenia* sp., were obtained. Twelve elements of Y, Nb, Fe, Ca, Cl, K, Cu, F, Ir, P, Mg and Si were detected within the 16 investigated microalgae species. This study illustrated that microalgae identification and classification, as well as their elemental characterisation, could be simultaneously and effectively analysed by SEM-EDX using a modified pre-treatment protocol.

Keywords: Microalgae, morphology, ultrastructure, elemental profiles, modified SEM-EDX pre-treatment protocol

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E-mail addresses:
sookkun_88@yahoo.com (Yau, S. K.),
fatimamy@upm.edu.my, fatimahyus@gmail.com (Yusoff, F. M.),
nmhkhong@gmail.com (Khong, N. M. H.),
suchern@gmail.com (Foo, S. C.),
dora_fly83@yahoo.com (Lai, J. I.)
* Corresponding author

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INTRODUCTION

Microalgae are photosynthetic microorganisms that are ubiquitous in aquatic ecosystems regardless of freshwater, brackish or marine environment, either drifting in lotic or lentic water columns (planktonic) or attaching on substrates (periphytic). Many research studies have revealed that bioactive compounds derived from microalgae possess several important pharmacological activities such as antioxidant (Natrah et al., 2007; Goh et al., 2010), antimicrobial (Suresh et al., 2014), anti-inflammatory (Jo et al., 2010) and anti-cancer (Ebrahimi Nigjeh et al., 2013; Goh et al., 2014). Large untapped resources of microalgae provide great opportunities to explore novel energy and bioactive compounds such as hydrocarbons of *Botryococcus* spp. and carotenoids i.e. astaxanthin of *Haematococcus* spp., beta-carotene of *Dunaliella* spp. and lutein of *Scenedesmus* spp. (Sawayama et al., 1994; Boussiba, 2000; Ben-Amotz, 2004; Sánchez et al., 2008). However, species identification and classification remain the priority before further studies on their biological characterisation and application can be made. Besides, species identification and classification are also important in ecological studies especially on interaction between environmental factors and microalgae biodiversity (Van Staden et al., 2010; Renuka et al., 2014).

Essentially, researchers identify and classify microalgae using the guidance of dichotomous keys based on phenotypic characteristics of morphological appearances such as unicellular or multicellular, cell shape, cell arrangement and specialised cell parts like spines and flagella. Compared to light microscopy, scanning electron microscopy (SEM) offers even higher resolution, magnification, contrast and large depth of focus, and it becomes an important tool for the study of cellular and molecular components. Thus, SEM overcomes the limitation of light microscopy by displaying the ultrastructure characteristics in more detail which facilitates the microalgae identification and classification tasks. In addition, SEM coupled with energy dispersive X-ray spectroscopy (SEM-EDX) allows ultrastructure/ morphological visualisation and elemental characterisation of samples that can be conducted simultaneously. However, the quality and accuracy of the phytoplankton ultrastructure images are highly dependent on the modification of the pre-treatment protocol.

In pre-treatment protocol of SEM, all volatile substances from the sample must be removed and its strength of radiation damage resistance and electrical conductivity must be increased before submitting to the high vacuum chamber. Additionally, pre-treatment protocol is established according to the sample types which could well-preserve their native morphology without cell deterioration and eventually produce high quality images. Chen (2001) provided specific SEM pre-treatment steps for *Scenedesmus quadricauda*; however, the separation of delicate and fragile cells from fluids was not emphasised in his study. On the other hand, long fixation period was reported to be 12 hours in *Botryococcus* sp. and 24 hours in *Coelastrum* sp and

Scenedesmus obliquus (Dayananda et al., 2010; Liu et al., 2013; Basu et al., 2013; Tanoi et al., 2014). As a result, it is essential to improve SEM pre-treatments for various genera of microalgae which can be effectively used in future studies.

Elemental characterisation is one of the crucial studies to evaluate the nutritional properties of microalgae. Previous studies have shown that marine microalgae were good element sources for fish nutrition (Fabregas & Herrero, 1986; Hussein et al., 2013). Moreover, essential elements from algae have good nutritional benefits for human, but many studies provided information on edible seaweeds instead of microalgae (Ortega-Calvo et al., 1993; Tokusoglu & Ünal, 2003; Krishnaiah et al., 2008; Rohani-Ghadikolaei et al., 2012). Hence, this study aimed to develop the modified pre-treatment protocol for SEM-EDX analysis in order to produce high quality images for identification and classification, as well as to characterise the elemental compositions of 16 selected tropical microalgae.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents used were of analytical grade. Chemicals such as glutaraldehyde, sodium cacodylate and osmium tetroxide were obtained from Agar Scientific (Agar Scientific, Elektron Technology, UK). Ethanol and acetone were purchased from Merck, (Merck, Darmstadt, Germany).

Microalgae Cultivation and Maintenance

Microalgae strains were obtained from the Microalgae Culture Collection of the Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia (Table 1). Purified freshwater microalgae were maintained in Bold Basal medium (Bold & Wynne, 1978), pH 6.8 ± 0.2, whereas marine diatoms in f/2 medium (Guillard, 1975), pH 8.3 ± 0.2 and salinity 30 ± 1‰. All microalgae (100 mL in 250 mL conical flasks) were batch-cultured in an environmental chamber (Versatile Environmental Test Chamber, Sanyo, Japan) under constant 120 µmol photons $m^{-2}sec^{-1}$ light intensity at 12 h light: 12 h dark at 25°C for 14 days. Purity of unialgal culture was examined routinely via light microscopy. Fourteen freshwater green microalgae and two marine diatoms were used in this study for SEM-EDX analyses (Table 1).

Sample Pre-treatment Steps

Generally, the flow of pre-treatment steps included monoalgal sample preparation, pre- and post-fixation, first and second washing, dehydration, drying, mounting and sputter-coating (Figure 1) before the samples were subjected to image viewing and elemental characterisation. One millilitre of monoalgal culture (1 x 10^6 of cell density) was sampled during its late exponential growth phase and centrifuged to separate cells from liquid medium. Green microalgae and diatoms (without acid washing step) were pre-fixed in 4% glutaraldehyde ($C_5H_8O_2$) that was prepared in 0.2M sodium cacodylate buffer ($C_2H_6AsNaO_2$) at pH 7.2 for 3 hours

at 4°C. After pre-fixation, samples were washed three times with 0.1M sodium cacodylate buffer for 10 min to remove excessive fixative. Cells were post-fixed in 1% osmium tetroxide (OsO_4) for 2 hours at 4°C. The washing step was repeated for the same purpose. Samples were dehydrated gradually using a series of ethanol (30%, 40%, 50%, 60%, 70%, 80% and 90%) for 10 min each, followed by 100% ethanol and 100% acetone, twice for 15 min each. They were centrifuged for 3 min at 3213 x *g* at each changing of buffer or chemical solution.

Samples inside small handmade baskets made from aluminium foil were critical-point dried at 42ºC under pressure of 85 bar using a critical point dryer (Leica EM CPD030). The samples were mounted on stubs using double-sticky tapes and then sputter-coated with gold at 20 mA for 180 s using sputter coater (BAL-TEC SCD005). The pre-treated samples were inserted into working chamber of scanning electron microscope prior to SEM-EDX analyses. Images of the samples were visualised and their elemental profiles were analysed using variable pressure scanning electron

Table 1
Microalgae used in scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDX) analysis

Collection code	Species	Freshwater (F)[1] / Marine (M)[2]
UPMC-A0002	*Scenedesmus* sp. A	F
UPMC-A0003	*Scenedesmus* sp. B	F
UPMC-A0004	*Scenedesmus* sp. C	F
UPMC-A0005	*Scenedesmus* sp. D	F
UPMC-A00049	*Scenedesmus* sp. E	F
UPMC-A0043	*Botryococcus* sp. A	F
UPMC-A0044	*Botryococcus* sp. B	F
UPMC-A0045	*Coelastrum* sp. A	F
UPMC-A0046	*Coelastrum* sp. B	F
UPMC-A0008	*Ankistrodesmus* sp.	F
UPMC-A0048	*Crucigenia* sp.	F
UPMC-A0042	*Desmodesmus* sp.	F
UPMC-A0006	*Kirchneriella* sp.	F
UPMC-A0009	*Selenastrum* sp.	F
UPMC-A0050	*Biddulphia sinensis*	M
UPMC-A0051	*Thalassiosira* sp.	M

[1]Freshwater species were maintained in Bold Basal Medium (BBM): chemical compositions of $NaNO_3$, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, K_2HPO_4, KH_2PO_4, NaCl, EDTA, KOH, $FeSO_4.7H_2O$, H_3BO_3, $ZnSO_4.7H_2O$, $MnCl_2.4H_2O$, MoO_3, $CuSO_4.5H_2O$ and $Co(NO_3)_2.6H_2O$ (Bold & Wynne, 1978).
[2]Marine species were maintained in f/2 medium: chemical compositions of $NaNO_3$, $NaH_2PO_4.H_2O$, $Na_2SiO_3.9H_2O$, $FeCl_3.6H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $Na_2MoO_4.2H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$ and $MnCl_2.4H_2O$ (Guillard, 1975).

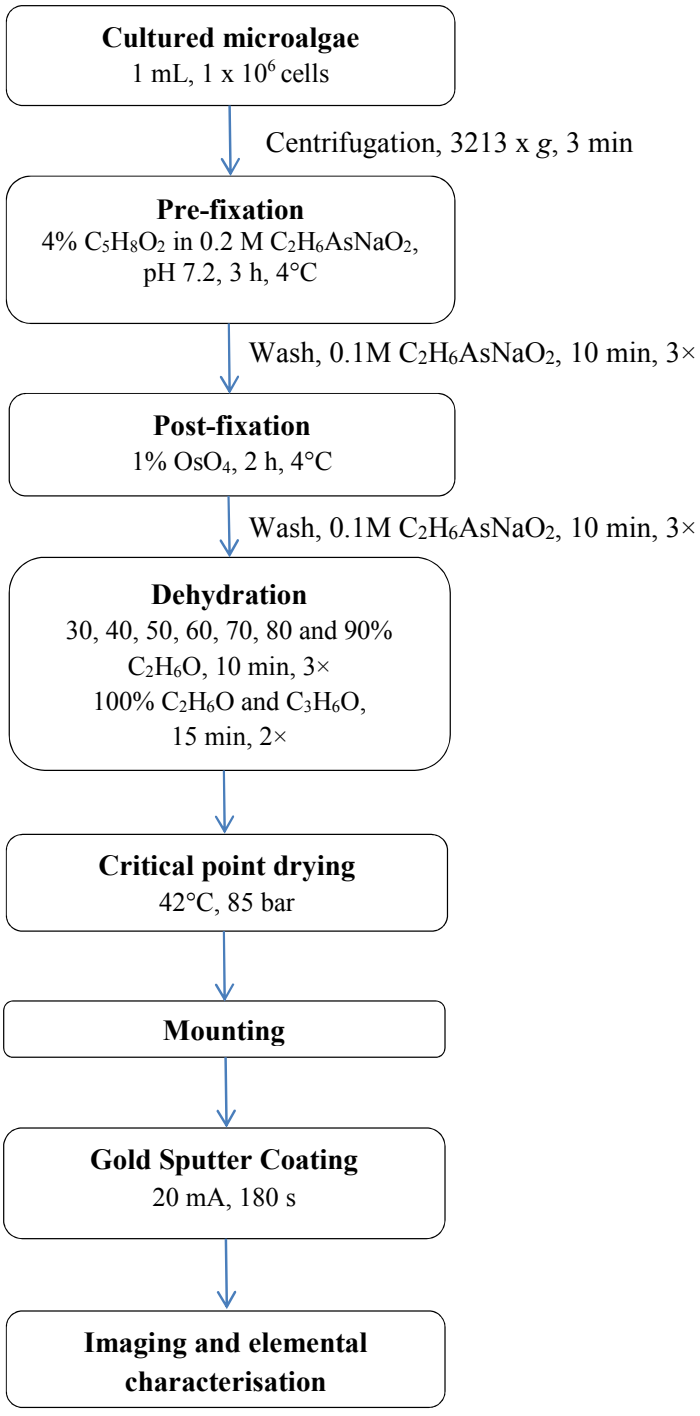

Figure 1. A modified scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDX) pre-treatment protocol for the analyses of microalgae.

microscopy (LEO 1455 VPSEM), coupled with energy dispersive X-ray spectroscopy (Oxford Inca EDX), at an accelerating voltage of 15-20 kV and working distance of 7-15 mm.

Statistical Analysis

All data were presented as mean ± standard deviation (n=3). One-way ANOVA (SPSS 21.0, USA) was applied to test the significant differences ($p<0.05$) for atomic % of microalgae elemental characterisation.

RESULTS AND DISCUSSION

Modification of Microalgae Pre-treatment Protocol for SEM-EDX Analysis

Environmental scanning electron microscopy (ESEM) is an alternative method to eliminate sample pre-treatments due to its capability to analyse hydrated samples. However, images of the hydrated microalgae obtained directly from both freshwater and marine culture media using the ESEM (Philips XL30 ESEM) equipped with water vapour were sub-standard. In our previous trials, ESEM failed to provide high quality images of *Scenedesmus* spp. that were not subjected to pre-treatments (Figure 2A-H). Cotton-like fine strands were widely spread over *Scenedesmus* sp. A, UPMC-A0002 (Figure 2A-B), indicating their instability in a high vacuum environment. A similar phenomenon was also reported for *Anabaena flos-aquae* due to improper dehydration step during sample pre-treatments (Bellinger & Sigee, 2010). Furthermore, cell shrinkage was also observed in *Scenedesmus* sp. B, UPMC-A0003 (Figure 2C-D) and *Scenedesmus* sp. D, UPMC-A0005 (Figure 2G-H), whereas blur images were observed in *Scenedesmus* sp. C, UPMC-A0004 (Figure 2E-F). On the other hand, crystallised salt from seawater culture medium appeared in the ESEM images of marine microalgae, which could affect their electron microscopy visualisation (Figure 2I). Hence, not all hydrated samples, especially microalgae, could be viewed and imaged as clear and distinctive cell structures under ESEM. Thus, the pre-treatment protocol prior to SEM analysis was modified for microalgae in order to preserve and stabilise their native structures in a high vacuum SEM chamber.

The major pre-treatment steps of fixation, dehydration, critical point drying and coating prior to SEM imaging were commonly applied and modified according to the biological samples. In microalgae, varieties of the pre-treatment steps have been published; these ranged from freeze fixation, single-step or two-step chemical fixation; freeze, air, chemical or critical point drying and carbon, gold, or ion coating. The present study was designed to modify, develop and describe complete series of pre-treatment steps for microalgae samples obtained directly from the culture medium prior to SEM analyses.

Chemical Fixation and Washing

Chemical fixatives are used in order to preserve cell shapes and structures by crosslinking their macromolecules (e.g., polysaccharides, proteins, lipids and nuclei acids) into a rigid form. Furthermore, fixatives induce the insolubility of

macromolecules so that they will not be extracted out during the dehydration step (Lee, 1993). According to some previous reports, fixation techniques such as single-step and two-step fixation have been mostly applied on microalgae. In this study, the samples were preserved and fixed using two-step fixation technique which involved a pre-fixation step in glutaraldehyde and a post-fixation step in osmium tetroxide. In order to achieve optimal fixation, two type-specific functional fixatives were utilised in this study for targeting and fixing the different macromolecules in microalgae (i.e. glutaraldehyde) for protein and polysaccharides while osmium tetroxide for lipids. In this study, silica was evidently absent in all the examined green microalgae species. In addition, previous studies revealed that silica of acid washed diatoms was stable under extreme vacuum SEM working environment. Therefore, we concentrated on the preservations of major and imperative compounds found in microalgae such as polysaccharides, proteins and lipids. It is similar to the two-step fixation technique of Chen (2001), where *Scenedesmus quadricauda* was pre-fixed in glutaraldehyde and post-fixed in osmium tetroxide. Additionally, *Coelastrum* sp. was pre-fixed in glutaraldehyde and post-fixed in GTGO solution, which is a combination of glutaraldehyde, tannic acid, guanidine hydrochloride and osmium tetroxide (Tanoi et al., 2014). However, Han et al. (2006) and Dayananda et al. (2010) demonstrated the single step fixation on *Chlorella miniata* and *Botryococcus* sp., respectively. Besides, *Chlorella* sp. and *Chlamydomonas* sp. were also reported to be fixed in McDowell-Trump's fixative consisting of formaldehyde and glutaraldehyde in phosphate buffer (Wan Maznah et al., 2012), whereas *Dictyosphaerium chlorelloides* was fixed in the combination of paraformaldehyde and glutaraldehyde in phosphate buffer saline (PBS) (Pereira et al., 2013). In comparison, the current study showed that glutaraldehyde and osmium tetroxide (only two fixatives) substantially fixed the 16 microalgae species with promising SEM-EDX outputs.

Fixation time of microalgae in this study was modified to 3 hours for 4% glutaraldehyde fixation and 2 hours for 1% osmium tetroxide fixation, respectively. Dayananda et al. (2010) subjected the samples of *Botryococcus* sp. to 12 hours fixation with 2% glutaraldehyde before viewing the images with SEM. Comparatively, *Botryococcus* sp. in this study was fixed in significantly shorter period (3 hours fixation instead of 12 hours) by increasing the concentration of glutaraldehyde to 4% instead of 2%. Liu et al. (2013) also reported long period of glutaraldehyde fixation (24 hours) for his sample of *Coelastrum* sp. By using the present modified pre-treatment protocol, the fixation time was successfully reduced in 8 folds to 3 hours, but with comparable SEM images of *Coelastrum* sp. (Figure 5). Besides, five hours of fixation period had effectively preserved five species of genus *Scenedesmus*. In comparison, the study of Chen (2001) chemically fixed *Scenedesmus quadricauda* in a shorter period (3 hours),

i.e. pre-fixed in 4% glutaraldehyde for 2 hours and post-fixed in 2% osmium tetroxide for 1 hour, which produced reliable images. Likewise, Han et al. (2006), Wan Maznah et al. (2012) and Pereira et al. (2013) reported short fixation period on *Chlorella miniata* (2 hours in 4% glutaraldehyde), *Chlorella* sp. and *Chlamydomonas* sp. (at least 2 hours in McDowell-Trump's fixative) and *Dictyosphaerium chlorelloides* (1 hour in a combination of 0.5% paraformaldehyde and 3% glutaraldehyde).

Fixation conditions such as fixative concentration, buffer types, pH and temperature mainly determined the effectiveness of fixatives. Improper fixation conditions create osmotic pressure between fixatives and cell cytoplasm and would destroy the samples. In this study, microalgae were optimally preserved in 4% glutaraldehyde prepared in 0.2M sodium cacodylate (pH 7.2) and 1% osmium tetroxide at 4°C. Colour changes of fixative and samples mixture from colourless to brown indicated that the fixation process had taken place. In comparison, Chen (2001) also fixed *Scenedesmus quadricauda* at 4°C with 4% glutaraldehyde prepared in 0.1M sucrose and 0.1M sodium cacodylate (pH 7.0) and 2% osmium tetroxide. The cold condition could decelerate the cellular metabolism processes, particularly enzymatic activity. Previous findings of Gündisch et al. (2015) established that the cold fixation technique enhanced the protein preservation in biological samples of breast cancer tissues. However, fixations of *Chlorella miniata* and *Dictyosphaerium chlorelloides* were conducted at room temperature instead of 4°C (Han et al., 2006; Pereira et al., 2013). After the fixation step, the samples were washed three times using sodium cacodylate for 10 min each to remove excess fixative. In this study, sodium cacodylate was used for both preparation of 4% glutaraldehyde and also extraction of excess glutaraldehyde at the end of the fixation step. It is similar to the study of Burgos et al. (2012), where Millonig's buffer was used to prepare 2.5% glutaraldehyde and also for sample washing of *Chroococcus* sp., *Spirulina* sp. and *Microcoleus* sp.

Solvent Dehydration

Solvent dehydration is the most critical step among other pre-treatments in SEM that could damage samples easily due to severe osmotic changes that will interrupt water interactions which hold and maintain the cellular shape of macromolecules or membranes. This step is to remove water content of the samples and substitutes it with organic solvent which is easily removed via critical point drying. In the present study, microalgal samples were gradually dehydrated with a series of ethanol (30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%) and 100% acetone to prevent any sudden osmotic shock. Microalgal-solvent was incubated in a series of ethanol dilution (30%-90%) for 10 minutes each and 15 minutes for 100% of ethanol and acetone. At this time, microalgae would clump together and its supernatants had to be discarded carefully. Both ethanol and acetone solvents were preferred to replace

water content in microalgae. However, ethanol was mostly utilised throughout the whole dehydration process because of its higher relative polarity than acetone. Thus, single solvent of ethanol was formerly and widely used in microalgae dehydration step (Burgos et al., 2012; Basu et al., 2013; Ho et al., 2013; Ponnuswamy et al., 2013). On the contrary, Pereira et al. (2013) reported that *Dictyosphaerium chlorelloides* was dehydrated using a series of acetone (25%, 50%, 75%, 85%, 95% and 100%) instead of ethanol. In order to obtain superb SEM images, 100% ethanol and acetone were used in the final stage of dehydration step for entirely replacing the water content in microalgae with organic solvent.

Critical Point Drying and Sputter Coating

After solvent dehydration step, solvents in microalgae samples were removed using critical point drying to ensure that liquid was passed to gas phase at critical point without creating surface tension forces which would damage the samples. Critical-point dried samples were mounted onto aluminium studs and then gold sputter-coated prior to SEM viewing and imaging. The samples were coated with a thin layer of gold in order to reduce or eliminate charging effects and further increase the effectiveness of electron beam activities. In this failed ESEM analysis, ultrastructure of fresh *Scenedesmus* spp. UPMC-A0002, UPMC-A0003, UPMC-A0004 and UPMC-A0005 with high water content was adversely destroyed. However, Dayananda et al. (2010) skipped the critical point drying step for their *Botryococcus* sp. and directly subjected the wet samples to gold sputter coating after alcohol dehydration. Despite of alcohol in *Botryococcus* sp., they still produced the SEM images in good feature. This was most probably due to the natural hardy attribute of *Botryococcus* sp. In contrast, Han et al. (2006) and Ponnuswamy et al. (2013) air-dried and sputter-coated *Chlorella miniata* and *Chlorella vulgaris* with carbon, whereby Chen (2001) critical point-dried and coated *Scenedesmus quadricauda* with ion beams. In addition, acid washed diatoms were air-dried and metal sputter-coated prior to SEM analyses (Govindasamy & Anantharaj, 2012; Lang et al., 2013). Nevertheless, in this present study, the acid washing step of diatoms was replaced by chemical fixation and organic solvent dehydration, and thus avoided the tedious and time-consuming procedures of repeated acid boiling and settling of samples in order to completely digest their organic matter. Furthermore, acid washed diatoms would interrupt their elemental profiles in the SEM-EDX analysis. On the other hand, chemical drying agent of hexamethyldisilazane (HMDS) is considered an alternative method to replace critical point drying which was applied on *Chlorella* sp., *Chlamydomonas* sp., *Verrucophora farcimen* and *Coolia* spp., but the extra process of removing excess HMDS needs to be included before sputter-coating (Edvardsen et al., 2007; Wan Maznah et al., 2012; Momigliano et al., 2013).

Mechanical Disturbances

Inputs of mechanical forces were unavoidable and yet constantly subjected onto samples during the mixing and separation procedures of fixative, buffer and solvent using vortex and centrifuge. Delicate and fragile structures of microalgae, especially their spines, cannot withstand strong forces and will get damaged easily. In this study, cells in 1.5 mL eppendorf tube centrifuged at 3213 x *g* for 3 min (Eppendorf Centrifuge 5810R, Germany) were separated from the chemical solutions with no induced damages (Figure 3A-B). In one of our failed trials, centrifugation of samples at 6797 x *g* for 10 min resulted in damaged spines of *Scenedesmus* sp. A, UPMC-A0002 (Figure 2J-K). Artefact images such as those in Figure 2J-K were not valid for scientific interpretation of morphological structures especially species-specific unique structures for taxonomic classification and identification of microalgae. Up to now, no study has highlighted or reported the optimum centrifugation speed, which is important to ensure the cellular structures of samples remain intact throughout the whole separation process.

Ultrastructure and Morphological Visualisation

In this study, SEM pre-treatment protocol was modified for microalgae samples obtained directly from the culture medium prior to the ultrastructure and element analyses. During the SEM pre-treatments, high preservation of ultrastructure and physical morphology, especially in fragile and delicate structures, is critical for accurate characterisation, description and identification of microalgae species. Species distinctive ultrastructure and morphological structures of 16 selected microalgae, particularly in cell ornamentation and interconnecting structures, are presented in Figure 3 to Figure 7, and the descriptions of their morphological characteristics are presented in Table 2. Size is one of the principal physical traits for phenotypic identification and classification of microalgae. Fundamentally, length is the longest distance of cell, width is the measurement gap from side to side and diameter is a straight line passing through the cell circle centre. On average, microalgae size regardless of marine or freshwater ranged from 3.42 to 80.00 µm in length, 0.42 to 11.38 µm in width and 3.5 to 13.92 µm in diameter. *Scenedesmus* sp. A, UPMC-A0002 in Figure 3A-B (10.42 µm in length and 9.21 µm in width) and *Scenedesmus* sp. D, UPMC-A00005 in Figure 3G-H (10.29 µm in length and 11.38 µm in width) formed the biggest colony, among others in the same genus. Among the spherical cells, *Botryococcus* sp. B, UPMC-A0044 (Figure 4C-D) was the smallest individual cell with the diameter of 3.50 µm whereby *Coelastrum* sp. A, UPMC-A0045 (Figure 5A-B) exhibited the largest colony with a diameter of 13.92 µm. Compared to non-spherical cells, the shortest length (3.42 µm) was observed in *Selenastrum* sp. UPMC-A0009 (Figure 6I-

J) with the width of 1.17 µm, whereby the diatom *Biddulphia sinensis* UPMC-A0050 (Figure 7A-B) has the longest length, 80.00 µm and second in width, 11.34 µm.

Scanning Electron Microscopy (SEM) images provide information on photogrammetric surfaces instead of volumetric (Friedrichs et al., 2012). Morphological characteristics such as fusiform or pointed cells of *Scenedesmus* spp., with or without curved spines and colony forming cells of *Scenedesmus* spp., *Botryococcus* spp., *Coelastrum* spp. and *Crucigenia* sp., can be observed in both light and SEM microscopy. Early developmental stage of *Scenedesmus* spp. were coccoid cells but in the mature stage they started to form colonies of 2 or 4 roughly cylindrical or pointed cells with or without curved spines (Figure 3A-J). Besides that, cells of *Botryococcus* spp. UPMC-A0043 and UPMC-A0044 (Figure 4A-D) were clustered together to form a dense colony. On the other hand, protuberance in *Coelastrum* spp. UPMC-A0045 and UPMC-A0046 (Figure 5A-D) and interconnecting structures in *Scenedesmus* sp. B, UPMC-A0003 (Figure 3F) and *Crucigenia* sp. UPMC-A0048 (Figure 6D) were minute structures and could only be observed clearly in SEM images. Additionally, the ultrastructure of cell ornamentations such as rosette structure (Figure 3A) and rib-like pattern (Figure 3J) in *Scenedesmus* spp.; two different areola patterns in *Biddulphia sinensis* UPMC-A0050 (Figure 7B) and marginal small spines of *Thalassiosira* sp. UPMC-A0051 (Figure 7C) were the results of appropriate pre-treatments prior to SEM analyses. It was observed that ornamental patterns on cell surface were unique and species-specific among the 16 selected microalgae.

Unicellular cells of *Ankistrodesmus* sp., *Kirchneriella* sp. and *Selenastrum* sp. were usually presented in clusters but they sometimes occurred in solitary. *Biddulphia sinensis* cells were normally found in solitary but sometimes they formed chains. *Thalassiosira* sp. cells usually joined to form a loose chain but would mostly be solitary in culture conditions. These images demonstrated the superiority of electron microscopy over light microscopy in the observation of unique cellular topography and minuscule structures of microalgae for their characterisations, discrimination and taxonomic classifications.

Elemental Characterisation

A total of 12 elements were detected and distributed within the 16 selected microalgae (Table 3). Yttrium (Y), a type of rare earth element (REE) was the most abundant element found in all microalgae. *Scenedesmus* sp. E, UPMC-A0049 had the highest Y content (57.56 ± 4.85% atom), while *Thalassiosira* sp. UPMC-A0051 had the lowest (7.39 ± 1.49% atom). Niobium (Nb) was another REE that was only found in green microalgae. The highest Nb content was observed in *Scenedesmus* sp. A, UPMC-A0002 (45.53 ± 3.08% atom) while the lowest in *Coelastrum* sp. A, UPMC-A0045 (15.22 ± 3.11% atom). Iridium (Ir), an element of the platinum

Table 2

Morphological characteristics of tropical microalgae as observed using scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDX) analysis. Mean cell sizes (µm) in column with different superscript letters are significantly different by Duncan post-hoc test ($P < 0.05$).

Species	Collection code	Average cell size[3] (µm)			Morphological characteristics (Prescott, 1978; Bellinger & Sigee, 2010)
		Length	Width	Diameter	
Scenedesmus spp. Meyen 1829	UPMC-A0002	10.42±4.36^{ab}	9.21±0.76^{e}	nd[4]	Cells are more or less oval, ellipsoidal or fusiform in shape (Figure 3A-J). Colonies of cells are arranged side by side in one row. Some cells have a curved spine that is projected out from each corner (Figure 3A-D) with rosette structure (Figure 3A-B). Some cells are pointed and narrowed at the end without spines (Figure 3G-J) with apparent cell wall ornamentation of rib-like pattern (Figure 3J).
	UPMC-A0003	6.79±0.06^{a}	5.04±0.30^{d}	nd	
	UPMC-A0004	7.13±0.06^{a}	3.38±0.53^{cd}	nd	
	UPMC-A0005	10.29±1.36^{ab}	11.38±1.94^{f}	nd	
	UPMC-A0049	7.13±1.59^{a}	8.09±0.83^{e}	nd	
Botryococcus spp. Kützing 1849	UPMC-A0043	nd	nd	5.80±0.28^{a}	Cells are ovoid or spherical in shape aggregated together to form a dense colony. UPMC-A0043 has larger cells compared with UPMC-A0044 (Fig.4A-D).
	UPMC-A0044	nd	nd	3.50±0.71^{a}	
Coelastrum spp. Nägeli 1849	UPMC-A0045	nd	nd	13.92±0.59^{b}	Cells are ovoid or polygonal in shape arranged to form a hollow, spherical and many-sided colony like a football. Cells are joined together by protuberances (Figure 5A-D).
	UPMC-A0046	nd	nd	13.17±0.23^{b}	
Ankistrodesmus sp. Corda 1838	UPMC-A0008	9.34±1.89^{a}	0.42±0.12^{a}	nd	Cells are sickle-shaped, fusiform and crescent-shaped. Cells twisted about one another and usually in clusters but can be solitary (Fig.6A-B).
Crucigenia sp. Morren 1850	UPMC-A0048	5.42±0.83^{a}	3.00±0.47^{bc}	nd	Cells are trapezoidal-shaped and arranged in four cells to form quadrate plates or in multiple of four (Figure 6C-D).
Desmodesmus sp. (Chodat) An, Friedl and Hegewald 1999	UPMC-A0042	17.25±7.42^{c}	11.25±1.06^{f}	nd	Physical structure is similar as *Scenedesmus* sp. A colony composed of 2, 4, or 8 cells with curved spines and a hollow structure in between the cells (Figure 6E-F).
Kirchneriella sp. Schmidle 1893	UPMC-A0006	3.92±0.12^{a}	2.34±0.47^{abc}	nd	Cells are sharply curved and lunate in shape. They are enclosed in mucilage and usually in clusters but can be solitary (Figure 6G-H).
Selenastrum sp. Reinsch 1867	UPMC-A0009	3.42±0.83^{a}	1.17±0.23^{ab}	nd	Cells are strongly crescent-shaped but not entangled. They are not or rarely enclosed in mucilage and usually in clusters but can be solitary (Figure 6I-J).
Biddulphia sinensis Greville 1866	UPMC-A0050	80.00±5.66^{d}	11.34±0.94^{f}	nd	Cells are rectangular-shaped in girdle view and oval-shaped in valve view. A long spine is projected out from each corner (labiate process) and a short protrude is projected out from both ends (ocellus) (Figure 7A-B).
Thalassiosira sp. Cleve 1873	UPMC-A0051	nd	nd	12.17±2.60^{b}	Cells are circular and gently undulating valve face with mesh like radiated punctae. Small spines found at valve margin. Cells usually joined to form a loose chain but can be solitary (Figure 7C-D).

[3]Average cell size was measured according to length, width and diameter of cells. [4]Not detected.

Table 3
Elemental characterisation of 16 microalgae species by scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDX) analysis. Data in column marked with different superscript letters are significantly different by LSD post-hoc test (P < 0.05).

Species	Elements (% atom)											
	Y	Nb	Fe	Ca	Cl	K	Cu	F	Ir	P	Mg	Si
UPMC-A0002	28.47 ±4.10^{d}	45.53 ±3.08^{e}	2.12 ±0.55^{ab}	2.47 ±2.33^{ab}	11.55 ±1.38^{c}	7.05 ±0.73^{bc}	1.58 ±1.43^{a}	nd	nd	nd	nd	nd
UPMC-A0003	46.59 ±16.40^{c}	27.89 ±4.38^{b}	2.93 ±0.47^{a}	2.58 ±0.26^{a}	nd^{d}	nd	2.98 ±0.71^{a}	nd	2.86 ±2.49^{a}	nd	nd	nd
UPMC-A0004	41.22 ±12.96^{c}	21.57 ±5.41^{b}	4.91 ±1.50^{a}	2.06 ±1.79^{a}	7.86 ±2.10^{a}	3.60 ±3.16^{a}	2.27 ±1.97^{a}	nd	nd	nd	nd	nd
UPMC-A0005	40.00 ±5.26^{e}	17.07 ±1.92^{d}	1.38 ±1.27^{a}	6.54 ±2.00^{b}	13.19 ±2.10^{c}	8.69 ±0.81^{b}	nd	nd	1.76 ±1.53^{a}	nd	nd	nd
UPMC-A0049	57.56 ±4.85^{c}	24.83 ±2.79^{b}	3.14 ±0.62^{a}	5.89 ±1.72^{a}	5.45 ±4.80^{a}	3.13 ±0.24^{a}	nd	nd	nd	nd	nd	nd
UPMC-A0043	46.15 ±6.34^{d}	16.41 ±4.92^{b}	1.33 ±0.35^{a}	nd	nd	nd	1.43 ±1.41^{a}	26.89 ±2.98^{c}	nd	nd	nd	nd
UPMC-A0044	45.35 ±5.79^{d}	18.12 ±4.00^{b}	nd	28.47 ±6.16^{c}	nd	nd	2.56 ±0.66^{a}	nd	2.91 ±0.54^{a}	nd	nd	nd
UPMC-A0045	35.54 ±3.86^{d}	15.22 ±3.11^{b}	nd	5.46 ±0.95^{a}	nd	nd	nd	nd	nd	30.74 ±4.38^{c}	11.32 ±4.34^{b}	nd
UPMC-A0046	15.08 ±3.33^{c}	27.20 ±2.29^{e}	nd	3.48 ±0.25^{a}	19.90 ±1.86	16.03 ±1.36^{c}	1.23 ±1.08^{a}	nd	3.32 ±3.38^{a}	nd	10.08 ±0.66^{b}	nd
UPMC-A0008	39.32 ±1.98^{f}	24.18 ±0.44^{e}	4.86 ±2.01^{ab}	2.87 ±2.56^{a}	9.1 ±0.31^{c}	13.03 ±0.66^{d}	2.36 ±2.08^{a}	nd	4.28 ±1.57^{a}	nd	nd	nd
UPMC-A0048	44.98 ±2.29^{d}	15.33 ±2.12^{b}	0.88 ±0.76^{a}	14.13 ±1.33^{b}	nd	nd	nd	nd	nd	21.11 ±2.45^{c}	nd	nd
UPMC-A0042	42.37 ±1.08^{e}	24.14 ±1.06^{d}	3.23 ±1.29^{a}	6.34 ±0.62^{b}	9.13 ±0.49^{c}	5.40 ±0.53^{b}	3.29 ±0.69^{a}	nd	6.10 ±1.38^{b}	nd	nd	nd
UPMC-A0006	44.99 ±6.14^{e}	20.78 ±1.01^{d}	3.24 ±0.86^{a}	3.88 ±0.15^{ab}	10.74 ±2.09^{c}	11.74 ±2.16^{c}	3.09 ±0.85^{a}	nd	nd	nd	nd	nd
UPMC-A0009	43.02 ±10.54^{c}	17.75 ±1.49^{b}	4.20 ±2.05^{a}	3.87 ±1.75^{a}	12.62 ±4.42^{b}	7.18 ±2.06^{ab}	nd	nd	nd	nd	nd	nd
UPMC-A0050	7.48 ±3.71^{ab}	nd	1.87 ±0.73^{a}	nd	nd	nd	nd	21.64 ±5.81^{c}	nd	nd	nd	65.95 ±12.5^{d}
UPMC-A0051	7.39 ±1.49^{ab}	nd	2.99 ±0.91^{a}	nd	nd	nd	0.73 ±0.63^{a}	nd	nd	nd	nd	76.10 ±8.89^{c}

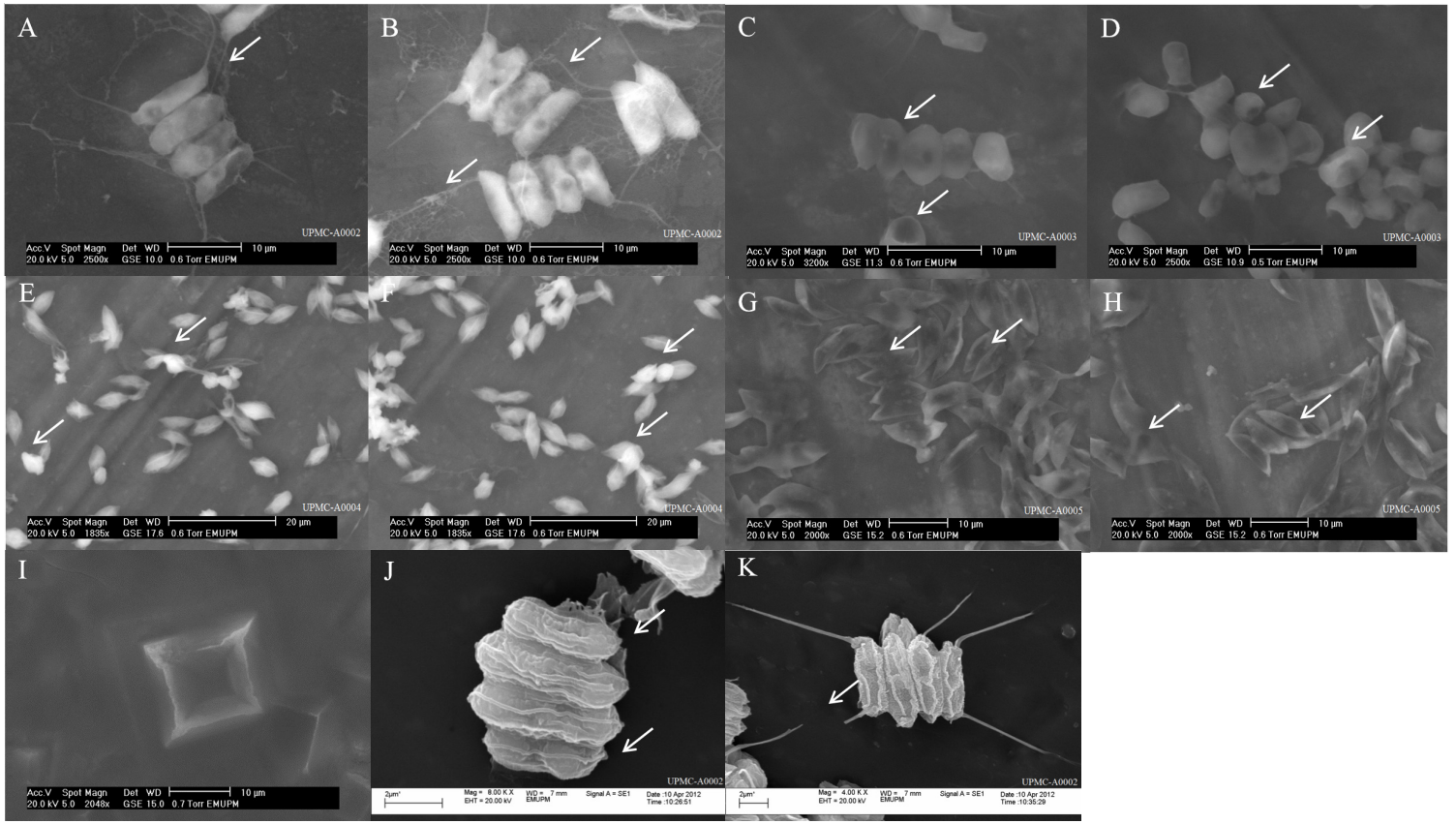

Figure 2. (A-H); Environmental scanning electron microscopy (ESEM) images of *Scenedesmus* spp. UPMC-A0002, UPMC-A0003, UPMC-A0004 and UPMC-A0005 without any pre-treatments show poor images with low resolution. Arrows in A and B elucidate cotton-like fiber strands. Arrows in C, D, G and H show cell shrinkage and damage of cell integrity that could affect the study of cell topography. Arrows in E and F show blur cells due to hydrated samples and their weak interaction with electron beams. (I); Environmental scanning electron microscopy (ESEM) image demonstrates the crystal salt derived from culture medium of marine microalgae. (J-K); Scanning electron microscopy (SEM) images of *Scenedesmus* sp. A, UPMC-A0002 which was centrifuged at high speed during pre-treatments. Arrows in J and K elucidate the absence of delicate spines that would deviate the interpretation.

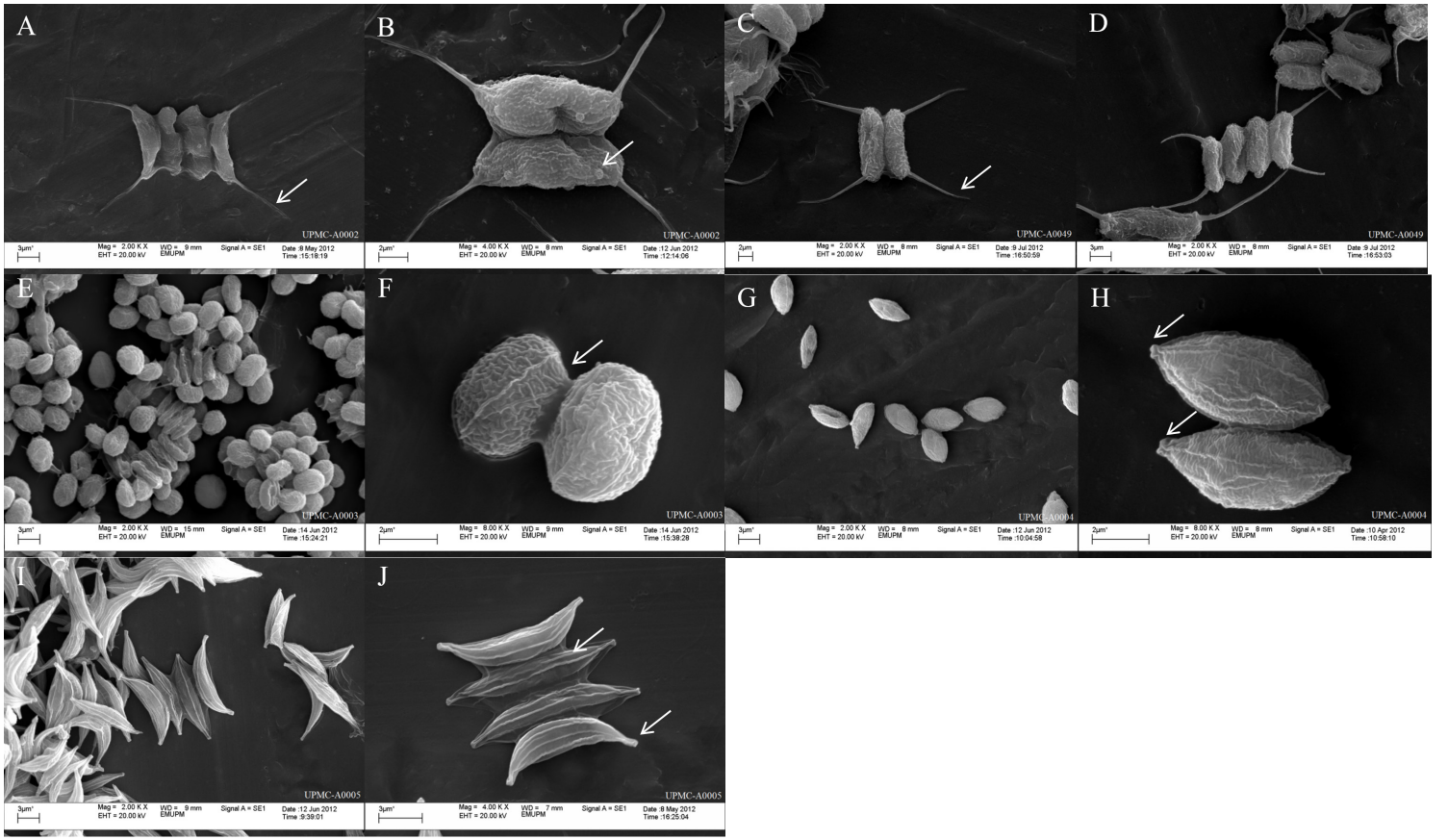

Figure 3. (A-B and C-D); *Scenedesmus* sp. A, UPMC-A0002 and *Scenedesmus* sp. E, UPMC-A0049. Arrows in A and C depict curved spine which is delicate to electron microscopy pre-treatments. Arrow in B depicts rosettes. (E-F); *Scenedesmus* sp. B, UPMC-A0003. Arrow in F depicts connecting structure. (G-H and I-J); *Scenedesmus* sp. C, UPMC-A0004 and *Scenedesmus* sp. D, UPMC-A0005. Arrows in H and J depict pointed cells and cell wall ornamentation of rib-like pattern

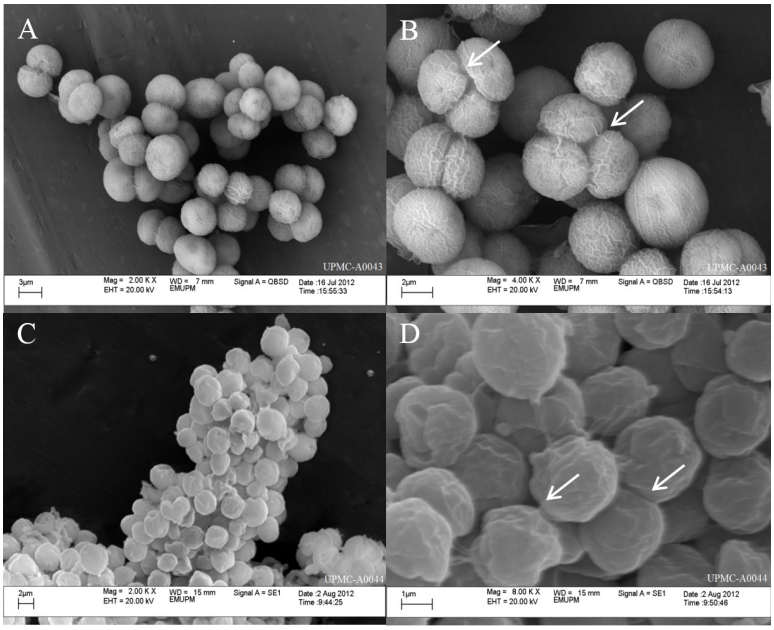

Figure 4. (A-B and C-D); *Botryococcus* spp. UPMC-A0043 and UPMC-A0044. Arrows in B and D show spherical cells are stuck together

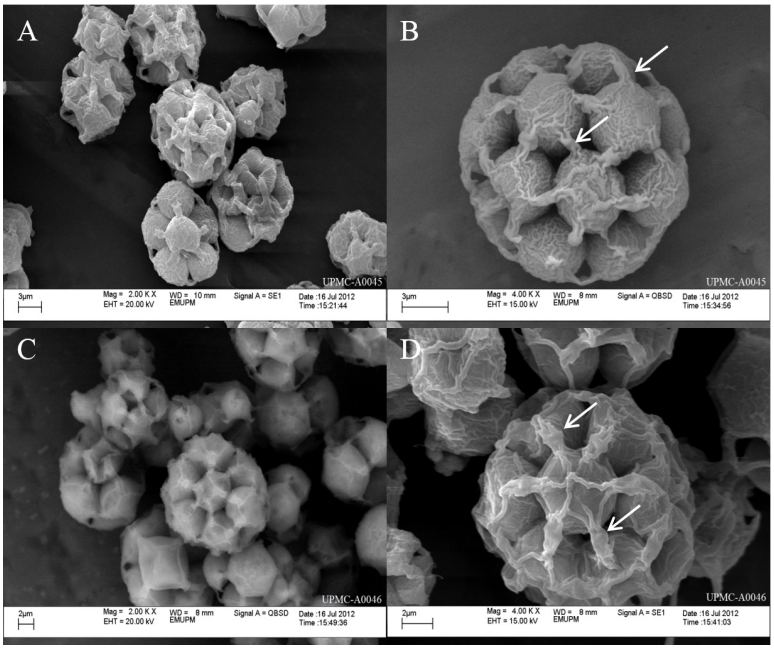

Figure 5. (A-B and C-D); *Coelastrum* spp. UPMC-A0045 and UPMC-A0046. Arrows in B and D demonstrate ovoid and hexagonal cells and their interconnecting structures of protuberances

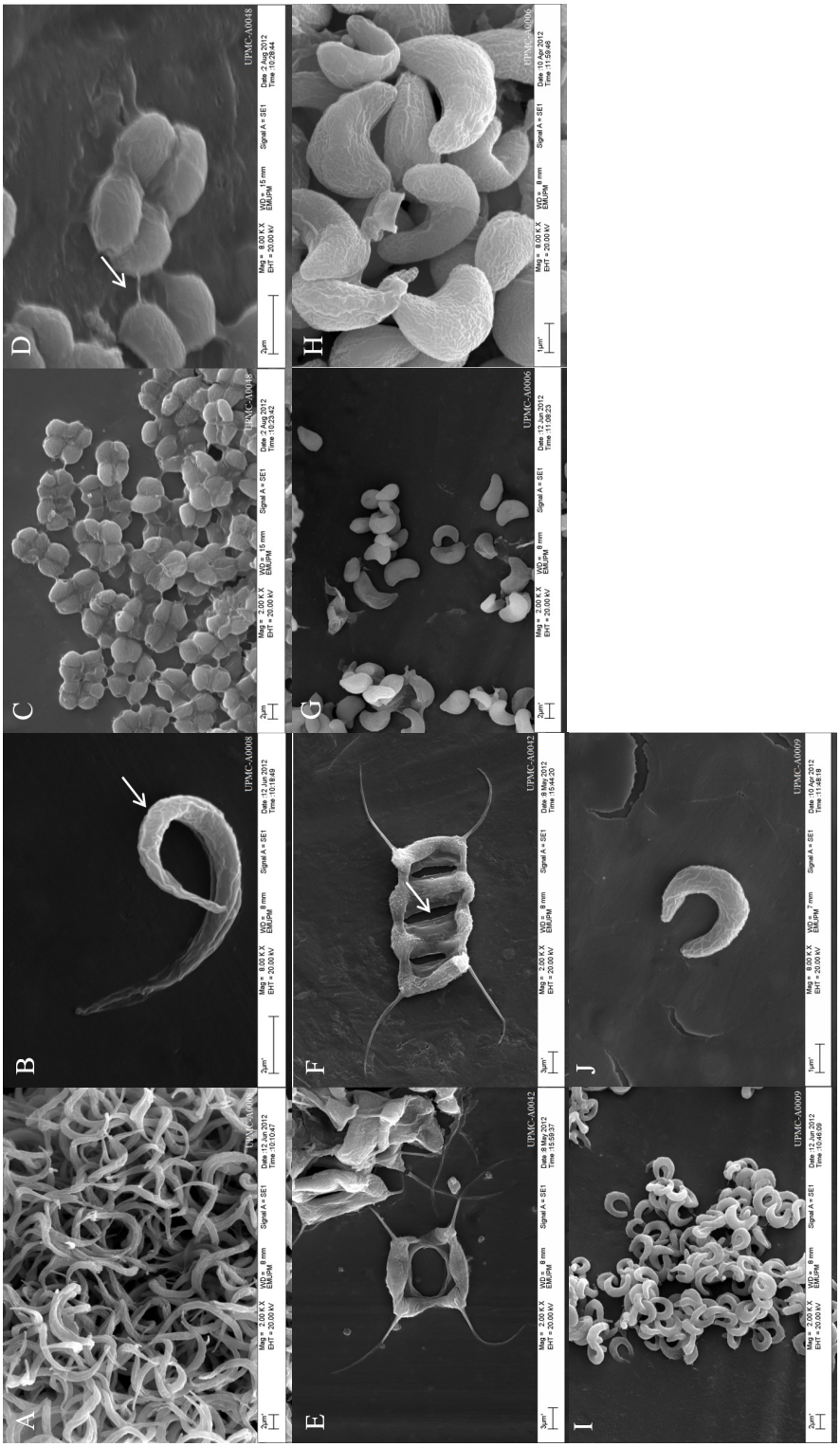

Figure 6. (A-B); *Ankistrodesmus* sp. UPMC-A0008. Arow in B shows twisted cell. (C-D); *Crucigenia* sp. UPMC-A0048. Arrow in D shows interconnecting structure. (E-F); *Desmodesmus* sp. UPMC-A0042. Arrow in F shows hollow structure. (G-H and I-J); *Kirchneriella* sp. UPMC-A0006 and *Selenastrum* sp. UPMC-A0009

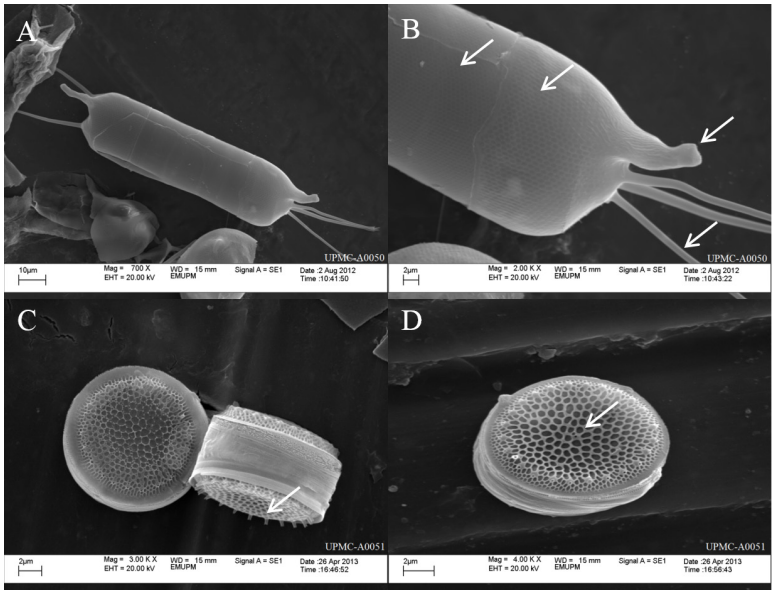

Figure 7. (A-B); *Biddulphia sinensis* UPMC-A0050. Arrows in B illustrate two different cellular areolae patterns with special parts of ocellus and long spine. (C-D); *Thalassiosira* sp. UPMC-A0051. Arrow in C illustrates marginal small spines. Arrow in D illustrates mesh-like punctae

group element (PGE), was found in six microalgal samples, namely *Desmodesmus* sp. UPMC-A0042 (6.10 ± 1.38% atom), *Ankistrodesmus* sp. UPMC-A0008 (4.28 ± 1.57% atom), *Coelastrum* sp. B, UPMC-A0046 (3.32 ± 3.38% atom), *Botryococcus* sp. B, UPMC-A0044 (2.91 ± 0.54% atom), *Scenedesmus* sp. B, UPMC-A0003 (2.86 ± 2.49% atom) and *Scenedesmus* sp. D, UPMC-A0005 (1.76 ± 1.53% atom). Meanwhile, silicon (Si) was only found in diatoms i.e. *Thalassiosira* sp. (76.10 ± 8.89% atom) and *Biddulphia sinensis* (65.95 ± 12.53% atom).

Twelve elements i.e. Y, Nb, Fe, Ca, Cl, K, Cu, F, Ir, P, Mg and Si were discovered in this microalgae SEM-EDX study. Similarly, Priyadarshani and Rath (2012) reported that microalgae were rich in elements such as Fe, Ca, K, and Mg, and these additional nutrients made them suitable for nutraceutical products such as *Chlorella* spp. and *Spirulina* spp. On the other hand, Fabregas and Herrero (1986) reported that Ca, Cl, Cu, Co, Fe, K, Mg, Mn, Na, P and Zn were found in *Tetraselmis suecica*, *Isochrysis galbana*, *Dunaliella tertiolecta* and *Chlorella stigmatophora*. In this study, three elements of Y, Nb and Ir are classified as metallic transition elements, whereby Y and Nb are rare earth elements, and Ir is platinum group element which has been detected in microalgae species being studied. Previous studies reported that the rare earth elements of lanthanum (La), gadolinium (Gd) and yttrium (Y) were found in green microalgae of *Chlorella vulgaris* and neodymium (Nd) in blue green algae of *Phormidium* sp. respectively (Sun et al., 1997; Kim et al., 2011). In comparison,

several studies of rare earth elements were intensively conducted on plants and their optimum quantities in fertilizers would enhance the plants' growth, development, chlorophyll content and photosynthetic rate (Emmanuel et al., 2010; Kastori et al., 2010; Zhang et al., 2013). However, such information on rare earth elements in microalgae is still lacking. Platinum group elements of rhodium (Rh), palladium (Pd) and platinum (Pt) were detected in *Chlorella stigmatophora* and *Ulva lactuca* (Turner et al., 2007; Shams et al., 2014). In this study, high amounts of Si were found in only diatoms, *Biddulphia sinensis* and *Thalassiosira* sp. Silicon is an essential element to diatoms and it is required in the construction of their disc-like frustules.

CONCLUSION

A modified SEM-EDX pre-treatment protocol was developed for 16 selected tropical microalgae, which could be applied on other microalgae species especially green algae and diatoms. The current protocol with shorter fixation time and optimum separation forces successfully preserved ultrastructure and morphological structures of samples especially in cell ornamentation and interconnecting structures for 10 genera of microalgae inclusive of both marine and freshwater species. Elemental characterisation of microalgae was simultaneously evaluated, whereby the rare earth elements of Y and Nb were found abundantly in most the microalgae species tested. In conclusion, this SEM-EDX study illustrates that microalgae can be characterised, classified and identified based on ultrastructure and morphological description, whereas their potential of certain element sources has also been evaluated.

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Population Fluctuation and Dispersion Patterns of Apple Snails, *Pomacea* spp. (Gastropoda: Ampullariidae) in a Rice Ecosystem

Arfan, A. G.[1,2], Muhamad, R.[1]*, Omar, D.[1], Nor Azwady, A. A.[3] and Manjeri, G.[1]

[1]*Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia 43400 Serdang, Selangor, Malaysia*

[2]*Department of Entomology, Faculty of Crop Protection, Sindh Agriculture University Tandojam, Sindh, Pakistan*

[3]*Department of Biology, Faculty of Science, Universiti Putra Malaysia 43400 Serdang, Selangor, Malaysia*

ABSTRACT

A field study was conducted for two consecutive rice-growing seasons from August, 2013 to May, 2014 to understand the population dynamics of exotic apple snails, *Pomacea* spp. (Ampullariidae), as affected by ambient weather and aquatic weeds. A one-acre rice field was divided into four blocks and eight samples per block were taken using a 0.5x0.5m quadrat. Collected snails were recorded as numbers of egg clutches, juveniles, adult females and males. Average rainfall, relative humidity, temperature and water pH, along with number of aquatic weeds and seedlings, were also recorded. Results confirmed the presence of only *Pomacea maculata*. The numbers of egg clutches, juveniles and adults were relatively high during the off-season as compared to the main-season. Meanwhile, relative humidity had a significant effect on the number of egg clutches, and rainfall affected the densities of juveniles and adults. Among the weeds, *Limnocharis flava* (Alismataceae) had significant effect on the densities of different snail stages. Different stages showed uniform dispersion pattern during both seasons due presumably to continuous availability of water and abundant food. Thus, results obtained could be helpful in understanding the population dynamics of *P. maculata* and devising appropriate management strategy.

Keywords: Apple snail, rice, weeds, population fluctuation, dispersion, weather, *Pomacea*

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E-mail addresses:
irfan_gilal@yahoo.com (Arfan, A. G.),
rita@upm.edu.my (Muhamad, R.),
zolkifli@upm.edu.my (Omar, D.),
azwady@upm.edu.my (Nor Azwady, A. A.),
manjeri@upm.edu.my (Manjeri, G.)
* Corresponding author

INTRODUCTION

Alien freshwater species often impose threats to lakes, streams, ponds, rivers and other freshwater bodies by altering their natural habitats and interacting with

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native fauna (Sala et al., 2000; Carpenter et al., 2011). Although the presence of such species can easily be recognised, quantifying and explaining their successful establishment along with their temporal population dynamics are difficult tasks (McCann, 2014). However, understanding patterns of population density dependence is important for forecasting the establishment and maintenance of an alien species population because some species have significant ability to regulate their growth and reproduction or both (Courchamp et al., 1999; Taylor & Hastings, 2005).

Invasive apple snails, *Pomacea* spp. (Ampullariidae), are one of the most successful invaders of freshwaters and this signifies their invasions because of the negative impacts on aquatic bodies and macrophytes, especially rice in Southeast Asia and taro in Hawaii and Florida in the continental USA (Hayes et al., 2008; Horgan et al., 2014). Apple snails were introduced in Malaysia around 1991 and spread to all rice growing areas of the country (Teo, 2003; Yahaya et al., 2006). Severe infestation of the snails can cause complete loss of rice crop in the field at an early growth of rice. In Malaysia, these snails are more devastating than in other countries due to the large scale direct seeding and flooded conditions in rice fields, either because of irrigation water or heavy rains. In case of severe snail damage, growers can lose more than RM425/hectare, either due to replanting of missing crop or application of control measures (Yahaya et al., 2006).

Many factors can contribute towards successful invasions of these *Pomacea* spp. in new regions such as high adaptability to stressful environmental conditions, high reproductive potential and lack of potential natural enemies (Cowie, 2002; Yusa et al., 2006). Considering the importance of *Pomacea* spp. as a major pest of rice, studies have been conducted on their population dynamics (Martin et al., 2001; Burlakova et al., 2010; Byers et al., 2013; Yoshida et al., 2013, 2014). However, studies on the population patterns of *Pomacea* spp., the role of environmental factors and rice weeds on their distribution in farmer-managed rice fields in the tropical regions are still lacking. Moreover, in currently available studies, none has focused on the dispersion patterns of *Pomacea* spp. in rice fields; an important factor in the application of any management strategy.

Therefore, considering the importance of *Pomacea* spp. to rice, this study was undertaken in a farmer-managed rice field to estimate the patterns of population fluctuation and dispersion patterns of different stages of *Pomacea* spp., as well as the effects of major aquatic-weeds and environmental factors on their population's fluctuation. The results obtained could be helpful in understanding the population dynamics of various life stages of *Pomacea* spp. in rice fields and the same could be helpful in the appropriate management of snails to reduce damages and improve rice productivity.

MATERIALS AND METHODS

Study site

The study was conducted at MARDI Rice Research Station (N 03° 27.335' E101° 09.541′) located in Tanjung Karang, Selangor, Malaysia, for two consecutive rice-growing seasons from August, 2013 to May, 2014. Off-season rice cultivation depends on the irrigation system, whereas during main-season, it is not totally dependent on irrigation system; instead it depends on monsoon rains. Moreover, in main-season, fields were properly levelled as compared to off-season one, hence ensuring proper management of water. Farmer applied molluscicide (Fentin acetate) twice; one week before transplanting and one week after transplanting to remove snails from the field. Note that other agronomic practices were the same during both seasons. Transplanted 21 days old seedlings of MR 263 variety were used by the farmer during both seasons.

Sampling Procedures

A rice field of 0.405 hectare size was selected and divided into four blocks in accordance to the layout of the field. Eight random samplings were taken from each block using a0.5x0.5m quadrat. The snails falling within each quadrat were carefully collected by hand or by using strainer to avoid any damage to the rice plants. The collected snails were differentiated into egg clutches, juveniles, adult females and males for individual species. The identification was done according to the external morphology of the apple snails as described by Marwato and Nur (2012), Cowie et al. (2006) and Hayes et al. (2012), whereas males and females were distinguished by observing the testicles through the translucent shell (Takeda, 1999) along with convex operculum for males and concave operculum for females (Estebenet et al., 2006). The snails with shell length of below 2 cm were considered as juveniles.

The number of rice seedlings and major aquatic weeds in each quadrat was also counted to ascertain their effects on the distribution of apple snails. HI1991300 pH Meter (HANNA Instruments, USA) was used to measure water pH in the field. The weather parameters of mean fortnightly temperature, relative humidity and average rainfall were obtained from the Pusat Pertanian station, *Tanjung Karang,* Metrological Department, Malaysia, to understand their influence on the distribution of snails at different sampling locations. Sampling was done from the first transplanting until harvesting of rice on a fortnightly basis, resulting in eight observations in one rice growing season.

Data Analysis

Population distribution of *Pomacea* spp. Student *t*-test at 0.05 level of probability was used to determine the significant difference in the population of individual stages of *Pomacea* spp. between two rice seasons using SAS version 9.2 (SAS Institute, 2009).

Population fluctuations of *Pomacea* spp. Population fluctuation of different stages of *Pomacea* spp. in each rice season

was determined by plotting the mean number of each *Pomacea* spp. stage against the population of the mean number of rice seedlings, major aquatic weeds and weather parameters of mean fortnightly temperature, relative humidity, rainfall and water pH. Meanwhile, stepwise regression was used to determine the most significant factors among weather parameters and aquatic weeds and seedlings which contributed towards the population fluctuation of different stages of *Pomacea* spp.

Population dispersion of *Pomacea* spp. In order to calculate the dispersion patterns, the simplest method of the variance to mean ratio (s^2/m) was used as all other methods basically include mean and variance. For variance to mean ratio, the value of $s^2/m < 1$ indicates a uniform dispersion, while $s^2/m = 1$ indicates random dispersion and $s^2/m > 1$ indicates an aggregated dispersion (Southwood & Henderson, 2000).

Moreover, Taylor's power regression, $Log\ S^2 = log\ a + b\ log$ m (Taylor, 1961) and Iwao's patchiness regression, $m = \alpha + \beta x$ (Iwao, 1970) were also used to assess the level of aggregation by means of slope b and β. Taylor's law is an empirical law in ecology that relates the between-sample variance in density to the overall mean density of a sample of organisms in a study area. In Taylor's power regression or Iwao's patchiness regression, the slope values (b or β) when = 1, indicates a random dispersion. When it is > 1, it indicates aggregated dispersion; and when it is < 1, it indicates regular dispersion. In Iwao's patchiness regression, α indicates the tendency to crowding (positive) or repulsion (negative) (Arnaldo & Torres, 2005; Vinatier et al., 2011).

RESULTS

Population Abundance of *Pomacea* spp.

During the two rice seasons, all the apple snails collected were identified as *Pomacea maculata* (synonym: *Pomacea insularum*) (Horgan et al., 2014) based on their shell morphology. Population distribution of different stages illustrated that a significantly higher mean number of egg clutches per m^2 (1.1 ± 0.1) was collected during off-season as compared to main-season (0.69 ± 0.07) ($P<0.001$). Juvenile *P. maculata* population also indicated the same trend with significantly higher ($P<0.001$) population recorded during off-season (5.68 ± 0.69 per m^2) in comparison to main-season (1.08 ± 0.16 per m^2). The populations of females and males recorded during main-season were 0.49 ± 0.08 per m^2 and 0.22 ± 0.04 per m^2, respectively, and they increased significantly ($P < 0.001$) during off-season to 1.29 ± 0.08 per m^2 and 0.85 ± 0.08 per m^2, respectively (Figure 1).

Population Fluctuation of Different Stages of *P. maculata* against Weather Parameters, Rice Seedlings and Weeds

Figures 2 and 3 show the population fluctuation of different stages of *P. maculata* during the two rice seasons against weather parameters, rice seedlings and weeds. Based on the results, the population of eggs first appeared on 31 August, 2013 in main-season and showed a gradual rise through the

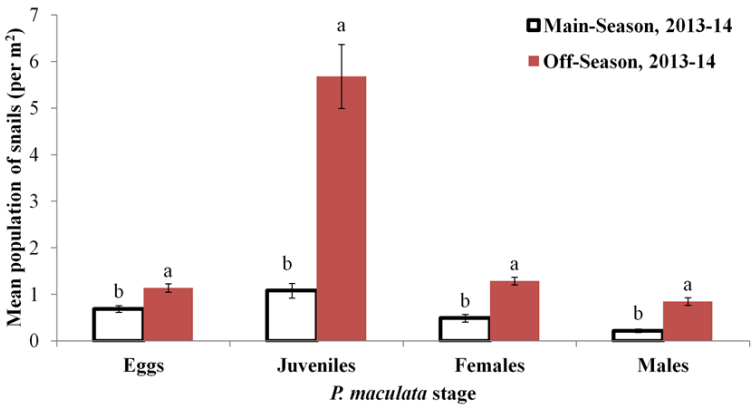

Figure 1. Mean number of different stages of *P. maculata* during two rice-growing seasons
*Means followed by the same letters against each stage are not significantly different (P < 0.001)

vegetative and reproductive growths of the rice plant. However, maximum population of egg clutches was recorded towards the maturity of rice in the month of November, 2013. During off-season, the population of egg clutches was recorded throughout the season. The egg clutch population gradually increased with rice growth with the maximum population recorded during March, 2014. Meanwhile, egg clutch population declined during the latter half of March, 2014, but it increased once again during the flowering and maturity period of rice from late April to May, 2014. The highest number of egg clutches during the two rice seasons was recorded at the ripening of rice in off-season during the month of May, 2014. The peak population of egg clutches throughout the two seasons seemed to correspond with the variation in the number of seedlings along with high relative humidity and low rainfall. Moreover, a relatively high population of aquatic weeds, particularly *Limnocharis flava* (Alismataceae), also corresponded with higher population of *P. maculata* egg clutches during the two rice seasons as females preferred these broad leaves for the oviposition. Results obtained for the population fluctuation of juvenile *P. maculata* showed that the first population of juveniles was recorded during active tillerring of rice at the end of September, 2013. The juvenile population showed a sharp rise afterwards during reproductive and ripening stages of rice in October and November, 2013. In contrast to main-season, the juvenile population was recorded since the off-season transplanting of rice. The population remained constant during the vegetative growth of the rice, i.e. during March and early April, 2014. A sharp rise in the juvenile population was observed afterwards with the peak population recorded during late April, 2014. However, the population declined slightly in May, 2014, but remained relatively high. No population of juveniles was recorded at the end of both rice-growing seasons due to the drainage of rice field for harvesting.

The results for population fluctuation of juveniles show that peak populations during two rice seasons are related to higher rainfall and relative humidity (Figure 2). Moreover, the peak juvenile populations during the two rice-growing seasons mainly correspond to relatively higher population of *L. flava*, along with *Monochoria vaginalis* (Pontederiaceae) and *Ischaemum rugosum* (Gramineae) (Figure 3). The population fluctuation of female *P. maculata* showed that the female population first appeared in the mid-October, 2013, i.e. in main-season. The population gradually increased through reproductive stage of rice with peak female population recorded at the ripening of rice in November, 2013. In contrast to main season, the female population was recorded with the transplanting of rice during off-season. The population then fluctuated throughout the rice-growing season, with a relatively higher population recorded at the end of February and April, and early May, 2014. During the two rice-growing seasons, no female population was recorded during harvesting of rice due to water removal from the field. Weekly rainfall appeared to contribute significantly towards the population development of females, along with higher weed populations of *L. flava* (Figures 2 and 3). Similar to the female population, the male population of *P. maculata* also appeared in the middle of October, 2013, in main-season. The male population showed a small rise with the maximum population of main-season recorded during the ripening of rice in the later half of November, 2013. However, the male population reappeared in the next season with the transplanting of rice. Their population then fluctuated throughout the remaining season with the peak populations recorded during late April and early May. Relatively higher rainfall and relative humidity seemed to contribute towards the higher male population in two rice seasons. Among weeds, *L. flava* showed significant contribution in the population fluctuation of male *P. maculata* in the two seasons, with *I. rugosum* might also have contributed in the higher male population during off-season (Figures 2 and 3).

Results showing stepwise regression of weather parameters, rice seedlings and weeds towards population fluctuation of different stages of *P. maculata* are given in Table 1. The results indicated that during both rice growing seasons, relative humidity showed significant effects on the population fluctuation of egg clutches (R^2= 0.106 and 0.239, respectively). Among the weeds, *L. flava* (R^2=0.392) and *I. rugosum* (R^2=0.0.70) showed effects on the population development of egg clutches during main and off-season, respectively. In addition, *Ischaemum rugosum* (R^2=0.174) and *L. flava* (R^2=0.326) were also the significant contributors in the population fluctuation of juveniles during main and off-seasons, whereas rainfall was the main weather parameter to influence juveniles' population during both seasons with R^2=0.241 and 0.111, respectively. During main-season, water pH (R^2=0.306) and *L. flava* (R^2=0.288) were the key contributing factors in the population fluctuation of

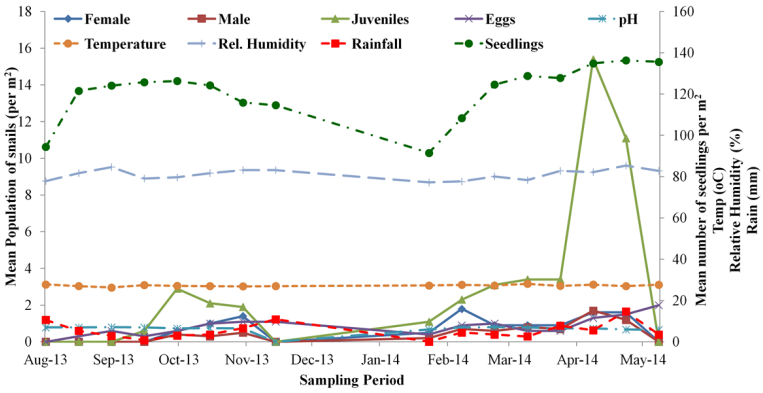

Figure 2. Population fluctuation of different stages of *P. maculata* against weather parameters and rice seedlings

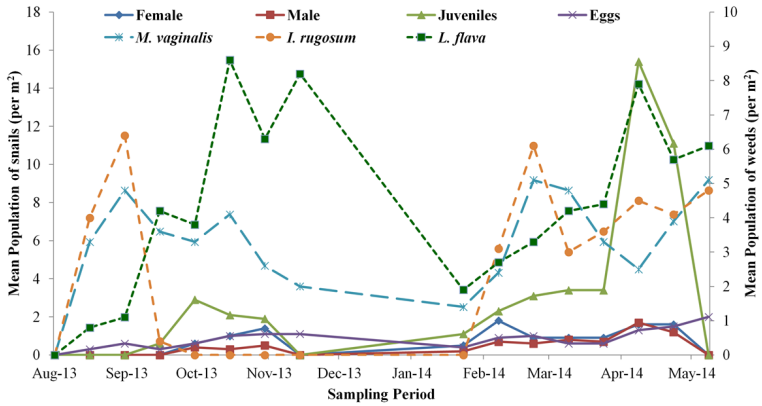

Figure 3. Population fluctuation of different stages of *P. maculata* against aquatic weeds

female snails, whereas relative humidity (R^2=0.254), rainfall (R^2=0.190) and *L. flava* (R^2=0.110) significantly affected the female population during off-season. Water pH (R^2=0.208) and *L. flava* (R^2=0.153) significantly regulated the population of male snails during main-season, whereas rainfall (R^2=0.194), temperature (R^2=0.089) and *M. vaginalis* (R^2=0.0944) were the key factors towards the population development of males.

Population Dispersion of *P. maculata*

The dispersion pattern of various stages of *P. maculata* was established in rice field by calculating different dispersion indices. The results from variance to mean ratio confirmed regular or uniform dispersion pattern for all stages of the *P. maculata* during the two rice growing seasons (Table 2). Moreover, Taylor's power law analysis for the main-season illustrated that the distribution of egg clutches and male *P.*

Table 1
Stepwise regression (R^2) of mean population of different stages of P. maculata *with rice seedlings, weeds and weather parameters*

Pomacea spp. stage	Rice season	Predictor variable	Intercept	R^2	Significance
Eggs per cluster	Main 2013-14	*L. flava*	-5.96	0.392	< 0.0001
		Relative Humidity		0.106	< 0.05
	Off 2013-14	Relative Humidity		0.239	< 0.05
		I. rugosum	4.78	0.070	< 0.05
		pH		0.142	< 0.05
Juveniles	Main 2013-14	*I. rugosum*	179.05	0.174	< 0.05
		Rainfall		0.241	< 0.05
		Temperature		0.102	< 0.05
		Relative humidity		0.095	< 0.05
		pH		0.071	< 0.05
	Off 2013-14	*L. flava*	-2.58	0.326	< 0.05
		Rainfall		0.111	< 0.05
Females	Main 2013-14	*L. flava*	24.79	0.288	< 0.05
		pH		0.306	< 0.001
		Rainfall		0.102	< 0.05
		Temperature		0.058	< 0.05
		I. rugosum		0.075	< 0.05
		Rainfall		0.190	< 0.05
		Relative humidity		0.254	< 0.05
	Off 2013-14	*L. flava*	11.03	0.110	< 0.05
		pH		0.047	< 0.05
		Seedlings		0.047	< 0.05
Males	Main 2013-14	*L. flava*	-0.37	0.153	< 0.05
		pH		0.208	< 0.05
	Off 2013-14	Rainfall		0.194	< 0.05
		Temperature	-21.69	0.089	< 0.05
		M. vaginalis		0.094	< 0.05

maculata showed significant ($P < 0.05$) relationships between their respective variance and mean, whereas no significant relationship was observed for juveniles and females. The slope values of Taylor's power law for different *P. maculata* stages (except for juveniles) were also greater than 1, indicating an aggregated or clumped distribution pattern. As compared to Taylor's power law, Iwao's patchiness regression showed a highly significant relationship between mean crowding index (m^*) and the mean (m) of all *P. maculata* stages ($P< 0.001$) during the main-season. Except for egg clutches, slope values (β) were greater than 1, indicating an aggregated disperion pattern (Table 3). Taylor's power law analysis for the off-season indicated

Table 2
Population dispersion (variance to mean ratio) of different stages of P. maculate

Fortnight	Rice-growing seasons							
	Main-Season 2013-14	Off-Season 2013-14	Main-Season 2013-14	Off-Season 2013-14	Main-Season 2013-14	Off-Season 2013-14	Main-Season 2013-14	Off-Season 2013-14
	Eggs per cluster		Juveniles		Females		Males	
1	-	0.04	-	0.61	-	0.08	-	0.31
2	0.17	0.24	-	0.41	-	0.17	-	0.20
3	0.10	0.13	-	0.23	-	0.24	-	0.18
4	0.18	0.17	0.57	0.06	-	0.06	-	0.06
5	0.30	0.17	0.79	0.00	0.11	0.11	0.06	0.33
6	0.17	0.58	0.05	0.87	0.13	0.20	0.18	0.38
7	0.25	0.11	0.09	3.85	0.07	0.14	0.08	0.12
8	0.25	0.52	-	-	-	-	-	-

Table 3
Regression data of Taylor's power law and Iwao's patchiness model analysis for different stages of Pomacea *spp. during main-season, 2013-14*

Species	Taylor's power law				Iwao's patchiness regression			
	a	b	R^2	P	α	β	R^2	P
Eggs per cluster	-0.53	1.50	0.669	< 0.05	-0.47	0.63	0.517	< 0.001
Juveniles	-0.20	-0.21	0.010	ns	-0.96	1.15	0.968	< 0.001
Females	-0.37	1.53	0.091	ns	-0.99	1.08	0.997	< 0.001
Males	0.04	3.25	0.899	< 0.05	-0.02	-1.15	0.867	< 0.001

that the distribution of different stages of *P. maculata* did not show significant ($P < 0.05$) relationships between respective variance and mean of the stages, except for egg clutches. The slope values of Taylor's power law for all *P. maculata* stages were also greater than 1, indicating an aggregated or clumped distribution pattern. However, Iwao's patchiness regression during the same season showed a highly significant relationship between mean crowding index (m^*) and the mean (m) of *P. maculata* egg clutches ($P < 0.01$) and a significant relationship ($P < 0.05$) between the females and males. Nevertheless, no significant relationship was observed for the juveniles during off-season. The slope values (β) for egg clutches and juveniles were greater than 1, showing an aggregated disperion and less than 1 for females and males indicating a regular disperion. Nevertheless, the constant α in the Iwao's model indicates the tendency to repulsion as its value is negative (-) and the tendency to crowding when it is positive (+); the same is defined by Iwao (1970) as the Index of Basic Contagion (Table 4). Based on the higher value of R^2 using Iwao's patchiness regression compared to

Table 4
Regression data of Taylor's power law and Iwao's patchiness model analysis for different stages of Pomacea *spp. during off-season, 2013-14*

Stage	Taylor's power law				Iwao's patchiness regression			
	a	b	R^2	P	α	β	R^2	P
Eggs per cluster	-0.73	2.31	0.775	< 0.05	-1.04	1.26	0.951	< 0.001
Juveniles	-0.72	1.34	0.286	ns	-1.80	6.50	0.400	ns
Females	-0.79	1.48	0.346	ns	0.42	0.68	0.732	< 0.05
Males	-0.62	1.06	0.395	ns	-0.50	0.75	0.696	< 0.05

Taylor's power law, we can say that Iwao's patchiness regression modal fitted the data better than Taylor's power law.

DISCUSSION

Population Distribution of *Pomacea* spp.

Results regarding population distribution of *Pomacea* spp. confirmed the presence of only *P. maculata* during both rice seasons. Previous research and genetic work confirmed the introduction of at least four apple snail species, namely, *P. canaliculata, P. maculata, P. scalaris* and *P. diffusa* in Southeast Asia, with the former two species being widely distributed and well established (Yahaya et al., 2006; Hayes et al., 2008). Moreover, studies have also confirmed the higher abundance and wide distribution of *P. canaliculata* in comparison to *P. maculata* in invaded areas including Malaysia (Yahaya et al., 2006; Rawlings et al., 2007; Hayes et al., 2008; Salleh et al., 2012). However, results of this study showed only the presence of *P. maculata* from the study site and this finding is supported by Arfan et al. (2014) who confirmed more abundance and wide distribution of *P. maculata* as compared to *P. canaliculata* in the rice fields of Peninsular Malaysia. The presence of only *P. maculata* could be due to its large scale and multiple introduction and tolerance to specific environmental stresses of Peninsular Malaysia as compared to *P. canaliculata*. This is supported by the findings of Hayes et al. (2008). Moreover, comparatively higher population of all the stages of *P. maculata* was recorded during the off-season crop as compared to main-season. The main reason identified for the low population during the main-season was proper management of water especially after the heavy rains through appropriate field levelling. However, continuous presence of water in the field by irrigation and heavy rains, along with lack of proper water management, augment the population of snails by induction of fresh population from the adjacent water channels. The same finding has also been confirmed by Salleh et al. (2012).

Population Fluctuation of Different Stages of *P. maculata* against Weather Parameters, Rice Seedlings and Weeds

The population fluctuation of different stages of *P. maculata* during the two rice-growing seasons showed high variation in the population density of different stages within and between two seasons. Relatively

higher populations of either stage were recorded in the latter period of the two rice-growing seasons. Burlakova et al. (2010) also reported highly variable pattern in the population of *P. canaliculata* in rice fields as compared to ponds and streams. Moreover, relatively lower populations of the snails in this study during the beginning of the seasons were mainly affected by the two applications of molluscicide (fentin acetate) by farmers before and after sowing that had almost eliminated snail population in the rice field. However, it was also observed in the study that higher relative humidity and rain fall contributed significantly towards the population build-up of different stages. Studies showed that the presence of water is key for snail movement and thus towards damages as it is the habitat that the snails used for their movement and feeding (Cowie, 2002: Teo, 2003; Joshi, 2007). Accordingly, higher rainfall during both cropping seasons helped to increase the population of *P. maculata* from the adjacent fields and water channels, the finding which had also been reported by Salleh et al. (2012). However, other weather parameters did not show any significant effect on the population regulation of *P. maculata* as it could tolerate high range of pH and temperature (Estebenet & Martín 2002; Ito 2003; Albrecht et al., 2005; Matsukura & Wada 2007; Ramakrishnan, 2007; Seuffert & Martín 2009; Seuffert et al., 2010; Byers et al., 2013). Moreover, constant temperature regime around 30^{o}C supported the steady population development of different stages of *P. maculata*.

A significant role of aquatic weeds especially broad leaf weeds was also identified to contribute towards the population fluctuation of *P. maculata* in the rice field. Snails were found to mostly depend on these weeds which are present at the corners of field for their survival and growth during the initial period of rice growth when famers apply molluscicides, and also during the latter period when rice crop becomes hard for their consumption. Joshi et al. (2006) also recorded the significant role of apple snails in reducing the weed density in transplanted rice when released after the early destructive period to rice. Other studies have also confirmed the significant role of *Pomacea* spp. in the management of weeds in the rice field and accordingly their role in the population fluctuation of *Pomacea* spp. (Wada et al., 2002; Yusa et al., 2003). High correlations of females and egg clutches of *P. maculata* with *L. flava* were also observed in the study, indicating the preference of females towards *L. flava* not only as source of food but also as a substrate for oviposition. The studies also confirmed the preference of female *Pomacea* spp. towards some particular plant species for their oviposition as compared to metal and concrete objects surrounding the water bodies (Burks et al., 2010).

Population Dispersion of *P. maculata*

The simplest dispersion index of variance to mean ratio confirmed a uniform dispersion for the different stages of *P. maculata*. However, Taylor's power law and Iwao's patchiness regression indicated the

aggregated dispersion pattern for most of the *P. maculata* stages. The difference in different indices seemed to be due to the applicability of individual indices, but the variable dispersion patterns of different stages of *P. maculata* populations might also be influenced by the availability of sufficient food in the forms of rice and aquatic weeds throughout the cropping seasons. Petney et al. (2012) also suggested an aggregated dispersion pattern for the snail population of genus *Bithynia* in Thailand. Studies by Ge et al. (2015) also obtained the aggregated distribution of the girdled horn snail *Cerithidea cingulata* (Caenogastropoda: Potamididae) mainly due to availability of food. Previous studies also showed the significant contribution of different stages of rice and weeds in the population development and dispersions of different stages of *Pomacea* spp. in rice fields (Sanico et al., 2002; Joshi et al., 2005). Another significant factor in the population dispersion of snails may be the availability of sufficient water due to irrigation and continuous rainfall that facilitate the movement of different stages of *P. maculata* in variable patterns (Cowie, 2002; Teo, 2003; Salleh et al., 2012). The dispersion indices used are commonly used to estimate the dispersion patterns of insects that mostly showed aggregated behaviour, which is either due to availability of food, mates, natural enemies, seasonal changes or role of microclimate (Tsai et al., 2002; Sule et al., 2012). Accordingly, the factors mentioned above might also have contributed towards the variable population distribution of *P. maculata* in the rice field.

In conclusion, this study confirmed the presence of only *P. maculata* in the rice field during two rice-growing seasons. Comparatively higher populations of egg clutches, juvenile, female and male *P. maculata* were recorded during off-season as compared to main-season. Among weather parameters, significant effects of rainfall and relative humidity were found to influence the population development of the various stages of *P. maculata* stages, along with aquatic weeds, *L. flava* and *I. rugosum*. Other weeds and weather parameters did not show any effects on the population of snails. Different dispersion indices indicated a variable pattern of uniform and aggregated dispersions for different stages of *P. maculate*, depending upon the applicability of individual dispersion indices. The results obtained could be practically applied in the field to properly devise various control measures, keeping in view not only the distribution pattern of different stages of snails but also considering the potential role of weather parameters and associated aquatic weeds.

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Urine versus Pre-mix (Sugar: Salt): Baits for Stingless Bees (Hymenoptera: Meliponini)

Kumara, T. K.[1]*, Farisya, M. S. N.[1], Wan Noor Aida, W. M.[1], Suhaimi Omar[1], Marcela, P.[2] and Aurifullah, M.[1]

[1]Faculty of Agro Based Industry, Universiti Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Malaysia
[2]School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

ABSTRACT

Stingless bees are dispersed throughout Malaysia and form an important group of pollinators in agriculture and natural ecosystems. A study was conducted at Lojing Highland in Kelantan, Malaysia on the preference of stingless bees towards pre-mix bait [sugar and salt (1:2) (sugar: water:v:v)] with 2.5g NaCl added per 500 ml of solution] and urine bait for two consecutive days. A total of 285 stingless bees of 15 species were sampled for this purpose. Overall, stingless bees showed no preference for either bait, but a closer examination showed species-level preferences. Five species preferred urine bait over pre-mix bait, and another eight preferred pre-mix bait over urine bait. No significant differences were found on stingless bees preferences towards pre-mix bait and urine baits ($p>0.05$). Five stingless bee species (*Lisotrigona scintillans, Pariotrigona pendleburyi, Lepidotrigona ventralis, Tetrigona apicalis,* and *Tetragonula collina*) were found to be frequently attracted to the urine bait compared with pre-mix bait. Meanwhile, eight stingless bee species attracted to pre-mix bait compared with urine bait. This study shows that stingless bees' preference for pre-mix or urine baits depends on species.

Keywords: Stingless bee, pre-mix, urine, bait preference

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E-mail addresses:
kumara_k2001@yahoo.com (Kumara, T. K.),
farisya_1991@yahoo.com (Farisya, M. S. N.),
aidamuhamad123@gmail.com (Wan Noor Aida, W.M.)
suhaimiomar@umk.edu.my (Suhaimi Omar)
marcela.fun@gmail.com (Marcela, P.)
aurifullah@umk.edu.my (Aurifullah, M.)
* Corresponding author

INTRODUCTION

Compared to *Apis*, stingless bees (Apidae: Meliponini) have 50 times more species and are very different biologically (Roubik, 2006). The stingless bees are dispersed throughout most parts of Malaysia and form an important group of pollinators in agriculture and natural ecosystems (Hannah

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et al., 2012). In Asia, there are 43 recognised species belonging to two genera, namely, Lisotrigona and Trigona (Michener, 2007). The genus *Trigona*, comprising of 120 species, were placed into 10 subgenera including *Homotrigona*, *Lepidotrigona* and *Heterotrigona* (Chinh et al., 2005). Recently, these subgenera have been upgraded to genera (Rasmussen, 2008). Malaysia hosts a rich diversity of stingless bees; 29 species have been identified so far (Eltz & Bru, 2003; Mohd Norowi et al., 2010; Hannah et al., 2012).

Many studies have been conducted in forest ecosystems to document the diversity of stingless bee species using different baiting methods (Boontop et al*.,* 2008). Baits include mixture of honey, water and salt (Boontop et al*.,* 2008), sugar solution with added lemon or rose essence (Hannah et al*.,* 2012), diluted honey (Salmah et al*.,* 1990) and odour baits (Pedro & Cordeiro, 2015). However, not all stingless bees are attracted to sugar baits. *Trigona necrophaga*, *T. hypogea* and *T. crassipes* have an obligate necrophagy habit (Camargo et al., 2012), whereas in Thailand, *Lisotrigona cacciae*, *L. furva* and *Pariotrigona klossi* (Meliponini, Apidae) workers drink tears from human eyes (Banziger et al*.,* 2009). Thus, when studying the diversity of stingless bees, using just one type of bait would make the sample bias to specific groups and fail to capture a more comprehensive representation of the stingless bee community of the study area. In order to better understand how bait types may affect the type of stingless bees sampled, this study examined the stingless bee preference towards pre-mix (sugar: salt) and urine baits.

MATERIALS AND METHODS

The study was conducted at Lojing Highland located in Kelantan, Malaysia, for the period of 2 days (23rd - 24th January 2014). This sampling site is located at an altitude of 800 to 1000 meters above the sea level, which is categorised as an upper dipterocarp forest or lower montane forest. Two parallel transects, each 100m long, were used. The distance between the parallel transect was 1.5 m. In each transect, 10 baiting stations were installed at the distance of 10 meter apart. This was made into two replicates [sampling location coordinates – (04º 38' 01.7" N 101º 30' 21.0" E) (04º 38' 09.4"N; 101º30' 19.2" E)].

The pre-mixed bait was prepared by mixing sugar and water (1: 2), added with 2.5g NaCl per 500 ml of solution (Boontop et al*.,* 2008). The urine was collected overnight from a person. At each baiting station, the pre-mix solution and urine were sprayed (~20 ml). The bait station was ~1 m in diameter. The spraying commenced between 0800-0900 hours. The bees attracted to these bait spots were sampled for a maximum duration time of five (5) minutes in each baiting station, twice daily (9 a.m. – 11 a.m. and 2 p.m. - 4 p.m.). The same observations were done for both days. Sweep net was used to capture and collect the stingless bees. The captured stingless bees were then placed in a killing jar and preserved in 70% alcohol. The specimens

were brought back to Biology Laboratory, Universiti Malaysia Kelantan; they were pinned and preserved and identified using taxonomic keys provided by Sakagami et al. (1990).

Statistical Analysis

Collected data were subjected for normality test and were found not normally distributed (Shapiro-Wilk test, $p<0.05$). Therefore, Wilcoxon Rank Sum was performed to analyse any significant differences in the frequency of stingless bee captured using both the baits. Statistical analysis was done using JMP 8.0 (SAS Ins.).

RESULTS AND DISCUSSION

A total of 285 stingless bee specimens of 15 species were sampled for the duration of 2 days. There was no significant difference in the frequency of the stingless bees sampled using the pre-mixed bait and urine ($p > 0.05$) although more specimens were sampled for the pre-mixed baits (see Table 1). In particular, *Tetragonula geissleri* attracted to the pre-mixed bait twice as much compared with urine bait, whereas *Lepidotrigona ventralis* was found to prefer urine bait compared to the pre-mixed bait (Table 1). Five stingless bee species (*Lisotrigona scintillans, Pariotrigona pendleburyi, Lepidotrigona ventralis, Tetrigona apicalis* and *Tetragonula collina*) were found attracted to the urine bait compared to the pre-mixed bait. Meanwhile, eight (8) species attracted to the pre-mixed bait compared to the urine bait (Table 1). Throughout the sampling period, the *Lepidotrigona ventralis* was also found to be attracted to wet socks and sweat. Baiting stingless bees using pre-mix bait is a commonly accepted technique (Boontop et al., 2008; Hannah *et al*., 2012) with the assumption that all stingless bees are attracted to sugar. A recent study in Thailand showed that using 50% (v/v) honey solution was able to attract 12 species of stingless bees (Jongjitvimol & Petchsri, 2015). Others experimented with odour baits (cineole, vanillin, benzyl acetate, methyl salicilate, eugenol and benzyl benzoate) to attract *Trichotrigona* but failed (Pedro & Cordeiro, 2015). However, this study found that some species preferred urine compared with pre-mixed baiting. Therefore, stingless bees might get attracted to the salt content in both the baits tested. Nevertheless, the subject matter needs to be further tested and scientifically validated. In addition, it was noticed during the sampling that the baiting stations which were exposed to sunlight attracted more stingless bees compared to those baiting stations located under the forest canopy. This could be due to the light intensity as a number of stingless bee catches were found to be positively correlated with transmission of light (Boontop et al*.,* 2008).

CONCLUSION

In sum, the stingless bees in our study did not show any preference for either urine or pre-mixed baits ($p > 0.05$). Certain species were sampled at one bait more than double the other, though statistical tests were not performed. For a well distributed stingless bee sample, we suggest that different baits

Table 1
The frequency of stingless bees sampled from the pre-mixed baits and urine baits

Species	Pre-mix (sugar: salt)	Urine
Geniotrigona thoracica	1	1
Lisotrigona scintillans	2	12
Pariotrigona pendleburyi	7	13
Heterotrigona erythrogastra	13	5
Lepidotrigona trochanterica	5	0
Lepidotrigona ventralis	18	25
Heterotrigona itama	9	2
Tetragonula geissleri	61	15
Tetrigona apicalis	1	6
Trigonella lieftinicki	3	0
Trigonella moorei	9	0
Tetragonula collina	4	10
Tetragonula laeviceps	13	0
Tetragonula reepeni	37	12
Tetrigona atripes	1	0
Total	184	101

are to be applied. In addition, biotic and abiotic factors should not be ignored as different stingless bees might have different foraging time, weather condition, and foraging distance.

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Effect of Dietary Soy Lecithin on Laying Performance, Egg Quality and Meat Texture of Aged Layer Hen

Akit, H.[1]*, Sazili, A. Q.[1,2,3], Ismail, N. A.[1], Atan, N. A.[1], Shazali N.[1] and Loh, T. C.[1,3]

[1]Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Halal Products Research Institute, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Laboratoty of Animal Production, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

This study was conducted to determine the laying performance, egg quality and meat texture of aged layer hens fed with soy lecithin. A total of 100 layer hens (Novogen Brown), aged 76-weeks old, were randomly assigned to five treatment groups and treated as follows: basal diet with 0% (control), 2%, 4%, 6% and 8% soy lecithin, respectively. After four weeks of treatment, lecithin showed no improvement in body weight gain, egg mass, egg production, feed intake, feed conversion efficiency, egg quality and meat texture . Meanwhile, egg weight increased in birds fed with 2% lecithin (P=0.02) and this might be attributed to the high linoleic acid content in soy lecithin. The results implied that feeding hens with 2% lecithin increased egg weight but it had no beneficial effects on other laying performance parameters. Thus, it is of interest to investigate potential benefits of lecithin on different dietary fat sources.

Keywords: Egg quality, layer hen, laying performance, lecithin, meat texture

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E-mail addresses:
henny@upm.edu.my, henny_akit@hotmail.com (Akit, H.),
awis@upm.edu.my (Sazili, A. Q.),
fifahpenang@gmail.com (Ismail, N. A.),
nuratiqahatan164138@yahoo.com.my (Atan, N. A.),
aira_hazirah87@yahoo.com (Shazali N.),
tcloh@upm.edu.my (Loh, T. C.)
* Corresponding author

INTRODUCTION

With increasing bird age, the rate of egg production reduces and the incidences of thin-shelled and cracked eggs markedly increase (Lillpers & Wilhelmson, 1993). Egg quality such as Haugh unit and egg weight were also negatively affected by increasing hen age (Akyurek & Okur, 2009). Consequently, the economic life of a bird is often as short as two years. Meanwhile,

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meat from spent layer hens (more than 1.5 years old) that have completed most of their egg production, is tougher, less acceptable by consumers and usually sold at a lower price than broiler meat (Kijowski, 1993). Tougher meat from older animals is a result of increased meat collagen content and collagen insolubility (Nakamura et al., 1975).

Soy lecithin is a by-product from the processing of soybean oil that contains a mixture of various phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Phospholipids are components of cell membranes found in all living cells. Lecithins (E322) are commercially used as emulsifier and anti-oxidant in various food applications, cosmetics and pharmaceutical industries. Lecithin involved in regulating lipid metabolism (Huang et al., 2008). Lecithin was also reported to be an important source of choline in broiler diets (Pena et al., 2014). Lecithin is an omega-6 polyunsaturated fatty acid and contains high level of linoleic acid (Soares & Lopez-Bote, 2002). The ability of chicken to utilise dietary fat increases with age (Krogdahl, 1985; Tancharoenrata et al., 2013) and this is more pronounced in diets containing higher polyunsaturated fatty acids (Wiseman et al., 1991; Smits et al., 2000). Lecithin provides energy and it has been used as a substitute for dietary fats and oil to improve broiler productivity (Azman & Ciftci, 2004; Huang et al., 2007). Attia et al. (2009) demonstrated that 47-weeks old dual-purpose crossbred hens, fed with 3% soy lecithin as an extra energy source, had improved laying rate, egg weight and egg mass. Despite the impact of lecithin on providing energy, essential fatty acids and improving lipid metabolism, soy lecithin has received little attention in layer hen nutrition. Hence, this study aimed to investigate the effects of dietary soy lecithin on laying performance, egg quality and meat texture of aged layer hens.

MATERIALS AND METHODS

Animals and Management

A total of 100 Novogen Brown hens were obtained from a commercial pullet-rearing farm at the age of 75 weeks with an average weight of 2.0 ± 0.06 kg (mean ± s.e.d.). Upon arrival, hens were adapted to the experimental diet for one week. The birds were randomly allocated in individual battery pens and assigned to five treatment groups. Each treatment group consisted of five hens, and with four replicates. The dietary treatments included: (i) basal diet without soybean lecithin as control; (ii) soybean lecithin at 2%; (iii) soybean lecithin at 4%; (iv) soybean lecithin at 6%; and (v) soybean lecithin at 8% of basal diet. The composition of the test diets are as displayed in Table 1. Experimental diets were fed to the birds starting from 76 weeks old to slaughter at 80 weeks old. All the birds had *ad libitum* access to feed and water via nipple drinker prior to reaching an average final slaughter live weight of 2.0 ± 0.06 kg (mean ± s.e.d.).

Laying Performance

Egg production was recorded daily and hen-day egg production was calculated using formula [1].

$$\text{Hen-day egg production} = \frac{\text{Total number of eggs produced during the period}}{\text{Total number of hen-days in the same period}} \times 100 \quad [1]$$

Table 1
Formulation and calculated composition of diets

Item	0% soy lecithin	2% soy lecithin	4% soy lecithin	6% soy lecithin	8% soy lecithin
Ingredients (%)					
Corn	47.80	45.16	44.20	42.13	40.30
Soybean	22.00	21.20	21.85	27.10	28.43
Fish meal	4.36	4.90	4.62	1.23	0.51
Wheat pollard	10.65	12.65	12.24	10.50	10.18
Crude palm oil	3.70	2.76	1.65	0.92	0.04
Soy lecithin	0.00	2.00	4.00	6.00	8.00
DL-methionine	0.07	0.07	0.07	0.09	0.10
Dicalcium phosphate	0.68	0.53	0.60	1.31	1.45
Calcium carbonate	10.09	10.13	10.12	10.07	10.06
Choline chloride	0.03	0.03	0.03	0.03	0.32
Salt	0.30	0.25	0.30	0.30	0.29
Mineral premix[1]	0.05	0.05	0.05	0.05	0.05
Vitamin premix[2]	0.03	0.03	0.03	0.03	0.03
Antioxidant[3]	0.14	0.14	0.14	0.14	0.14
Toxin binder[4]	0.10	0.10	0.10	0.10	0.10
Total	100	100	100	100	100
Calculated analysis (g/kg)					
ME (kcal/kg)	2833	2830	2831	2830	2830
Crude protein	17.11	17.11	17.10	17.12	17.11
Crude fat	5.92	6.00	5.92	5.97	6.06
Crude fibre	3.60	3.64	3.62	3.77	3.79
Calcium	4.40	4.40	4.40	4.40	4.40
Phosphorus available	0.34	0.34	0.34	0.34	0.34
Methionine	0.38	0.38	0.38	0.38	0.38
Lysine	0.95	0.95	0.95	0.97	0.97

[1] Provided per kg of diet: Fe 120 mg; Mn 150 mg; Cu 15 mg; Zn 120 mg; I 1.5 mg; Se 0.3 mg; Co 0.4 mg.
[2] Provided per kg of diet: Vitamin A 11494 IU; vitamin D 1725 IU; vitamin E 40 IU; vitamin K3 2.29 mg; cobalamin 0.05 mg; thiamine 1.43 mg; riboflavin 3.44 mg; folic acid 0.56 mg; biotin 0.05 mg; panthothenic acid 6.46 mg; niacin 40.17 mg; pyridoxine 2.29 mg.
[3] Butylated hydroxyanisole
[4] Natural hydrated sodium calcium aluminium silicate
ME: Metabolisable energy

Feed intake was recorded by calculating the average daily feed intake of the five hens in each replicate on a weekly basis. Using one egg from each replicate, egg weight was determined once a week, regularly in the same day of week. Egg mass (g/bird per day) and feed conversion efficiency (feed intake/egg mass) were calculated every week throughout the experimental period.

Egg Quality

Egg quality was measured using one egg from each replicate collected on the last day of each week. Eggshell thickness was measured using a vernier caliper after being dried off at room temperature for three days. Haugh unit was determined using Egg Analyzer™ (SANOVO, Denmark). The yolk colour, height, thickness of egg white and the Haugh unit were automatically measured, calculated and recorded. Haugh unit values were calculated from egg weight (*W*) and albumen height (*H*), using the following formula:

Haugh unit
= 100 log (H-1.7$W^{0.37}$+7.6), as described by Eisen et al. (1962).

Meat Cooking Loss and Shear Force analysis

At the end of the experiment, all the birds were slaughtered by severing the jugular vein. *Pectoralis major* muscles were dissected from the carcass after chilling at 4°C for 24 hr. Cooking loss was measured according to the method of Honikel (1998). Samples were weighed and recorded as the initial weight (*W1*). Then, the samples were placed in individual polyethylene plastic bags and cooked in a pre-heated water bath set at 80°C. When the internal temperature reached 80°C, the cooking was continued for another 20 min. After that, the cooked samples were equilibrated to a room temperature for about an hour, removed from the bag, blotted dry using paper towels without squeezing, and (*W2*). Percentage of cooking loss was calculated as: [(*W1*-*W2*)/*W1*] x 100. The samples used for cooking loss determination were used for determining meat tenderness. Strips [1cm (thickness) x 1cm (width) x 2 cm (length)] parallel to the muscle fibre were prepared and sheared perpendicular to the longitudinal direction of the fibres by Volodkevitch bite jaw attached to TA.HD plus® texture analyser (Stable Micro System, UK) (Nakyinsige et al., 2014). Shear force values were expressed as kilogram force (kg f).

Statistical Analysis

Data were analysed using one-way analysis of variance (ANOVA). All data were also analysed for linear and quadratic effects of soy lecithin supplementation using the Genstat software 13th ed. (VSN International Ltd, UK). Ninety five percentage level of confidence ($P<0.05$) was taken as significant.

RESULTS AND DISCUSSION

Laying performance, egg quality and meat quality data are as presented in Table 2. Lecithin showed no linear and quadratic

effects on all the parameters measured (P>0.05, data not shown). In particular, lecithin had no effect on egg quality such as Haugh unit, yolk colour and shell thickness (P>0.05, respectively). Lecithin also had no effect on body weight gain, egg mass, egg production, feed intake and feed conversion efficiency (P>0.05, respectively). The laying performance results are in agreement with Attia et al. (2009), who were unable to find any changes in the laying performance of dual-purpose crossbred hens fed increasing levels of soy lecithin within the isocaloric diets. On the other hand, a study using broilers showed that diets containing soybean oil and soy lecithin mixtures in the proportion of 75/25 improved average daily gain and feed conversion efficiency (Huang et al., 2007). In a separate study using broilers, diets containing soybean oil and soy lecithin mixtures in the proportion of 75/25 increased daily weight gain in the grower period but feed conversion efficiency was not affected (Azman & Ciftci, 2004). Both studies in broilers showed that lecithin had no effect on the utilisation of lipid. It was indicated that a lower ration of saturated fatty acid to polyunsaturated fatty acid had a better feed conversion ratio (Wongsuthavas et al., 2008). Saturated fatty acids were proven to be less digestible than unsaturated fatty acids (Smink et al., 2008). Palm oil used in the current study is of vegetable origin but it is rich in saturated fatty acids, particularly palmitic acid (Smink et al., 2008). The high content of saturated fatty acids in the palm oil could have influenced the benefits of lecithin in improving body weight gain and feed conversion efficiency. The effects of lecithin on different fat sources still need further research. Broilers are selected for rapid growth and high meat yield whilst layer chickens for egg production. Consequently, the body weight gain of broiler is higher than those of layers at a similar age (Zhao et al., 2004). In commercial poultry production, peak production usually occurs when birds reach 24-26 weeks of age and production steadily declines until the flock is taken out of production at approximately 80 weeks of age. A decline in body weight gain at the end of the production cycle was expected because the hens were no longer in the development stage of life. It may be possible that body weight gain and feed conversion efficiency could be improved if lecithin supplementation was initiated during the growing period (8 to 18 weeks old).

Egg weight increased in birds fed 2% lecithin (P=0.02). Attia et al. (2009) reported that increasing levels of soy lecithin within the isocaloric diets did not affect the laying performance including egg weight. However, when 3% lecithin was used as an extra energy source, it increased laying rate, egg weight, egg mass and body weight gain, as well as improved feed conversion efficiency. This was suggested to be attributed to the increase in the availability of energy, essential fatty acids and choline, and lipid absorption. Choline bioavailability of de-oiled soy lecithin was estimated to be equivalent to choline chloride, a common source of choline in poultry diet (Emmert et al., 1996). These

Table 2
Effects of feeding five levels of soy lecithin to layer hens aged between 76 to 80 weeks old on laying performance, egg quality and meat texture

	Dietary soy lecithin (%)					s.e.d.	P value
	0	2	4	6	8		
Laying performance							
Body weight gain (g)	-14	58	-35	-34	-48	49.4	0.240
Egg weight (g/egg)	66.86[a]	76.34[b]	67.7[a]	66.32[a]	64.38[a]	2.332	0.020
Hen-day egg production (%)	68.13	75.79	67.7	67.06	69.85	5.403	0.497
Egg mass (g/hen/day)	98.43	110.95	107.03	98.87	96.6	9.084	0.475
Feed intake (g/hen/day)	115.5	119.0	116.9	113.9	109.3	5.79	0.546
Feed conversion efficiency	4.78	4.68	4.47	4.62	4.87	0.348	0.800
Meat quality							
Cooking loss (%)	27.48	27.91	27.38	28.28	27.69	0.852	0.838
Shear force (kgf)	0.92	1.12	1.08	1.24	1.08	0.138	0.233
Egg quality							
Haugh unit (mm)	51.14	50.24	62.17	60.01	55.08	6.927	0.378
Yolk colour	5.16	5.27	5.36	4.64	4.88	0.316	0.218
Shell thickness (mm)	0.29	0.30	0.29	0.28	0.30	0.016	0.549

s.e.d: standard error of difference
[a,b] Means with different superscripts differ significantly ($P<0.05$)

authors reported that supplementation of the choline-free purified basal diet with choline or de-oiled lecithin induced linear increases in feed intake and weight gain of broilers. To the authors' knowledge, no one has investigated the effects of dietary choline on laying performance of aged layer hen. Egg weight increased when linoleic acid was supplemented in the layer diets containing linoleic acid that was below the NRC requirements (Grobas et al*.,* 1999). Laying hens do not need more than 1.0% of linoleic acid in their diet to maximise egg size (Pérez-Bonilla et al., 2011). Soy lecithin contains 83.98% of polyunsaturated fatty acid with linoleic acid being the dominant fatty acid (Huang et al., 2007). The high linoleic acid content in soy lecithin may contribute to the increased egg weight in birds fed with 2% lecithin in the current study. All the treatment groups lost weight except for hens fed with 2% lecithin ($P>0.05$), and this might explain the increased egg weight.

Cooked meat shear force values and cooking loss were not affected by lecithin ($P>0.05$, respectively) (Table 2). In contrast, Moraes et al. (1981) observed that soy lecithin supplementation in broiler chicken diet increased the toughness of dark meat, that was also proven to be tougher compared to white meat. Studies in pigs showed that soy lecithin supplementation had no effect on pork shear force values (D'Souza et al., 2012; Akit et al., 2014). Lecithin might influence meat texture if the treatment was

started as early as the grower stage since maturity of collagen increases with age (D'Souza et al*.,* 2012).

In conclusion, 2% soy lecithin increased egg weight of aged layer hens. However, lecithin did not improve egg production, feed conversion efficiency, as well as egg quality and meat texture. Therefore, it is of interest to investigate potential benefits of lecithin on different dietary fat sources.

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Preliminary Studies towards Identification of Ginger Wilt Disease in Sabah, Malaysia

Cosmas, L. L.[1,2]*, Atong, M.[2] and Poili, E.[3]

[1]Sabah Department of Agriculture, Wisma Pertanian Sabah , Jalan Tasik Luyang, Locked Bag No. 2050, 88632 Kota Kinabalu, Sabah, Malaysia
[2]Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Locked Bag No. 3, 90509 Sandakan, Sabah, Malaysia
[3]Agriculture Research Centre, Sabah Department of Agriculture, P.O Box 3, 89207 Tuaran, Sabah, Malaysia

ABSTRACT

Bacterial wilt is a major ginger disease in Sabah after rhizome rot. The disease has affected the production of ginger in Sabah since 2005. In this study, the ginger plants with foliar symptoms (yellowing and wilting), collected from six ginger-growing areas in the Tambunan and Ranau districts, were observed to have signs of bacterial pathogen (i.e., rhizome rot and bacterial ooze). A total of 19 bacterial strains were isolated, and all of the isolates were characterised as rod-shaped and Gram-negative by Gram-staining and potassium hydroxide test and microscopic examination. MALDI-TOF analysis identified six species from the isolates: *Enterobacter cloacae* complex (57.9%), *Ralstonia pickettii* (10.5%), *Agrobacterium tumefaciens* (10.5%), *Bacillus pumilus* (10.5%), *Stenotrophomonas maltophilia* (5.3%) and *Serratia marcescens* (5.3%). In pathogenicity test, *E. cloacae*, which constituted most of the isolates, induced mild rot symptoms (discoloration) on ginger rhizome slices, but no disease symptoms were produced in ginger plants. Further studies on the interaction of *E. cloacae* with other isolated species are required to confirm the causes of ginger wilt disease in Sabah, Malaysia.

Keywords: ginger, ginger wilt disease, *Enterobacter cloacae* complex

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E-mail addresses:
laverny_69@yahoo.com (Cosmas, L. L.),
xabex96@ums.edu.my (Atong, M.),
Evenni.Poili@sabah.gov.my (Poili, E.)
* Corresponding author

INTRODUCTION

Ginger (*Zingiber officinale* Rosc) is one of the important spices in Malaysia. It is a high-value crop, with a potential yield of 9 – 12 tonne per hectare; at the price of RM 3 – 5 per kilograms, farmers can earn net

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income per hectare of RM 20,595–53,595 (Department of Agriculture, 2011). The type of ginger that is grown in Sabah is Sabah cultivar (Mahdi et al. 2013). The main growing seasons are on March to April. Sabah ginger is known to be the best of its kind, and the main ginger-producing areas in Sabah are Ranau and Tambunan districts. Tambunan district is recognised as the main ginger producer in Malaysia (Daily Express, 2006), and the state government aimed to make Sabah the biggest ginger producer in the country (Daily Express, 2012). However, the bacterial wilt disease restricted the production of ginger in Sabah. The planting areas and production of ginger in Sabah declined from 458.3 hectares (5,848.4 tonnes) in 2006 to only 117.7 hectares (956.7 tonnes) in 2014 (Department of Agriculture, 2006–2014). This incident caused the increase of ginger import value from 1,185 tonnes (RM 1,953,000) in 2006 to 3,508 tonnes (RM5,691,698) in 2012 (Department of Statistic, 2006 - 2012). Bacterial wilt is the main ginger disease in Sabah after rhizome rot. The disease has affected the production of ginger in Sabah since 2005. The symptoms observed in the field included yellowing, wilting and rotting of rhizomes, suspected to be bacterial wilt disease caused by *Ralstonia solanacearum*. The objective of this study was to identify the bacteria that cause ginger wilt disease up to species level. Identification of the bacteria was done by culturing in nutrient agar and using MALDI-TOF technology. Identification of the causal agent of ginger wilt disease is important for proper control of the disease in order to improve ginger production in Sabah.

MATERIAL AND METHODS

Pathogen isolations were based on the standard procedure described by Johnston and Booth (1968). Ginger plants with foliar symptoms (yellowing and wilting) (Figure 1) were collected from six different ginger-growing areas in the Tambunan and Ranau districts which were affected by wilt disease in January to February 2014. The samples in each area were collected by random sampling. The ginger plants were inspected for signs of bacterial pathogen (i.e., rhizome rot and bacterial ooze). Bacterial strains were then isolated from rhizome as the suspected bacteria (*R. solanacearum*) is a seed rhizome borne, and bacterial population is generally larger in roots compared to stems and leaves (Lamb et al., 1996; Nelson, 2013). The ginger rhizomes were washed with tap water to remove soil, and then air-dried. The rhizomes were then aseptically cross-sectioned with approximately 5 mm^3 tissue sections cut out from the central cylinder. The tissue sections were macerated in sterile distilled water (SDW) in a glass cube before one loop-full of the suspension was streaked onto a nutrient agar plate and incubated at 28°C to 30°C for 2 days. The single colonies forming after that were transferred to new plates and incubated. Purified cultures were characterised based on their morphological characteristics, Gram staining, potassium hydroxide testing and microscopic examination (Suslow et al., 1982; Breakwell et al., 2007). Identification

of the bacterial strains was completed using matrix-assisted laser desorption ionisation–time of flight (MALDI-TOF) technology (Bizzini et al., 2010). Pathogenicity tests were carried out based on the method of Nishijima et al. (2004). Inoculation tests were performed on ginger rhizome slices and young ginger plants grown in polybags. Pathogenicity tests on the slices were done using mature ginger rhizomes purchased from the local market. The rhizomes were cleaned with tap water and dried at room temperature. After that, they were cut into approximately 3 mm slices with a sterilised knife.

The slices were sterilised on both surfaces by flame, then immersed in SDW for rehydration and placed on moistened filter paper in petri plates. The rhizome slices in each petri plate were inoculated with bacterial suspension. Rhizomes inoculated with SDW served as a control. Inoculations were made using two methods. In the first, a sterile toothpick dipped in either bacterial culture or SDW was punctured into the centre of each rhizome slice. In the second method, 100 ul of bacterial suspension at approximately 10^{11}, 10^{10}, 10^{9}, 10^{8} and 10^{7} colony-forming units (CFU)/ml or SDW were pipette-inoculated into the puncture-wounds of the rhizome slices. Each inoculated rhizome slice in the petri plate of both methods was incubated at 30°C until visible symptoms were observed for evaluation. Pathogenicity tests on the ginger plants were carried out using tissue-culture-initiated ginger plants that were grown in polybags containing sterilised Sahara potting media (90% organic matter). The 3-month-old plants were inoculated by pouring 18 ml of bacterial suspension at approximately 10^{11}, 10^{10}, 10^{9}, 10^{8} and 10^{7} CFU/ml or SDW into the media in three replicates. Monitoring of foliar symptoms was done every week.

RESULT AND DISCUSSION

The ginger plants with foliar symptoms (yellowing and wilting) collected from six ginger-growing areas in the Tambunan and Ranau districts was observed to have signs of bacterial pathogen (i.e. rhizome rot and bacterial ooze). A total of 19 bacterial strains were isolated, and all of the isolates

Figure 1. Ginger plants with foliar symptoms (yellowing and wilting)

were characterised as rod-shaped and Gram-negative by Gram-staining and potassium hydroxide test, and microscopic examination. MALDI-TOF analysis identified six species from the isolates: *Enterobacter cloacae* complex (57.9%), *Ralstonia pickettii* (10.5%), *Agrobacterium tumefaciens* (10.5%), *Bacillus pumilus* (10.5%), *Stenotrophomonas maltophilia* (5.3%) and *Serratia marcescens* (5.3%).

The bacteria with the highest abundance were *E. cloacae* with 57.9% isolates, while *R. solanacearum* which was thought to be the cause of ginger wilt disease was not detected among the isolates. *E. cloacae* is a Gram-negative, facultative anaerobic, rod-shaped bacterium that has been reported to cause ginger rhizome rot in Hawai and Brazil (Nishijima et al., 2004; Moreira et al., 2013). *Enterobacter* species have also been reported to cause ginger rhizome rot in Australia (Stirling, 2004). *E. cloacae* had been reported to have antagonistic effects and to effectively suppress the growth of *R. solanacearum* (Xue, 2009; Liu et al., 2013). Previous study showed that two species of *Enterobacter, E. asburiae* and *E. cloacae,* were frequently isolated from ginger rhizomes, and in fact there was overgrown *R. solanacearum* in culture (Alvarez et al., 2003). Moreover, *E. cloacae* had been reported to replace *R. solanacearum* as the causal pathogen for bacterial wilt of mulberry in China (Wang et al*.,* 2010). This result suggested that *R. solanacearum* could have been suppressed or overgrown by *E. cloacae*.

The other four plant pathogenic bacteria, *R. pickettii*, *A. tumefaciens, B. pumilus* and *S. marcescens*, were also isolated from the samples. *B. pumilus* is a secondary pathogen of ginger rhizomes that causes rhizome rot and foliar symptoms at later growth stages (Peng et al*.,* 2013). *R. pickettii, A. tumefaciens* and *S. marcescens* are not known to be the pathogen for ginger. *R. pickettii* is a pathogen for leaf spot and blight disease of Bird of Paradise tree (Polizzi et al*.,* 2008). *R. pickettii* also known as a biocontrol for *R. solanacaerum* and it can be found in soil, water and plants (Stelzmueller et al*.,* 2006; Wei et al*.,* 2013). *A. tumefaciens* is a pathogen for crown gall disease of many plant species (Suma et al*.,* 2008; Gohlka & Deeken, 2014). *S. marcescens* is a pathogen for cucurbit yellow vine disease and corn whorl rot (Besler & Little, 2015; Wang et al*.,* 2015).

S. marcescens also known as a biocontrol agent and a rhizobacteria that can be found in the ginger rhizosphere (Bini et al*.*, 2011; Yang et al*.* 2012). The other bacteria *S. maltophilia* is not a plant pathogenic bacteria. *S. maltophilia* is a biocontrol agent and a rhizobacteria that can be found in the ginger rhizosphere (Bini et al*.,* 2011; Yang et al*.,* 2012).

Pathogenicity test of *E. cloacae* was conducted on rhizome slice and ginger plants to determine it as the causal pathogen. Results showed that obvious disease symptoms in rhizome slice were observed in both inoculation methods that were used. However, rhizome slices inoculated using

Method 1 exhibited disease symptoms earlier (i.e., after 10 days of inoculation) compared to rhizome slices inoculated using Method 2 (i.e., after 13 days of inoculation). Rhizome slices infected with *E. cloacae* showed discoloration and decaying at the punctured wound, which are common symptoms of rotting, while control slices showed no rotting symptoms (Figure 2).

There was no disease symptoms observed in any ginger plants inoculated with *E. cloacae*. A previous study by Nishijima et al. (2004) reported that *E. cloacae* did not produce disease symptoms in ginger plants when the temperature, humidity, aeration and soil moisture are not optimal for infection. Environmental condition such as high temperature (35 to 37^{0}C), high humidity and low oxygen atmosphere (i.e. stagnant and waterlogged soil) are needed for optimal infection (Nishijima et al*.,* 2004). The soil used during the time of the test was aerated (90% organic matter), the average air temperature was 33^{0}C and the plant was placed under shade. This environment condition was not optimal for infection.

The bacteria from the infected rhizome slices in the pathogenicity test was re-isolated and cultured in nutrient agar and

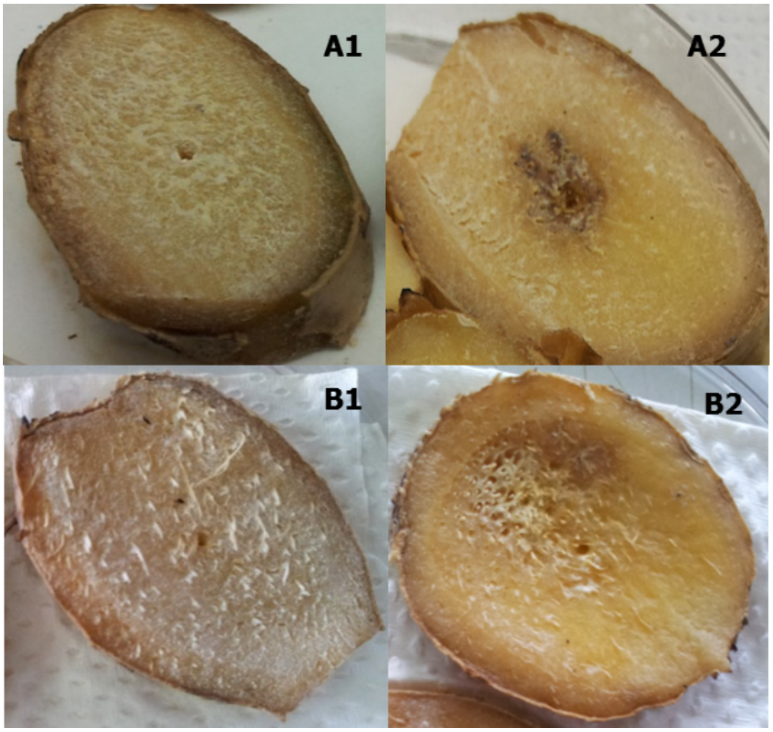

Figure 2. Symptoms produce by *Enterobacter cloacae* on rhizome slices. A: Rhizome slices inoculated with bacteria using toothpick (A1: Control inoculated with sterile distilled water, A2: Infected rhizome showing decaying symptoms). B: Rhizome slices inoculated with 10^{11} CFU/ml bacteria using pipette (B1: Control inoculated with sterile distilled water, B2: Infected rhizome with discoloration which is a mild rotting symptoms)

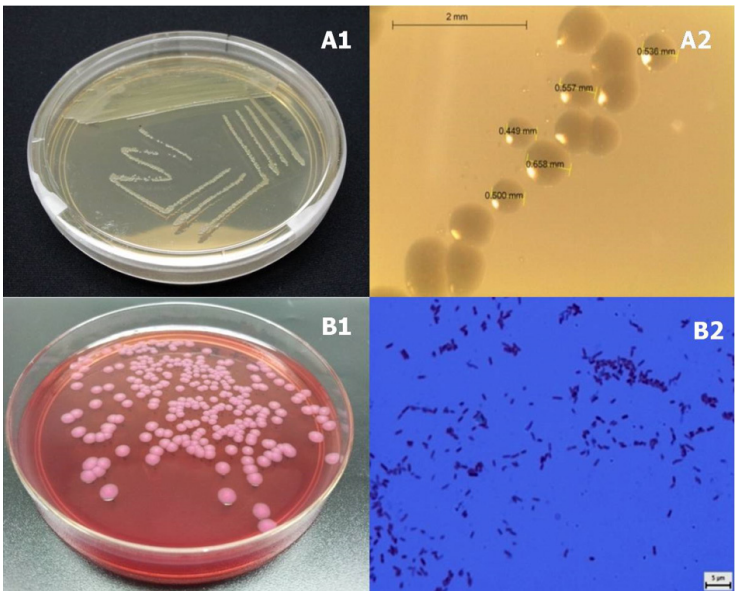

Figure 3. *Enterobacter cloacae* colony characteristics. A: Nutrient agar (A1: Colony form in nutrient agar; A2: Colony view under stereo microscope). B: MacConkey agar (B1: Colony form in MacConkey agar, B2: Microscope view showed Gram-negative and rod shape bacteria, 1.4 um at 100x magnification)

selective media agar for *E. cloacae* (i.e., MacConkey agar). The isolated bacteria were indeed *E. cloacae* because they were able to grow in the selective agar and confirmed by MALDI-TOF analysis. The *E. cloacae* colonies were cream-colored in nutrient agar, and pink in MacConkey agar, irregular in form with raised elevations and an entire margin size of 0.45 to 0.66 mm. The bacteria were Gram-negative and rod-shaped with a size of 1.0 to 2.0 µm (Figure 3).

CONCLUSION

The bacterial pathogen isolated from the ginger plants with yellowing and wilting symptoms had been identified up to species level as a species of *Enterobacter cloacae* complex instead of *Ralstonia solanacearum,* as expected. However, further studies on the interaction of *E. cloacae* with other isolated species are required to confirm the causes of ginger wilt disease in Sabah, Malaysia.

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Estimation of Grain Quality Components and their Correlation of Basmati Rice (*Oryza sativa* L.)

Mahmuda Ratna[1], Shahnewaz Begum[2], Md Abu Kawochar[3], Shiuli Ahmed[4] and Jannatul Ferdous[5]*

[1]Spices Research Centre, Bangladesh Agricultural Research Institute, Gazipur-1701, Bangladesh
[2]Plant Breeding Division, Bangladesh Agricultural Research Institute, Gazipur-1701, Bangladesh
[3]Tuber Crops Research Centre, Bangladesh Agricultural Research Institute, Gazipur-1701, Bangladesh
[4]Biotechnology Division, Bangladesh Agricultural Research Institute, Gazipur-1701, Bangladesh
[5]Biotechnology Division, Bangladesh Rice Research Institute, Gazipur-1701, Bangladesh

ABSTRACT

Variability and correlation for twelve grain characters (before cooking) and eight characters (after cooking) on distinct six lines viz. S1, S2, S5, 42(i), 42(ii) and 44(i) of Basmati rice were studied. Before cooking, the maximum hulling (%), milling outturn (%) and head rice recovery were recorded in S2 genotype. The highest kernel length and breadth of rice were also found in S2, whereas the highest kernel length and breadth ratio (L/B) of brown, rough and milled rice were observed in S2, S1 and S5 genotypes, respectively. After cooking, the highest kernel length and breadth were recorded in S2, while the highest length and breadth ratio was noted in 42(ii) genotype. The highest kernel elongation ratio and volume expansion were recorded in S5. The maximum alkaline spreading value and the minimum cooking time were found in S2. The L/B ratio of rough rice exhibited significant positive relationship with L/B of brown, milled and cooked rice.

Keywords: Variability, correlation, grain quality components, Basmati rice

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E-mail addresses:
mahmuda.ratna@yahoo.com (Mahmuda Ratna),
shahnewaz_ctg1952@yahoo.com (Shahnewaz Begum),
kawocharpgrc@yahoo.com (Md Abu Kawochar),
dey_shiuli@yahoo.com, shiuli_a@bari.gov.bd (Shiuli Ahmed),
kbdjannatul@yahoo.com (Jannatul Ferdous)
* Corresponding author

INTRODUCTION

Rice (*Oryza sativa* L.; 2n=24) belonging to the family Gramineae is one of the important cereal crops. Bangladesh is the fourth largest producer and consumer of rice in the world, with annual production of 33.834 million metric tons (BBS, 2013). It occupies 74.77% of total cropped areas and it alone constitutes about 90% of the total

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food grain produced annually in the country (BBS, 2010).

Consumer demand for the fine rice varieties is high due to its good nutritional quality, palatability, aroma and taste. The demand of basmati rice has been increasing in Bangladesh as the country is approaching more prosperous in rice production (BRRI, 2004). The climatic condition of Bangladesh is also suitable to produce quality Basmati rice. Besides yield, the grain quality of rice is the most important factor for deciding the profitability of the farmers as the grain quality decides the price in the market. Juliano and Duff (IRRI, 1991) reported that grain quality is second after yield as the major breeding objective for crop improvement. Quality of rice may be considered from the view point of size, shape and appearance of grain, milling quality and cooking properties (Dela Cruz & Khush, 2000). Quality of rice mainly depends on its intended end use by the consumers. All consumers want the best quality that they can afford. Traditionally, plant breeders concentrated on breeding for high yields. The quality of rice grain is not only dependent on the variety or genotype, but it also depends on the crop production environment, harvesting, processing and milling systems. As for example, the rice millers prefer varieties with high milling whereas consumers consider physicochemical characteristics (Meca & Juliano, 1981). The amylose content of rice is considered as the main parameter of cooking and eating quality (Juliano, 1972). Intermediate to high amylose rice with low to intermediate gelatinization temperature is preferred. Therefore, grain quality should be acceptable to farmers. However, there is very few information on qualitative data on before and after cooking in rice.

Conceiving the above scheme in mind, the research work has been undertaken in order to study the milling, cooking and eating quality and grain appearance of different advance lines of Basmati rice.

MATERIALS AND METHODS

The experiment was conducted at the Sher-e-Bangla Agricultural University, Dhaka, Bangladesh. Six different lines viz. S1, S2, S5, 42 (i), 42(ii), 44 (i) of Basmati rice and one check variety namely BRRI dhan29 were used as experimental material. The experiment was laid out in a randomized complete block design (RCBD) with three replications. A comparative laboratory analysis on quality characteristics was completed at the laboratory of Bangladesh Rice Research Institute and at the laboratory of Sher-e-Bangla Agricultural University, Bangladesh. From each entry 200 g well dried paddy was hulled in a mini "Satake Rice Machine" to get brown rice. The brown rice was passed through "Satake Rice whitening and caking machine" to obtain uniform polished grains. The polished samples were sieved to separate whole kernels from the broken ones. The samples comprising of full shape grains were used to proceed for the study. Kernels length and breadth of rough rice, brown rice, polished rice and cooked rice were measured by digital slide calipers. Ten whole kernels

from each entry were used in each case. The size of polished grain was determined on the basis of average length viz. extra long (>7.50 mm), long (6.61 to 7.50 mm), medium (5.51 to 6.60 mm), short (5.50 mm to less) and shape was determined on the basis of length and breath ratio viz. slender (over 3.0), medium (2.1 to 3.0), bold (1.1 to 2.0) and round (1.0 or less) (Ahuja et al., 1995). Grain elongation ratio was computed by dividing the average length of cooked rice to the average length of raw rice. One gram milled rice kernels were used for the study of water absorption (uptake) percentage. The water absorption ratio was determined by weight of the sample was recorded before and after cooking. Volume expansion ratio was calculated as volume of cooked rice to volume of raw rice. The same procedure was repeated for each sample. For the measuring Gelatinizationtemperature sample from each entry was placed in small petriplates (5 cm wide) containing 10 ml of 1.7% potassium hydroxide (KOH) solution. The petriplates were covered and placed in an incubator maintained at 30 ± 1°c for 16 hours as suggested (Zaman, 1981). After 16 hours of incubation, the petriplates were gently taken out from the incubator. Alkali spreading values of six grains of each entry were recorded separately and mean was calculated on a seven point numerical scale viz. high (1 to 3), intermediate (3.1 to 5.9) and low (6 to 7) (Jennings et al., 1979). For determining cooking time stop watch was used. The analysis of variance for different quality characters was performed following analysis of variance technique. When F was significant at the $p < 0.05$ level, treatments means were separated using Duncan's multiple range test (Steel & Torii, 1960).

RESULTS AND DISCUSSION

From the analysis of variance, it was observed that highly significant variation existed for all the characters studied (Table 1). All the lines and check were showed clear-cut translucent endosperm appearance (Table 2). Grain appearance is largely determined by the endosperm opacity, the amount of chalkiness. IRRI (1979) classified the endosperm of rice based on endosperm opacity as waxy or non waxy. Waxy rice devoid of or have only trace of amylose content and are opaque. Non waxy rice has varying amylose level (2.1 to 32%) and are dull, hazy or translucent. Chalkiness is undesirable in all segments of rice industry. Breeders select intensively for clear, vitreous kernels. Chalky kernels break easily, reducing milling yields. The present results for quality traits are in agreement with the findings of Sandeep (2003). Grain size and shape are the first criteria for the quality of rice that breeders consider in developing new varieties for commercial production (Adair et al., 1973). All the genotypes studied were slender in shape and extra long in size (Table 2).

From the study of mean performance of quality characteristics before cooking (Table 3) the line S2 had the maximum hulling percent (76.20%), milling percent (66%) and Head Rice Recovery (HRR) percent (79.34%). The minimum hulling percent (63.85%) and milling percent (51%) were

recorded in S1 line. The minimum HRR percent (63.01%) was also foud in S1. The milling per cent ranged from 51 to 66% but Ahuja et al. (1995) reported a range of 67 to 71 % for milling recovery in Basmati varieties. It assumes importance because it tells the actual yield of consumable product. A good milling quality includes high whole kernel recovery and less of broken rice. For the commercial success of a rice variety it must possess high total milled rice and whole kernel (HRR) turnout. If a variety possesses high broken percentage, its marketability will be reduced. The highest kernel length (12.61 mm) and breadth (2.63 mm) of rough rice were recorded in line S2, while the highest length and breadth (L/B) ratio was found in S1. The lowest kernel length (8.65 mm), breadth (2.19 mm) and L/B (4.13) ratio of rough rice were observed in check variety BRRI dhan29. In case of brown rice, the maximum kernel length and L/B ratio were recorded inS2 but the maximum breadth (1.92 mm) was observed in 42(i). The minimum Kernel length (6.20 mm), Breadth (1.81 mm) and

Table 1
Analysis of variance (ANOVA) for different quality traits (before and after cooking)

Sl. no.	Characters	df		Mean sum of square	
		Genotypes	Error	Genotypes	Error
	Before cooking				
1.	Hulling (%)	6	12	49.865**	7.56
2.	Milling outturn (%)	6	12	63.719**	9.667
3.	Head Rice Recovery (%)	6	12	95.862**	9.667
4.	Grain length of rough rice (mm)	6	12	5.661**	0.042
5.	Grain breadth of rough rice (mm)	6	12	0.084**	0.009
6.	Grain length/breadth ratio	6	12	0.556**	0.031
7.	Grain length of brown rice (mm)	6	12	3.312**	0.006
8.	Grain breadth of brown rice (mm)	6	12	0.005^{ns}	0.003
9.	Grain length/breadth ratio of brown rice	6	12	0.778**	0.013
10.	Grain length of milled rice (mm)	6	12	1.530**	0.021
11.	Grain breadth of milled rice (mm)	6	12	0.015**	0.001
12.	Grain length/breadth ratio of milled rice	6	12	0.490**	0.011
	After cooking				
1	Length of cooked rice (mm)	6	12	12.522**	0.098
2	Breadth of cooked rice (mm)	6	12	0.236**	0.041
3	Length/breadth ratio of cooked rice	6	12	1.762**	0.058
4	Cooking time (minutes)	6	12	5.857*	1.286
5	Elongation index	6	12	0.032*	0.009
6	Water uptake (%)	6	12	2913.714**	329.33
7	Volume expansion (%)	6	12	0.065**	0.008
8	Alkali spreading value	6	12	0.195**	0.024

* Significant at 5% level, ** Significant at 1% level, ns-non significant

Table 2
Endosperm appearances, size, shape, Alkali spreading value (ASV), Gelatinization Temperature (GT) of six advanced lines of basmati rice and check variety

Lines/ check	Endosperm appearance	Shape	Size	ASV	Alkali digestion	GT types
S1	Translucent	Slender	Extra long	6.00	High	Low
S2	Translucent	Slender	Extra long	7.00	High	Low
S5	Translucent	Slender	Extra long	4.25	Intermediate	Intermediate
42 (i)	Translucent	Slender	Extra long	4.59	Intermediate	Intermediate
42 (ii)	Translucent	Slender	Extra long	4.25	Intermediate	Intermediate
44 (i)	Translucent	Slender	Extra long	4.25	Intermediate	Intermediate
BRRI dhan29	Translucent	Slender	Extra long	3.83	Intermediate	Intermediate

Table 3
Mean performance of quality characteristics before cooking in different lines and check

Lines/ check	Hulling (%)	Milling (%)	Head Rice Recovery (%)	Rough rice			Brown rice			Milled rice (uncooked rice)		
				Length (mm)	Breadth (mm)	L/B ratio	Length (mm)	Breadth (mm)	L/B ratio	Length (mm)	Breadth (mm)	L/B ratio
S1	63.85 d	51.00 c	63.01 d	12.25 a	2.24 cd	5.48 a	8.57 d	1.85 ab	4.64 b	7.25 c	1.59 c	4.57 ab
S2	76.20 a	66.00 a	79.34 a	12.61 a	2.63 a	4.79 c	9.48 a	1.90 ab	5.00 a	8.05 a	1.79 a	4.50 b
S5	67.30 cd	56.00 bc	65.82 bcd	12.43 a	2.43 b	5.11 bc	9.06 b	1.87 ab	4.84 a	7.77 b	1.64 bc	4.75 a
42 (i)	69.20 bc	56.75 bc	67.50 bcd	12.27 a	2.34 bc	5.25 ab	8.73 c	1.92 a	4.55 b	7.60 b	1.69 b	4.49 b
42 (ii)	69.00 bc	56.50 bc	73.18 ab	11.45 b	2.25 bcd	5.08 bc	8.43 d	1.89 ab	4.45 bc	7.26 c	1.66 b	4.39 b
44 (i)	69.45 bc	56.93 bc	65.07 cd	11.66 b	2.31 bc	5.04 bc	8.19 e	1.91 ab	4.29 c	7.22 c	1.58 c	4.56 ab
BRRI dhan29	73.80 ab	60.50 ab	71.05 bc	8.65 c	2.19 d	4.13 d	6.20 f	1.81 b	3.43 d	5.82d	1.66b	3.51 c
Range	63.85-76.20	51-66	65.07-79.34	12.61-8.65	2.19-2.63	4.13-548	6.20-9.48	1.81-1.92	3.43-5.00	5.82-8.05	1.58-1.79	3.51-4.75
Mean	69.83	57.67	69.28	11.62	2.33	4.98	8.38	1.88	4.46	7.28	1.66	4.39
CV (%)	6.94	5.39	5.57	5.77	4.10	7.54	6.89	7.80	9.54	5.98	6.95	7.37

In a column, means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

L/B ratio (3.43) of brown rice were noticed in check variety BRRI dhan29. In case of milled rice, the utmost kernel length (8.05 mm) and breadth (1.79 mm) were found in line S2 whereas, the utmost L/B ratio (4.75) was observed in S5. On the other hand the minimum kernel length (5.82 mm) and L/B ratio (3.51) of milled rice were found in check variety BRRI dhan29 and the minimum breadth (1.58) of milled rice was recorded in 44(i). Grains with short to medium length break less than the long grains during milling. Thus grain size and shape have direct effect on the yield of head rice. Shobha Rani (2003) reported that bold grains give low head rice recovery because of high breakage. Viraktamath (1987) observed that kernel breadth enhanced the milling output and HRR was strongly associated with milling percentage.

From the study of mean performance of quality characteristics after cooking (Table 4) the maximum length of kernel of cooked rice (13.38 mm) was obtained from line S5 which is statically identical to line S2 (13.36 mm) and the minimum length was observed (6.68 mm) in check variety BRRI dhan29. The range of kernel length of cooked rice of studied genotype was 10.15 to 13.38 mm. Shobha Rani (2003) reported that the kernel length after cooking of nine released hybrids of India ranged from 10.2 to 12.4 mm and Soroush et al. (2005) showed that the cooked kernel length varied from 10.62 to 12.32 mm, which were almost similar with the present study. These literatures supported the present study. During cooking rice grains absorb water and increase in volume through increase in length or breadth alone or length and breadth both.

Breadth wise splitting is not desirable whereas length wise splitting (grain elongation) on cooking without increase in girth is considered trait in high quality premium rice such as basmati, which elongate almost 100 per cent on cooking (Khush et al., 1986; Sidhu, 1989). The utmost kernel breadth of cooked rice (3.12 mm) was recorded from S_2 and the shortest (2.19 mm) from BRRI dhan29. The highest ratio of kernel L/B (5.21) was found in 42(ii) and the lowest ratio (3.05) was in BRRI dhan29. Sandeep (2003) reported that kernel L/B ratio of 20 new genotypes ranged from 2.04 to 3.95 after cooking. Considering this review, the studied genotypes were more superior. The highest kernel elongation ratio (1.72) was recorded in S5 and the lowest ratio (1.14) was in BRRI dhan29. Kernel elongation was primarily influenced by the kernel shape and size. The highest water absorption (294%) was recorded in line 44(i). The lowest water absorption (210%) was found in BRRI dhan29 which was statistically identical to S1 and S2 (Table 4). Water uptake is considered an important economic attribute of rice as it gives indirect measure of volume increase on cooking. Zaman (1981) reported that the good cooking rice varieties have water absorption value ranging between 174 and 275%. This result partially supported the present finding. He also reported that the majority of those showing pasty appearance have value as high as from 300 to 570%. The high water absorption is relatively less

desirable characteristics and it would be desirable to select a variety or hybrid with moderate water absorption. According to Zaman (1981) water absorption rate of line S2 and S1 (214%) were moderate. The maximum volume expansion (4.41%) was found in S5 and the minimum (4.00%) was recorded in line 44(i). Volume expansion of kernels on cooking is considered another important measure of consumer preference. More volume of cooked rice from a given quantity is a matter of great satisfaction to an average rice consumer irrespective of the fact whether the increased volume is due to length-wise or breadth-wise expansion. Volume expansion by and large is determined by water uptake, however, subject to the influence of kernel texture (Zaman, 1981). The varieties which tend to show high volume expansion are sticky and give a pasty appearance on cooking. Invariably all the pasty cooking types have been found to be associated with higher water absorption. The pasty cooking closely related to high water absorption. Therefore, hybrids with low water absorption and high volume expansion are more desirable. Statistically significant variation was also recorded for alkaline spreading value for different advanced line of basmati rice (Table 4). The highest alkaline spreading value (7.00) was found in S2 and the lowest (3.83) was recorded in BRRI dhan29. Alkali spreading value is inversely related to gelatinization temperature (GT). GT is the range of temperature within which granules begin to swell irreversibly in hot water. Rice varieties having low GT start to swell

Table 4
Mean performance of quality characteristics after cooking in different lines and check

Lines/check	Cooked rice			Kernel el. ratio	Water uptake (%)	Volume expansion (%)	Alkali spreading value	Cooking time (minute)
	Length (mm)	Breadth (mm)	L/B ratio					
S1	11.95 c	2.96 a	4.04 b	1.64 a	214 c	4.30 a	6.01 b	17 b
S2	13.36 a	3.12 a	4.28 b	1.66 a	214 c	4.10 b	7.00 a	16 b
S5	13.38 a	2.97 a	4.50 b	1.72 a	254 b	4.41 a	4.25 d	17 b
42 (i)	12.67 b	2.44 b	5.21 a	1.67 a	250 b	4.10 b	4.59 cd	18 ab
42(ii)	10.15 d	2.39 b	4.25 b	1.39 b	260 b	4.29 a	4.25 d	17 b
44 (i)	11.80 c	2.75 ab	4.32 b	1.63 a	294 a	4.00 b	4.25 d	17 b
BRRI dhan29	6.68 e	2.19 a	3.05 c	1.14 c	210 c	4.30 a	3.83c	20 a
Range	6.68-13.36	2.19-3.12	3.05-5.21	1.14-1.72	210-294	4.00-4.41	3.83-7.00	16-20
Mean	11.57	2.792	4.18	1.55	242.27	4.214	5.026	17.429
CV (%)	5.70	7.21	5.74	8.60	7.49	6.10	5.38	6.51

In a column, means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

at low temperature during cooking than the rice varieties having intermediate or high GT (Nagato & Kishi, 1966). Rice varieties having intermediate GT produces good quality cooked rice. The lowest cooking time (16 minutes) was found in line S2 and the highest (20 minutes) was recorded from BRRI dhan29. In order to find out the elongation ratio, the linear kernel elongation after cooking is compared to the original length of kernel before cooking (Irshad, 2001).

The nature and extent of association between various characters pairs relating to quality attributes are presented in a sample linear correlation analysis (Table 5). Correlation study reveals that in line hulling per cent had highly significant positive relationship with milling per cent, HRR per cent and with kernel breadth of milled rice. However, a significant negative relationship was observed with the L/B ratio of rough rice. Viraktamath (1987) found a similar correlation among these traits, which supports the finding of the present study. Milling per cent showed highly significant positive relationship with HRR per cent and Kernel breadth of milled rice. HRR per cent showed highly significant positive correlation with kernel breadth of milled rice and insignificant negative association with L/B ratio of rough rice, milled rice and cooked rice. Yadav and Singh (1989) also found HRR was negatively associated with the length/breadth ratio of rough rice, which supports the present study. Kernel length of rough rice exhibited highly significant positive relationship with the kernel length of brown rice, milled rice and cooked rice and the L/B ratio of rough rice, brown rice, milled rice and cooked rice. In addition, it also showed significant positive relationships with kernel breadth of rough rice and brown rice but with a significant negative relationship with elongation ratio. The kernel breadth of rough rice showed highly significant positive linear relationships with length and L/B ratio of brown rice, milled rice and cooked rice. The kernel L/B ratio of rough rice exhibited a significant positive relationship with the length and L/B ratio of brown rice, milled rice and cooked rice, although it showed a significant negative relationship with elongation ratio. The kernel length of brown rice exhibited highly significant positive relationships with the kernel length of milled rice and cooked rice and L/B ratio of brown rice, milled rice and cooked rice, although it showed a significant negative relationship with elongation ratio. The L/B of brown rice exhibited highly significant positive relationships with the length of milled rice, cooked rice and L/B of milled rice; it also showed significant positive relationship with the L/B ratio of cooked rice. The kernel length of milled rice exhibited highly significant positive relationships with the kernel length of cooked rice, L/B ratio of milled rice and cooked rice. The L/B ratio of milled rice exhibited highly significant positive relationships with kernel length and L/B ratio of cooked rice, but it showed a significant negative relationship with elongation ratio. The kernel length of cooked rice exhibited highly significant

Table 5
Genotypic and phenotypic correlation of various quality characters

		M	HRR	KLRR	KBRR	l/b RR	KLBR	KBBR	l/b BR	KLMR	KBMR	l/b MR	KLCR	KBCR	l/b CR	WU	VE	ER	ASV
H	r_g	0.978**	0.845**	-0.345	0.345	-0.777*	-0.183	0.005	-0.215	-0.123	0.768*	-0.497	-0.21	0.249	-0.327	-0.273	-0.361	0.259	0.344
	r_p	0.921**	0.487	-0.293	0.33	-0.671*	-0.156	-0.01	-0.176	-0.118	0.562	-0.402	-0.193	0.166	-0.282	-0.194	-0.185	0.212	0.269
M	r_g		0.874**	-0.162	0.528	-0.668*	0.011	0.098	-0.015	0.070	0.837**	-0.323	-0.021	0.324	-0.199	-0.26	-0.351	0.119	0.428
	r_p		0.493	-0.136	0.499	-0.604*	0.01	-0.02	0.007	0.069	0.662*	-0.252	-0.011	0.223	-0.161	-0.243	-0.21	0.162	0.341
HRR	r_g			-0.087	0.466	-0.503	0.113	0.108	0.097	0.133	0.876**	-0.275	-0.087	0.119	-0.157	-0.347	-0.162	-0.006	0.478
	r_p			-0.077	0.331	-0.39	0.106	0.102	0.082	0.110	0.742*	-0.253	-0.07	0.093	-0.121	-0.152	-0.238	0.007	0.407
KLRR	r_g				0.731*	0.822**	0.976**	0.689*	0.966**	0.965**	0.160	0.956**	0.951**	0.024	0.862**	0.247	-0.207	-0.821**	0.311
	r_p				0.666*	0.773*	0.968**	0.469	0.939**	0.930**	0.150	0.908**	0.929**	0.026	0.817**	0.23	-0.206	-0.648*	0.31
KBRR	r_g					0.212	0.83**	0.553	0.82**	0.860**	0.657*	0.608*	0.818**	0.375	0.54	0.01	-0.301	-0.583	0.543
	r_p					0.043	0.744*	0.528	0.679*	0.742*	0.563	0.506	0.735**	0.373	0.439	0.055	-0.292	-0.312	0.467
l/b RR	r_g						0.706*	0.522	0.700*	0.663*	-0.315	0.859**	0.674*	-0.274	0.777*	0.337	-0.051	-0.702*	0.008
	r_p						0.663*	0.181	0.679*	0.610*	-0.284	0.786*	0.614*	-0.285	0.717*	0.261	-0.035	-0.603*	0.024
KLBR	r_g							0.684*	0.992**	0.994**	0.329	0.906**	0.933**	0.064	0.819**	0.184	-0.172	-0.792*	0.373
	r_p							0.451	0.976**	0.977**	0.306	0.880**	0.919**	0.056	0.784*	0.169	-0.156	-0.617*	0.365
KBBR	r_g								0.584	0.732*	0.246	0.665*	0.638*	-0.477	0.872**	0.649*	-0.660*	-0.537	-0.07
	r_p								0.249	0.419	0.076	0.401	0.434	-0.025	0.476	0.406	-0.404	-0.353	-0.038
l/b BR	r_g									0.975**	0.312	0.895**	0.926**	0.152	0.759*	0.096	-0.073	-0.789*	0.422
	r_p									0.958**	0.303	0.861**	0.892**	0.075	0.731*	0.084	-0.061	-0.589	0.405
KLMR	r_g										0.365	0.896**	0.946**	0.047	0.844**	0.233	-0.229	-0.752*	0.335
	r_p										0.342	0.885**	0.922**	0.028	0.799**	0.173	-0.18	-0.577	0.317
KBMR	r_g											-0.086	0.23	0.163	0.136	-0.42	-0.218	-0.038	0.567
	r_p											-0.134	0.184	0.011	0.164	-0.313	-0.277	0.080	0.527
l/b MR	r_g												0.902**	-0.024	0.837**	0.449	-0.141	-0.786*	0.089
	r_p												0.882**	0.022	0.762*	0.339	-0.056	-0.645*	0.073
KLCR	r_g													0.198	0.818**	0.211	-0.241	-0.702*	0.325
	r_p													0.196	0.784*	0.167	-0.208	-0.526	0.318
KBCR	r_g														-0.399	-0.537	0.166	-0.153	0.630*
	r_p														-0.443	-0.44	0.189	-0.276	0.527
l/b CR	r_g															0.491	-0.342	-0.541	-0.058
	r_p															0.425	-0.329	-0.285	-0.047
WU	r_g																-0.373	-0.169	-0.685*
	r_p																-0.408	-0.021	-0.625*
VE	r_g																	0.363	-0.18
	r_p																	0.128	-0.172
ER	r_g																		-0.517
	r_p																		-0.409

H=Hulling %, M=Milling outturn%, HRR=Head rice recovery %, KLRR=Kernel length of rough rice, KBRR=Kernel breadth of rough rice, l/b RR=l/b ratio of rough rice, KLBR=Kernel length of brown rice, KBBR=Kernel breadth of brown rice, l/b BR=l/b ratio of brown rice, KLMR=Kernel length of milled rice, KBMR=Kernel breadth of milled rice, l/b MR=l/b ratio of milled rice, KLCR=Kernel length of cooked rice, KBCR=Kernel breadth of cooked rice, l/b CR=l/b ratio of cooked rice, WU=Water uptake %, VE=Volume expansion %, ER=Elongation ratio, ASV=Alkali Spreading

positive relationship with the L/B ratio of cooked rice but it showed a significant negative relationship with elongation ratio at genotypic level. Chauhan et al. (1995) pointed out a significant positive correlation between cooked kernel length and kernel elongation, which is contradictory with the present study. The kernel breadth of cooked rice showed a significant positive relationship with alkali spreading value at genotypic level. Water uptake % exhibited a significant negative relationship with alkali spreading value but an insignificant negative relationship with volume expansion per cent and elongation ratio. Sood and Siddiq (1996) reported that water uptake showed positive and significant influence on volume expansion, and this finding is contradictory with the present study.

CONCLUSION

Considering overall performance in relation to cooking and eating quality point of view line S2 performed better and it can be used for further breeding purpose. From Correlation coefficient study, it can be concluded that L/B ratio of rough rice have strong positive relationship with the L/B ratio of brown rice, milled rice and cooked rice. Therefore, for the development of fine rice variety length and breadth of rough rice should be considered.

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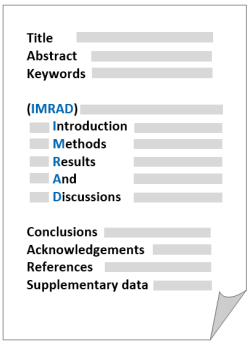

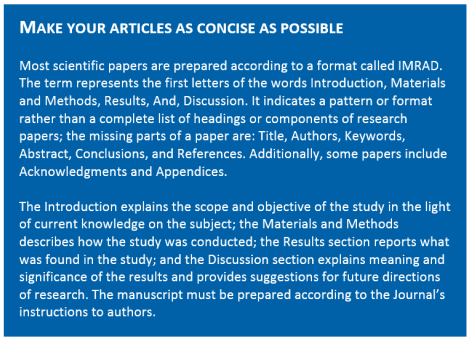

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Pertanika Editorial Office, Journal Division
Office of the Deputy Vice Chancellor (R&I)
1st Floor, IDEA Tower II
UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Malaysia

http://www.pertanika.upm.edu.my/
E-mail: executive_editor.pertanika@upm.my
Tel: +603 8947 1622 / 1619

PENERBIT UPM PRESS

http://penerbit.upm.edu.my
E-mail : penerbit@putra.upm.edu.my
Tel : +603 8946 8855 / 8854
Fax : +603 8941 6172

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