Pertanika Journal of

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JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science is the official journal of Universiti Putra Malaysia. It is an open-access online scientific journal. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognised 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.

Pertanika Journal of Tropical Agricultural Science 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 for submission by authors from all over the world.

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The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci*.

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and provides suggestions for future directions of research. The manuscript must be prepared according to the journal's **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_Authors.pdf).

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2. The Chief Executive Editor sends the article-identifying information having been removed, to 2 or 3 reviewers. They are specialists in the subject matter of the article. The Chief Executive Editor requests that they complete the review within 3 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 research field.

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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 authors may also submit a rebuttal if there is a need especially when the authors disagree with certain comments provided by reviewers.

5. The Chief Executive Editor sends the revised manuscript out for re-review. Typically, at least 1 of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the Editor-in-Chief examines their comments and decides whether the manuscript is ready to be published, needs another round of revisions, or should be rejected. If the decision is to accept, the Chief Executive Editor is notified.

7. The Chief Executive Editor reserves the final right to accept or reject any material for publication, if the processing of a particular manuscript is deemed not to be in compliance with the S.O.P. of *Pertanika.* An acceptance notification is sent to all the authors.

   The editorial office ensures that the manuscript 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 editorial office. 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 manuscript appears in the pages of the journal and is posted on-line.

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Contents

Foreword

Welcome to the Third Issue of 2020 for the Journal of Tropical Agricultural Science (JTAS)!

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

This issue contains 12 articles; all are regular articles. Articles submitted in this issue cover the scope of animal production; biotechnology; crop and pasture production; food and nutrition development; forestry sciences; horticulture; plant physiology; and soil and water sciences. The authors of these articles come from different countries namely Indonesia, Malaysia, Nigeria and Thailand.

A regular article entitled "Bioactivity Evaluation of *Melaleuca cajuputi* (Myrtales: Myrtaceae) Crude Extracts against *Aedes* Mosquito" discussed on the insecticidal properties of *Melaleuca cajuputi* crude extracts, which were in four different solvents *viz* dichloromethane, ethyl acetate, hexane, and methanol, against *Aedes aegypti* and *Aedes albopictus* mosquito. It concluded that the extract of *M. cajuputi* could potentially be the plant-based product in controlling dengue *Aedes* vectors, particularly in the adult mosquito. The detailed information of this article is presented on page 303.

Piyanan Nualhnuplong and Chaiyawan Wattanachant from Prince of Songkla University investigated on the effects of age at slaughter and sex on carcass characteristics and meat quality of Betong chickens. In order to control the quality of the meat, they found out that the males should be slaughtered at 20 weeks, while the females should be slaughtered when they reach the age of 24 weeks. Details of this study is available on page 343.

Nurul Najwa Mohamad and Nor Azizun Rusdi from Universiti Malaysia Sabah observed the morphological changes of flower initiation and early development by apical dissection and scanning electron microscopy (SEM). This study demonstrated ten stages of the early flower development pattern of *Renanthera bella*. The identification of the essential genes that may be involved and significant for the floral development process is needed. The further details of the study are found on page 377.

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

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

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of JTAS, who have made this issue possible.

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

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Studies on Genotype by Environment Interaction (GEI) and Stability Performances of 43 Accessions of Tropical Soybean (*Glycine max* (L.) Merrill)

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ABSTRACT

Soybean is the one most important oil-producing crop in Nigeria and the world. Genotype by environment interaction has been a major hindrance to effective selection and production. This study was conducted to determine the response of 43 soybean accessions to three environments to identify accessions that are adapted to the specific location and those that have wide adaptation. The 43 accessions were collected from the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria, and tested during the growing seasons of the years 2013, 2014, and 2015 in Ibadan. The data were analyzed using the additive main effects and multiplicative interaction (AMMI) and genotype main effect plus genotype-by-environment interaction (GGE) biplot methods. The AMMI analysis showed significant G x E interaction and identified accessions TGm-107, TGm-1200, and TGm-802 as the most desirable genotypes, whereas, TGm-868 and TGm-1209 were the least stable. The first two PC of the GGE analysis were able to capture 88.8% of the total variability due to G x E interaction. Accessions TGm-107, TGm-1200, and TGm-802 were the best performing and stable accessions due to their shortest projections in GGE biplot.

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Keywords: Adaptation, AMMI, environments, GGE biplot, soybean, stability

INTRODUCTION

Soybean is one of the leading oil crops in the world, which produces significantly higher protein per hectare when compared to many other crops. Nigeria ranks second among

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soybean-producing countries in sub-Saharan Africa. In 2014, Nigeria recorded production of 679,000 metric tons (Food and Agriculture Organization [FAO], 2016). It is cultivated by small- and large-scale farmers majorly for human consumption and livestock feed in various agro-ecological zones in Nigeria. Changes in climate can have a strong impact on agriculture, i.e. climatic conditions determine not only crop growth but also yield, so even little change of climatic conditions required for production can seriously reduce yield (Kang et al., 2009). Therefore, it is important to understand the effect of environmental factors on crop growth and development. This knowledge would reduce the G × E interactions and improve the selection of genotypes for specific and wide adaptations in the target environments. The genotypic performance of soybean germplasm in many environments and seasons can assess the stability and adaptations of genotypes (Gedif et al., 2014). Interaction between genotype and environment interaction (GEI) complicates evaluations/trials, selection, and release and recommendation decisions of superior and improved genotypes, and consequently, reduces genetic progress from the selection because breeders need to identify different genotypes from the evaluation (Rincent et al., 2017; Tariku, 2017). As a result, GEI alters the genotype rankings from one environment to the other, and genotypes selected from one environment may not do well in another environment. Hence, there is a need to conduct trials over a wide range of environments to ascertain the selection of superior and stable genotypes. To this end, breeders usually conduct multi-environmental trials (MET) to identify high yielding and stable genotypes.

Many statistical models have been employed to detect and quantify the GEI. Currently, additive main effects and multiplicative interaction (AMMI) analysis models developed by Gauch (1992) and Zobel et al. (1988); and genotype main effect plus genotype-by-environment interaction (GGE) biplot developed by Yan and Kang (2003) and Yan and Rajcan (2002) are the most frequently used statistical models. However, before the advent of the two models mentioned above, breeders also used principal component analysis (PCA) developed by Hill and Godchild (1981), joint regression analysis developed by Eberhart and Russel (1966) as well as Finlay and Wilkinson (1963), and ANOVA developed by Snedecor and Cochran (1980).

Several studies reported on stability studies that focused on soybean. Cucolotto et al. (2007) found four cultivars out of thirty that combined good adaptation and stability, while Gurmu et al. (2009) reported that high yielding cultivars were more likely to have lower stability and *vice versa*. Jandong et al. (2011) examined seven genotypes grown in six different soil pH regimes for adaptability and stability and observed specific adaptation, implying that each genotype had specific soil requirements. Therefore, the main objective of the study was to evaluate Genotype × Environment Interaction (GEI) and the level of yield stability of the 43 accessions of soybean.

MATERIALS AND METHODS

Forty-three (43) soybean accessions collected from the Genetic Resources Center, International Institute for Tropical Agriculture (IITA) (Table 1), Nigeria were evaluated during the three years of 2013, 2014, and 2015. The trials were laid out in the research farms of the Department of Seed GenBank Unit, National Center for Genetic Resources and Biotechnology (NACGRAB), Ibadan (7.23 ′47″N 3.55 ′0″ E) in 2013 and 2014; and International Institute of Agriculture (IIA) (8.0'N 4.0'E), Ibadan, Nigeria in 2015, respectively. NACGRAB is situated at moor plantation, Apata along Abeokuta Ogun State, Nigeria while IITA is situated at Moniya along Oyo town in Oyo State, Nigeria. The meteorological data of the three years are shown as appendix I, II, and III. The 43 accessions were planted in single-row plots with 60 cm between-row and 5 cm within-row spacing, with three replications using a 1-m alley between blocks in a randomized complete block design. Data were collected on five yield characters: number of days to 50% flowering, number of days to maturity, number of pods per plant, 100 seed weight (gm), and seed yield per plant (g). They were analyzed using AMMI analysis, MATMODEL version 2.0 (Gauch & Zobel, 1996). In this analysis, each planting season was considered an environment. Thus, there were three environments in this study. The analysis was done to estimate the magnitude of the GE interaction.

The AMMI statistical model equation used was:

$$Y_{ger} = \mu + \alpha_g + \beta_e + \Sigma\lambda_n\, y_{gn}\, \delta_{en} + P_{ge} + \epsilon_{ger}$$

AMMI's Stability Value (ASV) was also estimated by using the formula of Purchase (1997):

$$ASV = \sqrt{\left[\frac{SS_{IPCA1}}{SS_{IPCA2}}\left(IPCA1_{score}\right)\right]^2 + \left(IPCA2_{score}\right)^2}$$

ASV = AMMI's stability value, SS = sum of squares, IPCA = interaction principal component axis.

Likewise, Yield Stability index (YSi) was also calculated by adding up the ranks obtained from ASV and mean yield according to Farshadfar et al. (2011):

$$YSi = RASVi + RGYi$$

where; RASVi = rank of AMMI stability value of the ith genotype and RYGi = rank of the mean of seed yield of the ith genotype. The collected data also underwent a GGE biplot analysis to view the GEI. This analysis was carried out according to Mandel's site regression model ($SREG_{m+1}$ biplot) for MET data (Yan et al., 2001). In this biplot, the genotype main effect is the primary effect. The secondary effect comes from the first principal component (PC1) that comes from applying singular value decomposition (SVD) of the environment-centered data to the residual (Mandel, 1961).

According to Mandel (1961), the following model was used for the analysis:

$$Y_{ij} - \beta_j = b_j\alpha_i + \lambda_1\eta_{j1} + \Sigma_{ij}$$

GGE biplots were used to compare and

contrast among the performances of different genotypes in an environment as well as a genotype in different environments. It identifies the highest yielding genotypes at the different mega-environments and identifies ideal genotypes and test locations.

Table 1
The accession names and origin of 43 genotypes of soybean

S/N	Accession	Origin
1	TGm-107	Nigeria
2	TGm-109	Nigeria
3	TGm-1106	Taiwan
4	TGm-1200	Burkina Faso
5	TGm-1209	Burkina Faso
6	TGm-1215	Nigeria
7	TGm-136	Nigeria
8	TGm-138	Uganda
9	TGm-14	Nigeria
10	TGm-142	Uganda
11	TGm-150	Uganda
12	TGm-27	Nigeria
13	TGm-553	Nigeria
14	TGm-569	Nigeria
15	TGm-570	Nigeria
16	TGm-574	Nigeria
17	TGm-577	Nigeria
18	TGm-579	Nigeria
19	TGm-584	Taiwan
20	TGm-658	Indonesia
21	TGm-669	Indonesia
22	TGm-682	Indonesia
23	TGm-686	Indonesia
24	TGm-802	Burkina Faso
25	TGm-861	Taiwan
26	TGm-863	Taiwan
27	TGm-864	Taiwan
28	TGm-865	Taiwan
29	TGm-866	Taiwan
30	TGm-867	Taiwan
31	TGm-868	Taiwan
32	TGm-869	Taiwan
33	TGm-93	Nigeria
34	TGm-94	Nigeria
35	TGm-947	Nigeria
36	TGm-948	Nigeria
37	TGm-95	Nigeria
38	TGm-96	Nigeria
39	TGm-961	Nigeria
40	TGm-97	Nigeria
41	TGm-98	Nigeria
42	TGm-99	Nigeria
43	TGm-946	Nigeria

RESULTS AND DISCUSSION

The AMMI analysis results are presented in Table 2. The treatments (accessions + environments + interactions) accounted for 81.23% of the total sums of squares using approximately 33.16% of the total degrees of freedom. The accessions captured 38.39% of the total sums of squares explained and 47.26% of the total sum of treatment explained, while the environments explained 8.1% of the total sums of squares and 10.0% of the treatment sums of squares. The interactions explained 34.73% of the total sums of squares and 42.75% of the sums of squares for treatment (Table 2). Therefore, the accessions accounted for more variation, followed by the interactions

Table 2
Analysis of Variance for AMMI model

Source	df	SS	MS	% interaction explained	F	% total SS explained	% total treatment explained
Treatments	128	30034	234.6		9.29**	81.23	
Accessions	42	14193	337.9		13.38**	38.39	47.26
Environments	2	3000	1499.8		15.61**	8.1	10
Block	6	576	96.1		3.80**		
Interactions	84	12841	152.9		6.05**	34.73	42.75
IPCA	43	9792	227.7	76.26	9.02**		
IPCA	41	3049	74.4	23.74	2.95**		
Residuals	0	0					
Error	252	6363	25.3				
Total	386	36973	95.8				

Note. *, ** significant at 5% and 1% levels, respectively.
df = degrees of freedom; SS = sum of squares; MS = mean squares

and the environment captured the least variation. These results suggest that the 43 accessions and the three environments used were significantly different from each other. The significant differences showed for genotype by environment interaction indicated that the 43 accessions responded to the 3 environments differently. Furthermore, the results revealed that the accession component had more influence on the performance of soybean accessions, indicating less environmental influence for the test years and also showed that the largest source of variation observed was mainly due to genetic component probably because the genotypes are evaluated in the same geographical locations through different years.

The seed yield, environment, year, and first IPCA scores are shown in Table 3. The range of genotype mean yields was between 12.32 g in TGm-14 and 39.15 g in TGm-868. The environment means ranged from 21.62 g in environment 1 to 27.67 g in environment 3. Genotype TGm-868 recorded the largest IPCA score of 3.01 while genotype TGm-107 recorded the lowest IPCA1 score of –0.05. However, the largest environmental IPCA1 score was observed in environment 3 (6.07), while the lowest was recorded for environment 2 (–2.09). Accessions with IPCA1 scores close to zero had less interaction across the environments. It follows that out of the 43 accessions considered, TGm-1200 = G4 (0.10), TGm-570 = G15 (–0.07), TGm-579 = G18 (0.31), TGm-686 = G23 (0.16), TGm-802 = G24 (–0.06), TGm-865 = G28 (0.42) and TGm-869 = G32 (0.40) had negligible interaction with the test environments. All

the remaining 36 accessions had high IPCA1 scores and were highly interactive with the environments.

The AMMI biplot for the 43 accessions of soybean is presented in Figure 1. In AMMI analysis, the IPCA scores of a genotype either positive or negative suggest its stability. The higher the IPCA score, the more adapted The IPCA scores of genotypes in the AMMI analysis indicate the stability of a genotype over environments. The greater the IPCA score of a genotype, either positive or negative, the more specifically adapted that genotype is to a specific environment. Also, the closer an IPCA score is to zero, the more stable the genotype is over all environments (Gauch & Zobel, 1996). Figure 1 indicates that G31 (TGm-868) gave the highest yield followed by G26 (TGm-863) and G1 (TGm-107). The lowest yielding among the 43 accessions was G9 (TGm-14) due to its placement on the top left corner in the biplot. Accessions G1 (TGm-107), G4 (TGm-1200), and G24 (TGm-802) were most stable and high yielding considering their IPCA score being the closest to zero and can be considered adaptable to all the environments.

On the other hand, G31 (TGm-868) was the least stable as it was the farthest from the IPCA1 score of zero, however, due to its high mean seed yield, it can be considered a responsive accession for a specific environment. The most undesirable accession was G9 (TGm-14) as it combined low yield with instability. Accession G1 (TGm-107) was considered the most desirable.

Table 3

Seed yield of forty-three (43) soybean accessions grown in three environments, mean values and the first PCA scores

Genotype	code	E1	E2	E3	GM (g)	IPCA 1
TGm-107	G1	34.51	35.44	40.30	36.75	–0.05
TGm-109	G2	14.83	17.33	34.40	22.19	1.36
TGm-1106	G3	25.14	19.00	36.13	26.76	0.76
TGm-1200	G4	21.13	32.67	31.97	28.59	0.10
TGm-1209	G5	29.59	18.67	6.90	18.39	–2.64
TGm-1215	G6	16.21	20.67	16.37	17.75	–0.78
TGm-136	G7	27.28	26.00	14.47	22.58	–1.95
TGm-138	G8	19.55	30.67	32.40	27.54	0.33
TGm-14	G9	17.17	14.63	5.17	12.32	–1.82
TGm-142	G10	18.37	24.33	21.57	21.42	–0.51
TGm-150	G11	24.14	16.00	6.50	15.55	–2.21
TGm-27	G12	22.48	20.92	29.67	24.36	0.19
TGm-553	G13	20.61	**35.67**	36.57	30.95	0.51

Table 3 *(Continued)*

Genotype	code	E1	E2	E3	GM (g)	IPCA 1
TGm-569	G14	21.75	14.60	11.10	15.82	–1.50
TGm-570	G15	15.63	13.14	20.07	16.28	–0.07
TGm-574	G16	24.43	22.36	32.20	26.33	0.27
TGm-577	G17	**38.09**	27.67	23.37	29.71	-1.82
TGm-579	G18	16.07	27.00	28.73	23.94	0.31
TGm-584	G19	20.76	18.97	37.47	25.73	1.21
TGm-658	G20	18.75	20.67	29.83	23.08	0.48
TGm-669	G21	17.78	24.07	31.30	24.38	0.57
TGm-682	G22	17.79	19.67	34.67	24.04	1.10
TGm-686	G23	23.73	21.38	30.40	25.17	0.16
TGm-802	G24	35.48	17.00	34.47	28.98	–0.06
TGm-861	G25	23.67	17.67	24.00	21.78	–0.38
TGm-863	G26	35.69	31.00	46.83	37.84	0.72
TGm-864	G27	15.99	15.67	31.13	20.93	0.99
TGm-865	G28	12.02	16.00	23.27	17.10	0.42
TGm-866	G29	22.83	14.33	31.00	22.72	0.55
TGm-867	G30	18.88	16.15	15.00	16.68	–0.94
TGm-868	G31	22.09	34.67	**60.70**	39.15	3.01
TGm-869	G32	20.24	15.67	28.37	21.42	0.40
TGm-93	G33	16.46	17.67	31.97	22.03	0.97
TGm-94	G34	15.48	24.00	41.33	26.94	1.80
TGm-946	G35	22.11	22.97	22.13	22.40	–0.66
TGm-947	G36	10.79	15.87	11.83	12.83	–0.71
TGm-948	G37	23.48	14.97	31.40	23.28	0.52
TGm-95	G38	18.25	21.33	30.27	23.28	0.53
TGm-96	G39	27.73	24.67	38.33	30.24	0.61
TGm-961	G40	25.48	34.67	15.30	25.15	–2.05
TGm-97	G41	13.27	22.97	10.53	15.59	–1.28
TGm-98	G42	16.14	18.01	32.33	22.16	1.02
TGm-99	G43	27.70	25.78	38.03		
Mean		21.62	21.92	27.67	30.51	0.54
PCA 1 score		–3.98	–2.09	6.07		

Note. E 1(NACGRAB) = 2013; E 2 (NACGRAB) = 2014; E 3 (IITA) = 2015; GM = grand mean; IPCA = interaction principal component axis

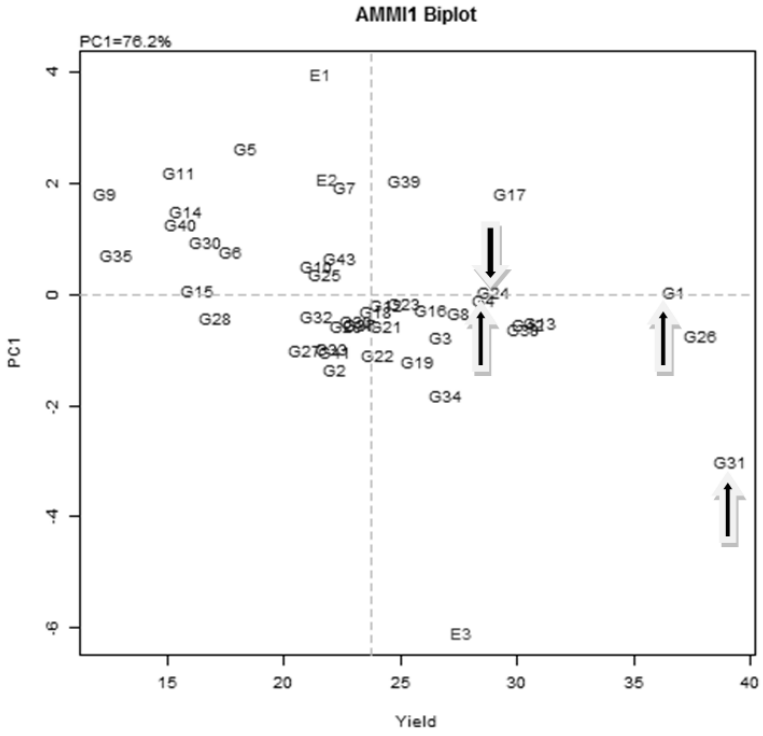


Figure 1. AMMI biplot of yield for 43 soybean accessions in three environments

Therefore, AMMI revealed that TGm-107 (G1), TGm-1200 (G4), TGm-802 (G24), TGm-138 (G8), TGm-686 (G23), TGm-553 (G13), TGm-869 (G32), and TGm-574 (G16) were the most desirable as they combine stability with high yield. This made them the most suitable variety for cultivation across seasons. However, accessions TGm-868 (G31), TGm-1209 (G5), and TGm-150 (G11) had high IPCA values indicating that they were responsive to changes in environments, a sign of high interaction with environments. The accessions that had more interaction with environments were found to be unpredictable in performance (unstable) and hence could be recommended for specific adaptation. Mohammadi et al. (2009) described a genotype exhibiting dynamic stability as one that responded to improved conditions and management practices with increased yield. Therefore, it would not be logical to recommend it for growing across environments. However, it would be better to recommend it for production in optimum growing conditions or environments. The differences among the test environments could be explained by climatic conditions, season length, and seasonal effects. Environment 1 (2013) was the least in terms of yield while environment 2 was the best in terms of stability in this study and therefore had little interaction effect with the 43 accessions studied. These results were consistent with

numerous studies (Gurmu et al., 2009; Rao et al., 2002; Yothasiri & Somwang, 2000). Accession TGm-868 (G31) was identified to be the highest yielding and most unstable accession and therefore not reliable while TGm-107 (G1) was the best candidate in terms of stability and yield. These results were in agreement with the reports of Mut et al. (2009). Studies have shown that seed yield is heritable and conditioned by additive gene action (Spehar, 1999). Thus, simple selection methods could be applied to advance yield stability and plasticity for cultivation over a wide range of environments. These results suggested that seed yield could be maximized through selecting accessions showing consistently high yield performance across heterogeneous growing environments.

The AMMI stability value (ASV) and yield stability index (YSi) are presented in Table 4. The genotypes with a larger ASV score, either positive or negative will be better adapted to a specific environment while those with a smaller ASV score indicate a more stable genotype across environments. Accordingly, TGm-107 with the lowest ASV (0.020 followed by TGm-570 (0.08) and TGm-686 (0.20) were the most stable accessions, whereas, TGm-868 (47.10) followed by TGm-1209 (36.27) and TGm-150 (25.35) were identified as more adapted and sensitive to environmental changes. Yield stability Index (YSi) (Farshadfar et al, 2011) measures stability and can be calculated by summing of genotype rank of mean seed yield across environments and rank of AMMI stability value of genotypes. The genotypes with the lowest value are desirable genotypes with high mean grain yield and stability. Hence, YSi identified TGm 107 and TGm 1200 as the most desirable accessions among all the 43 accessions of soybean.

Table 4

Ranking of 43 accessions of soya bean by AMMI stability value (ASV) and yield stability index (YSi)

Accession	Code	MY	RANK	ASV	RANK	YSi
TGm-107	G1	36.75	3	0.02	1	4
TGm-109	G2	22.19	27	9.54	33	60
TGm-1107	G3	26.76	12	3.48	26	38
TGm-1200	G4	28.59	9	1.05	8	17
TGm-1209	G5	18.39	35	36.27	42	77
TGm-1215	G6	17.75	34	3.40	25	59
TGm-136	G7	22.58	25	19.66	39	64
TGm-138	G8	27.54	10	1.41	12	22
TGm-14	G9	12.32	43	17.09	37	80
TGm-142	G10	21.42	32	1.69	15	47
TGm-150	G11	15.55	41	25.35	41	82

Table 4 *(Continued)*

Accession	Code	MY	RANK	ASV	RANK	YSi
TGm-27	G12	24.36	18	0.23	4	22
TGm-553	G13	30.95	4	2.87	22	26
TGm-569	G14	15.82	39	11.78	35	74
TGm-570	G15	16.28	38	0.08	2	40
TGm-574	G16	26.33	13	0.44	5	18
TGm-577	G17	29.71	7	17.52	38	45
TGm-579	G18	23.94	20	1.32	11	31
TGm-584	G19	25.73	14	7.71	32	46
TGm-658	G20	23.08	23	1.19	10	33
TGm-669	G21	24.38	17	1.87	16	33
TGm-682	G22	24.04	19	6.32	31	50
TGm-686	G23	25.17	15	0.20	3	18
TGm-802	G24	28.98	8	2.83	21	29
TGm-861	G25	21.78	30	1.00	7	37
TGm-863	G26	37.84	2	3.00	24	26
TGm-864	G27	20.93	33	5.11	29	62
TGm-865	G28	17.10	36	0.98	6	42
TGm-866	G29	22.72	24	2.34	20	44
TGm-867	G30	16.68	37	4.57	27	64
TGm-868	G31	39.15	1	47.10	43	44
TGm-869	G32	21.42	31	1.08	9	40
TGm-93	G33	22.03	29	4.86	28	57
TGm-94	G34	26.94	11	16.90	36	47
TGm-946	G35	22.40	26	2.27	19	45
TGm-947	G36	12.83	42	2.90	23	65
TGm-948	G37	23.28	21	2.18	18	39
TGm-95	G38	23.28	22	1.48	13	35
TGm-96	G39	30.24	6	2.08	17	23
TGm-961	G40	25.15	16	22.99	40	56

Note. ASV = AMMI stability value; YSi = yield stability index; MY = mean yield

The GGE biplot was also constructed for the 43 accessions. One of the important characteristics of a GGE biplot is its ability to reveal top-performing genotypes in a specific environment and it can also display low yielding genotypes across environments. Figure 2 illustrates the association of the 43 accessions of soybean within the three test environments. Five sectors were displayed in the biplot, which were generated by the perpendicular line that originated from the center of the biplot and runs perpendicular to the side of the polygon. Among the five sectors displayed, two had environments included within them. Accession(s) that fall in sectors where the environment(s) are included indicate the association of the accession(s) with that specific environment(s). The accession at the various vertices of the polygon is expected to be responding well as they are the furthest from the origin. However, the responsive vertex accession is the best performing accession at the specific environments where it is found (Rakshit et al., 2012; Yan & Rajcan, 2002). Accession G17 (TGm-577) was the most suitable accession at E1 (2013) whereas accessions G26 (TGm-863), G1 (TGm-107), G31 (TGm-868), and G34 (TGm-94) were found to perform well in E2 (2014) and E3 (2015). However, G31 was the best performer and most suitable in E2 and E3.

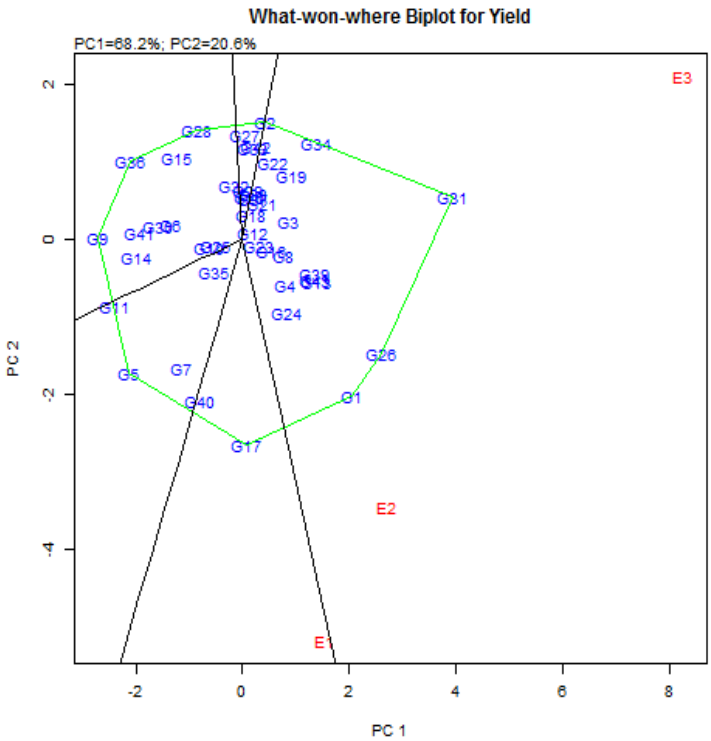


Figure 2. Which-won-where polygon view of the GGE biplot analysis

Figure 3 shows the biplot of stability and mean performance of the 43 accessions evaluated under three environments. The small circle indicates an average environment, which is defined by the mean IPC1 and IPC2 scores of the environments and the line that passes through the biplot and the average environment may be called the average axis (the ordinate). Projections of accession markers onto this axis approximate the mean yield of the accession. Thus, the accessions were ranked along the ordinate, with the arrow pointing to higher mean performance. Accession G31 was the highest yielding accession followed by G26. The abscissa is the double-arrowed line that passes through the biplot origin and is perpendicular to the ordinate (orthogonal). The double-arrowed line illustrates that a longer projection onto the abscissa, regardless of the direction, indicated greater instability. Given this, accessions G31 (TGm-868), G17 (TGm-577), and G34 (TGm-94) had the longest projections and were therefore the most variable across environments and less stable than others.

In contrast, accessions G4 (TGm-1200), G24 (TGm-802), and G1 (TGm-107) with shortest projections were relatively most stable over the three environments. Figure 4 shows the representativeness and

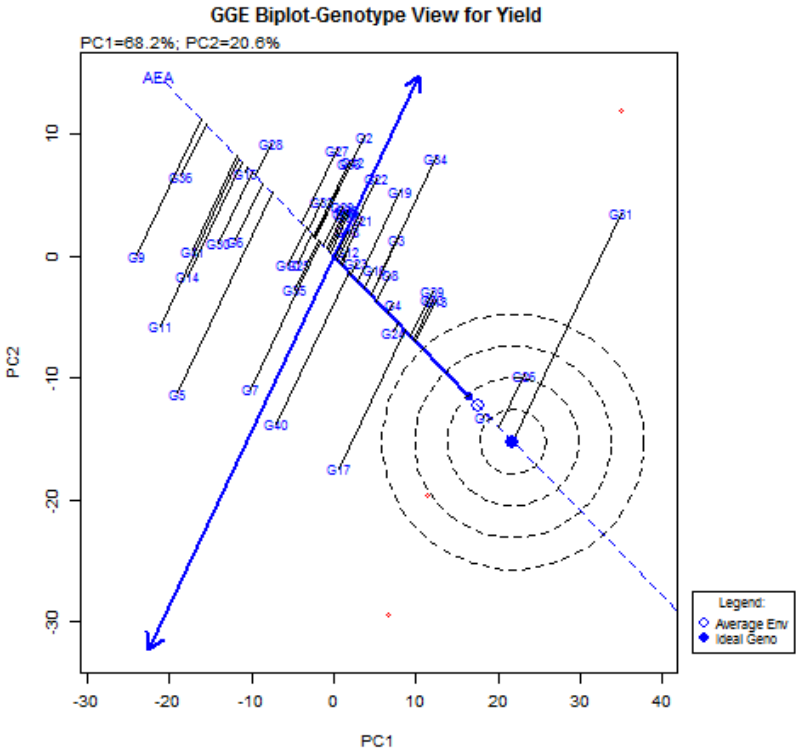
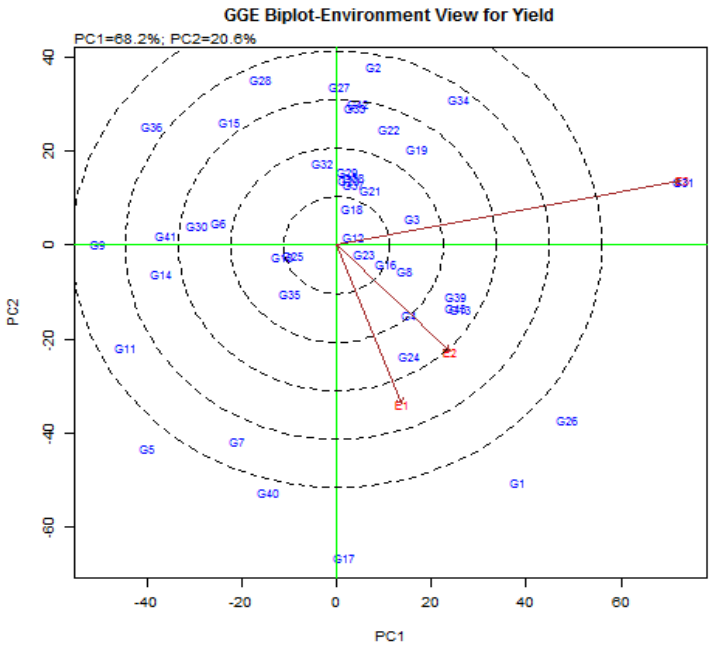


Figure 3. The mean performance and stability of the 43 genotypes of soybean across the three test environments

discriminating ability of the environments. The biplot explains 88.80% of the total variation. In a biplot analysis the vector length of an environment indicates its discriminating power; the longer the vector from the plot origin, the more discriminatory the environment. The longer the projection, the less representative the environment. Thus, E3 (2015) was the most discriminating environment due to its longest distance from the origin of the biplot while E2 (2014) was the least discriminating. Environments with small vector angles tend to have closer similarity and those with wide vector angles show a minimum association. Environments E1 and E2 were displayed close to each other as the association between them was small. However, the wider angle between E3 and E2; as well as E3 and E1 environments indicated the absence of association among them.

Similarly, accessions projected further from the ATC y-axis are considered less stable. The center of the concentric circle in a biplot is where an ideal accession should be. An ideal accession is considered as one with the highest yield and stable performance across test environments. Hence, the shorter the distance of accession to the ideal/virtual accession, the more suitable the accession (Yan & Kang, 2003). GGE also picked G31 (TGm-868) as the highest yielding in the E1 and E2 environments. The accessions that combined high yield with stability included G1 (TGm-107), G4 (TGm-1200), and G24 (TGm-802) because of their short projection on the genotype marker lines.



E = Environment, G = Genotype

Figure 4. Discriminating ability versus representativeness of the test environments

CONCLUSION

AMMI and GGE biplot revealed that accessions G1 (TGm-107), G4 (TGm-1200), and G24 (TGm-802) were the best and stable accessions across environments. This made them the most suitable variety for cultivation across the years. Among the environments, E3 (2015) was found to be the most discriminating, and E2 (2014) was found to be the most representative environment. Both AMMI and GGE agreed on the grouping of environment and the ideal test environment, as well on winner genotypes in this study, although, GGE biplot is believed to be superior to AMMI because it eliminates the environmental components in the analysis.

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APPENDIX

Appendix I

Monthly meteorological data for the year 2013 at NACGRAB, Ibadan

Month	Rainfall (mm)	No. of rain day	Temperature (°C)	Humidity (%)
January	0.0	NIL	27	70
Febuary	2.1	1	29	66
March	14.2	3	29	71
April	120.1	5	29	78
May	183.4	10	25.6	78
June	223.1	10	25.0	78
July	161.7	11	24.0	87
August	151.5	9	26	87
September	232.8	12	25	81
October	248.5	16	26.0	87
November	11.9	3	27.5	85
December	0.0	NIL	26.0	78

Appendix II

Monthly meteorological data for the year 2014 at NACGRAB, Ibadan

Month	Rainfall (mm)	No. of rain day	Temperature (°C)	Humidity (%)
January	15.3	2	28.0	60
Febuary	0.0	Nil	25.0	79
March	127.3	7	28.5	78
April	261.1	6	24.9	85
May	121.1	9	23.8	86
June	185.6	13	26.2	88
July	243	14	23.5	88
August	101	10	24.2	84
September	206.4	13	24.7	88
October	211.6	14	25.9	87
November	22.0	3	27.5	87
December	3.0	2	26.8	80

Appendix III

Monthly meteorological data for the year 2015 at IITA, Moniya

Month	Rainfall (mm)	No. of rain day	Temperature (°C)	Humidity (%)
January	12.9	2	30	85
Febuary	23.2	3	27.5	83
March	90.3	6	27.4	83
April	115.6	7	28.1	83
May	117	10	26	84
June	85.3	7	25.2	85
July	462	19	26.6	86
August	154.8	5	24.6	87
September	345.9	18	24.3	88
October	324.1	18	25.8	87
November	43.4	3	27.4	82
December	0.0	NIL	26.1	83

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Analysis of Qualitative and Quantitative Trait Variability among Black Pepper (*Piper nigrum* L.) Cultivars in Malaysia

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ABSTRACT

This project comprehensively documented the morphological characteristics of ten black pepper cultivars in Malaysia, focusing on diagnosing the morphological difference among the cultivars via qualitative traits. These cultivars are cv. 'Semongok Aman', cv. 'Kuching', cv. 'Semongok Emas', cv. 'Semongok Perak', cv. 'Semongok 1', cv. 'Nyerigai', cv. 'India', cv. 'Lampung Daun Lebar', cv. 'Sarikei', and cv. 'Yong Petai'. The morphological characteristics had been evaluated via field-grown vine where the randomized complete block design (RCBD) was adopted and potted vine evaluation via completely randomized design (CRD). Cv. 'Semongok 1' showed ovate shaped leaf and anthocyanin free shoot tip; cv. 'Semongok Aman' had rounded shape of leaf apex and base; cv. 'Lampung Daun Lebar' had an oblique shape in leaf base and 'Nyerigai' showed erect type branching; cv. 'Semongok Emas' had leaf colour of Green group 137 series (RHS code) and fruit colour of Green group 141 series (RHS); cv. 'India' had a lanceolate shaped leaf. At the same time, this study also revealed the key differences in quantitative traits that included leaf area, length-width ratio, inflorescent length, fruit spike length, and fresh to dried berry conversion rate. The study showed that cv. 'India' had a low length-width ratio (Lw-1) at 1.52 and lightest seed weight at 4.07(x 10-2) g; cv. 'Sarikei' had the smallest leaf area (36.90 cm²), shortest inflorescence (6.06 cm), shortest fruit spike (8.07 cm), smallest fruit diameter (5.78 cm), smallest seed diameter (3.84 cm), and thinnest pericarp (1.73 cm); cv. 'Kuching' had the greatest number of inflorescence per branch per node

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(ca.58.67) and the greatest number of node/ feet of the stem (ca.4.73); cv. 'Yong Petai' had the longest inflorescence (12.75 cm), longest fruit spike (17.07 cm), but thinnest fruit spike (2.90 mm); and, lastly, cv. 'Semongok Perak' had the conversion rate (from fresh to dried black) (36.12 %) and conversion rate (from fresh to dried white) (24.21 %). The comprehensive evaluation of both qualitative and quantitative traits of all the black pepper cultivars has ensured the efficiency of cultivar identification.

Keywords: Black pepper cultivar, qualitative and quantitative traits

INTRODUCTION

Black pepper, scientifically called *Piper nigrum* L., is known as the 'King of Spice' and is the most commonly used spice in the world. The plant is woody perennial climber required support, living or non-living to promote normal growth; leaves alternate and petiolate type, with shape commonly elliptical, lanceolate or ovate; inflorescence of catkin types and the flower is minute, bracteates, bisexual or unisexual and protogynous; the fruit of drupe type with thin pericarp and seed spherical shaped with diameter 3-5 mm; nodal stem with internode ranged from 8-13 cm when mature; shoot tip purplish-green or whitish green (Chen, 2011; Ravindran et al., 2000). In India, the morphological analyses of black pepper cultivars were comprehensively studied by Ravindran et al. (1997) through morphometric analysis. Whilst in Malaysia, Chen et al. (2018) had comprehensive analysis on the morphology of ten important cultivars while Noorasmah et al. (2018) had also recorded the inflorescence characteristics of some important pepper variety.

The plant was introduced to Malaysia as early as 1856 (Dalton, 1912), with cultivation focus in the state of Sarawak. However, the diversity of the black pepper cultivar in Malaysia is unidentified because varietal control is not practised. The most common black pepper cultivars are cv. 'Kuching' and cv. 'Sarikei', both widely planted throughout Sarawak (Sim, 1993), while Paulus (2007) reported three important cultivars in his publication, i.e. cv. 'Semongok Perak', cv. 'Semongok Aman', and cv. 'Kuching'. Through the International Pepper Community (IPC) exchange program, cv. 'Lampung Daun Lebar' and cv. 'Lampung Daun Kecil' was introduced to Malaysian farmers (Sim, 1993). A manual entitled 'Pepper production technology in Malaysia' was recently released by the Malaysian Pepper Board, mentioning the existence of seven cultivated varieties as common cultivars in Malaysia, including cv. 'Semongok Aman', cv. 'Semongok Emas', cv. 'Kuching', cv. 'Semongok Perak', cv. 'Uthirancotta', cv. 'Nyerigai', and cv. 'PN129' (Paulus, 2011). A total of 47 accessions of black pepper varieties and 46 accessions of wild *Piper* were reported, conserved in form of a living plant in the Agricultural Research Centre (ARC) in Sarawak, Malaysia, from 1957 until 1992 (Sim, 1993).

Black Pepper Test Guideline for Plant Variety Protection Act implementation has been established by the Department of Agriculture Malaysia (2009). This guideline listed the entire important characteristic for the diagnosis of black pepper variety. However, the existing documentation on cultivated black pepper in Malaysia is less comprehensive, and none of the cultivars is registered under the National Crop List of Malaysia. The importance of this study is to comprehensively document the morphological characteristics of all the important black pepper cultivars in Malaysia, focusing on the diagnosis of the distinctness among the cultivars. The Malaysian government strategized a new policy to ensure the sustainability of the industry by strengthening the quality of peppercorn. A mono-varietal farm concept is believed able to strengthen the quality of peppercorn. This can be achieved through varietal control, and a pre-requisite to this policy is comprehensive documentation on all important cultivars in the country.

MATERIALS AND METHODS

Extensive fieldwork has been undertaken by the first author since January 2014, to cover all the possible black pepper cultivation areas throughout Malaysia, to verify the diversity of black pepper cultivars. The black pepper farm distribution info was sourced from the Department of Crop, Extension, and Farmer's Development, from the Malaysian Pepper Board. Photography data particularly on the leaf, inflorescence, fruit spike, and shoot tip were comprehensively generated for the preliminary cultivar's diagnosis. The preliminary diagnosis must show at least one distinct character to be eligible for further cultivars verification study. The pepper germplasm centre situated at Agricultural Research Centre Semongok (ARC) Sarawak, Malaysia was referred for verification on the cultivar designation.

To develop a comprehensive guide for cultivar identification, a thorough assessment of morphological characteristics (qualitative or quantitative traits) had been conducted. Both potted peppers and field-grown vines were assessed in this study. Vine growing morphology or vigour was assessed on field-grown mature vines at the three field experimental plots while leaf, inflorescence, fruit, and seed morphology studies were based on samples collected from potted mature vines grown under a controlled environment. Data collection was carried out from January 2016 to December 2017. Microscopy assessment and data analysis were performed at the Malaysian Pepper Board, Kuching.

The field experiments were conducted at three locations, namely Kampung Jagoi, Serikin; Kampung Karu, Padawan and Kampung Belawan, Sri Aman. The plots were laid out in a Randomized Complete Block Design (RCBD) (Figure 1) having ten treatment with 5 replications, which are T1: 'Semongok Aman' vine; T2: 'Kuching' vine, T3: 'Semongok Emas' vine; T4: 'Semongok Perak' vine; T5: 'Semongok 1' vine; T6: 'Nyerigai' vine; T7: 'India' vine; T8: 'Lampung Daun Lebar' vine; T9: 'Sarikei' vine and T10: 'Yong Petai'

vine. Each trial plot at a different location containing ten treatments consists of 50 vines. Whilst, a pot experiment was conducted under a controlled environment at the Agriculture Research Center (ARC) Semongok, Department of Agricultural Sarawak, using Completely Randomized Design (CRD) (Figure 2) that consist of a total of 50 potted vines, with 5 replicates for each treatment, i.e. T1: 'Semongok Aman' vine; T2: 'Kuching' vine, T3: 'Semongok Emas' vine; T4: 'Semongok Perak' vine; T5: 'Semongok 1' vine; T6: 'Nyerigai' vine; T7: 'India' vine; T8: 'Lampung Daun Lebar' vine; T9: 'Sarikei' vine and T10: 'Yong Petai' vine. The pot was arranged 1m x 1m (between vine x between row). The data collection was initiated at 2 years old vine.

T2	T8	T6	T7	T1
T1	T3	T7	T6	T10
T10	T4	T1	T5	T9
T9	T7	T2	T1	T8
T8	T6	T9	T2	T3
T3	T5	T8	T4	T7
T4	T1	T3	T3	T2
T7	T2	T10	T8	T4
T6	T9	T4	T10	T5
T5	T10	T5	T9	T6

Figure 1. Randomized complete block design (RCBD) for the field-grown vine of ten cultivars. T1: 'Semongok Aman' vine; T2: 'Kuching' vine; T3: 'Semongok Emas' vine; T4: 'Semongok Perak' vine; T5: 'Semongok 1' vine; T6: 'Nyerigai' vine; T7: 'India' vine; T8: 'Lampung Daun Lebar' vine; T9: 'Sarikei' vine, and T10: 'Yong Petai' vine. Each block is differenced by topography elevation

T1	T4	T2	T4	T7	T6	T7	T5	T7	T2
T3	T8	T3	T8	T8	T2	T8	T5	T8	T6
T10	T10	T1	T6	T1	T2	T10	T1	T3	T8
T2	T10	T8	T3	T5	T4	T6	T3	T1	T10
T4	T5	T6	T8	T5	T8	T7	T8	T4	T7

Figure 2. Completely randomized design (CRD) pot arrangement for experimental plot under a controlled environment. T1: 'Semongok Aman' vine; T2: 'Kuching' vine; T3: 'Semongok Emas' vine; T4: 'Semongok Perak' vine; T5: 'Semongok 1' vine; T6: 'Nyerigai' vine; T7: 'India' vine; T8: 'Lampung Daun Lebar' vine; T9: 'Sarikei' vine, and T10: 'Yong Petai' vine

A total of 26 morphology characteristics, consisting of both qualitative and quantitative traits, had been assessed in this study, as listed in Table 1. A dichotomous key for diagnosing the cultivars was constructed as the outcome for this study.

Table 1

Morphological characteristic used for diagnosis of cultivar distinctness

	Morphological character	Measurement methods
1.	**Leaf characters**	
	Leaf shape; leaf apex and leaf base	Description based on UPOV standard
	Leaf area (cm^2); blade width (w) mm; blade length (L) mm and blade length-width ratio (Lw^{-1})	Measured by WinFOLIA image analysis system
	Leaf colour (fully expanded leaf)	RHS colour codes used
2.	**Inflorescence characters**	
	Inflorescence length at stigma withering stage (cm) and Inflorescence thickness at stigma withering stage (mm)*	Measured by Vernier calliper
	Inflorescence colour	RHS colour codes used
	Number of flowers per inflorescence	Counted via stereomicroscope
	Number of inflorescence (spike) per branch per node	Counted manually
3.	**Fruit characters**	
	Fruit spike length (cm) and fruit size in diameter (mm)	Measured by Vernier calliper
	Fruit weight (single fresh berry) (g)	Measured by analytical balance
	Fruit colour (hard dough stage)	RHS colour codes used
	Per cent fruit set (%)	Counted manually. Percent = (Number of developed fruit)/ (Number of developed fruit + number of underdeveloped fruit) x 100%.
	Conversion rate % (fresh to black pepper)	Measured by analytical balance (Drying specification: Oven dry at 40°C; moisture content ≤12%)
	Conversion rate % (fresh to white pepper)	Measured by analytical balance (Drying specification: Oven dry at 40°C; moisture content ≤12%)
	Pericarp thickness (mm)	Measured by Vernier calliper (Horizontal diameter of fresh berry - Horizontal diameter of seed)
4.	**Seed characters**	
	Seed diameter (mm)	Measured by Vernier calliper (Horizontal diameter of seed)
	Seed weight (g)	Measured by analytical balance

Table 1 *(Continued)*

	Morphological character	Measurement methods
5.	**Vigour**	
	Branch column	By observation
	Internode length (cm)	Measurement by a ruler (Node to node distance)
	Number of node /1feet stem	Counted manually
6.	**Shoot tips**	
	Anthocyanin: Absent or present	By observation on shoot tip colouration Greenish colour = Absent of anthocyanin; Purplish colour = Present of anthocyanin

Note. UPOV- International Union for the Protection of New Varieties of Plants; RHS - Royal Horticultural Society

RESULTS AND DISCUSSIONS

In this study, a total of ten black pepper cultivars have been assessed, including cultivars 'Semongok Aman' (SA), 'Kuching' (KCH), 'Semongok Emas' (SE), 'Semongok Perak' (SP), 'Semongok 1' (S1), 'Nyerigai' (NYE), 'India' (IND), 'Lampung Daun Lebar' (LDL), 'Sarikei' (SAR), and 'Yong Petai' (YP). Comprehensive assessment consisting of both qualitative and quantitative traits had been carried out to reveal key diagnostic morphology for each of the cultivars. The results of the assessment are shown in Table 2.

Table 2

Qualitative and quantitative traits used to diagnose the differences among black pepper cultivars

No.	Morphological characteristic	Cultivars				
		SA	KCH	SE	SP	S1
	Leaf (Refer to Figure 3)					
1.	Leaf shape	Elliptical	Ovate	Elliptical	Elliptical	Cordate
2.	Leaf apex	Mucronate	Acute	Acute	Acute	Acute
3.	Leaf base	Acute	Rounded	Acute	Oblique	Cordate
4.	Leaf area (cm^2)	45.40^{abc}	37.70^{ab}	46.60^{bc}	62.80^{d}	132.60^{f}
5.	Blade width (w)	6.36^{b}	5.37^{a}	5.67^{a}	7.47^{c}	11.87^{e}
6.	(cm)	10.70^{a}	10.83^{a}	13.31^{c}	13.20^{c}	16.67^{e}
7.	Blade length (L) (cm)	1.70^{b}	2.02^{d}	2.35^{ef}	1.77^{bc}	1.41^{a}
8.	Blade length-width ratio (Lw^{-1})	Green group 139 series	Green group 139 series	Green group 137 series	Green group NN137	Green group 139 series
	Leaf colour (fully expanded leaf)					

Table 2 *(Continued)*

No.	Morphological characteristic	Cultivars				
		NYE	IND	LDL	SAR	YP
	Leaf (Refer to Figure 3)					
1.	Leaf shape	Elliptical	Lanceolate	Ovate	Elliptical	Elliptical
2.	Leaf apex	Acute	Acuminate	Acute	Acute	Acute
3.	Leaf base	Oblique	Rounded	Oblique	Acute	Acute
4.	Leaf area (cm^2)	53.60^{c}	50.90^{c}	81.40^{e}	36.90^{a}	66.50^{d}
5.	Blade width (w)	6.49^{b}	5.75^{a}	8.85^{d}	5.26^{a}	6.62^{b}
6.	(cm)	12.07^{b}	13.63^{c}	13.50^{c}	10.93^{a}	14.75^{d}
7.	Blade length (L) (cm)	1.86^{c}	2.39^{f}	1.52^{a}	2.10^{d}	2.24^{e}
8.	Blade length-width ratio (Lw^{-1})	Green group 139 series	Green group 139 series	Green group NN137	Green group 139 series	Green group 139 series
	Leaf colour (fully expanded leaf)					

No.	Morphological characteristic	Cultivars				
		SA	KCH	SE	SP	S1
	Inflorescence (Refer to Figure 4)					
9.	Inflorescence length (cm)	7.84^{d}	7.03^{bc}	7.95^{d}	6.93^{b}	12.40^{e}
10.	Inflorescence thickness (mm)	3.50^{d}	3.56^{d}	3.47^{d}	3.73^{e}	3.85^{f}
11.	Inflorescence colour	Green group 144 series	Green group N144	Green group N144	Green group 145 series	Green group N144
12.	Number of flowers per	88.30^{de}	67.57^{ab}	86.33^{de}	72.70^{abc}	127.90^{g}
13.	inflorescence (average)	12.47^{a}	58.67^{f}	19.57^{abc}	22.20^{bc}	17.60^{ab}
	Number of inflorescence (spike) per branch per node (average)					

Table 2 *(Continued)*

No.	Morphological characteristic	Cultivars				
		NYE	IND	LDL	SAR	YP
	Inflorescence (Refer to Figure 4)					
9.	Inflorescence length (cm)	7.06^{bc}	7.80^{d}	7.68^{cd}	6.06^{a}	12.75^{e}
10.	Inflorescence thickness (mm)	3.10^{c}	3.00^{b}	3.75^{e}	3.54^{d}	2.90^{a}
11.	Inflorescence colour	Green group N144	Green group N144	Green group 144 series	Green group N144	Green group 145 series
12.	Number of flowers per inflorescence (average)	73.77^{bc}	80.40^{cd}	100.53^{f}	65.10^{a}	93.57^{ef}
13.	Number of inflorescence (spike) per branch per node (average)	35.83^{e}	27.37^{cd}	21.47^{bc}	34.73^{de}	16.20^{ab}

No.	Morphological characteristic	Cultivars				
		SA	KCH	SE	SP	S1
	Fruit (Refer to Figures 5 and 6)					
14.	Fruit spike length (cm)	10.38^{c}	9.37^{b}	10.62^{c}	9.27^{b}	16.48^{d}
15.	Fruit size in diameter (mm)	6.68^{e}	6.75^{e}	6.76^{e}	6.86^{e}	7.27^{f}
16.	Fruit weight (single fresh berry) (g)	0.20^{d}	0.17^{b}	0.17^{b}	0.19^{c}	0.20^{d}
17.	Fruit colour (hard dough stage)	Green group NN137 series	Green group NN137 series	Green group 141 series	Green group NN137 series	Green group NN137 series
18.	Per cent fruit set (%)	70.68^{f}	61.10^{bc}	68.75^{ef}	61.51^{bc}	64.24^{cde}
19.	Conversion rate (%) (fresh to black pepper)	37.35^{ab}	41.68^{cd}	42.24^{cd}	36.12^{a}	42.35^{d}
20.	Conversion rate (%) (fresh to white pepper)	30.37^{de}	31.08^{e}	31.68^{e}	24.21^{a}	27.70^{bc}
21.	Pericarp thickness (mm)	2.00^{bc}	2.20^{c}	2.16^{bc}	2.22^{cd}	2.46^{de}

Table 2 *(Continued)*

No.	Morphological characteristic	Cultivars				
		NYE	IND	LDL	SAR	YP
	Fruit (Refer to Figures 5 and 6)					
14.	Fruit spike length (cm)	9.39^{b}	10.38^{c}	10.22^{c}	8.07^{a}	17.07^{d}
15.	Fruit size in diameter (mm)	6.48^{d}	6.02^{b}	6.30^{c}	5.78^{a}	7.27^{f}
16.	Fruit weight (single fresh berry) (g)	0.14^{a}	0.14^{a}	0.15^{a}	0.17^{b}	0.19^{cd}
17.	Fruit colour (hard dough stage)	Green group NN137 series	Green group NN137 series	Green group 139	Green group NN137 series	Green group NN137 series
18.	Per cent fruit set (%)	66.93^{def}	65.76^{cdef}	55.10^{a}	64.28^{cde}	56.26^{ab}
19.	Conversion rate (%) (fresh to black pepper)	41.06^{bcd}	40.51^{bcd}	38.31^{abc}	39.55^{abcd}	36.25^{a}
20.	Conversion rate (%) (fresh to white pepper)	31.89^{e}	28.16^{cd}	29.62^{cde}	29.69^{cde}	25.70^{ab}
21.	Pericarp thickness (mm)	2.25^{cd}	1.94^{ab}	2.06^{bc}	1.73^{a}	2.69^{e}

No.	Morphological characteristic	Cultivars				
		SA	KCH	SE	SP	S1
	Seed (Refer to Figure 6)					
22.	Seed diameter (mm)	4.80^{e}	4.44^{c}	4.60^{d}	4.60^{d}	4.82^{e}
23.	Seed weight (g) (x 10^{-2})	6.11^{g}	5.13^{e}	5.40^{f}	4.85^{d}	5.46^{f}

No.	Morphological characteristic	Cultivars				
		NYE	IND	LDL	SAR	YP
	Seed (Refer to Figure 6)					
22.	Seed diameter (mm)	4.44^{c}	3.90^{a}	4.32^{b}	3.84^{a}	4.45^{c}
23.	Seed weight (g) (x 10^{-2})	4.91^{d}	4.07^{a}	4.30^{b}	4.56^{c}	4.98^{de}

Table 2 *(Continued)*

No.	Morphological characteristic	Cultivars				
		SA	KCH	SE	SP	S1
	Vigour					
24.	Branch column types	Horizontal	Horizontal	Drooping	Horizontal	Horizontal
25.	Internode length (cm)	11.42^{e}	8.33^{a}	11.40^{e}	10.10^{cd}	8.73^{ab}
26.	Number of node /feet of stem (average)	3.67^{ab}	4.73^{f}	3.33^{a}	4.13^{cdf}	4.17^{cdf}

No.	Morphological characteristic	Cultivars				
		NYE	IND	LDL	SAR	YP
	Vigour					
24.	Branch column types	Erect	Horizontal	Drooping	Horizontal	Horizontal
25.	Internode length (cm)	9.77^{bc}	9.57^{bc}	11.23^{de}	9.83^{bc}	12.7^{f}
26.	Number of node /feet of stem (average)	3.83^{bc}	4.30^{de}	4.43^{ef}	3.97^{bcd}	3.33^{a}

No.	Morphological characteristic	Cultivars				
		SA	KCH	SE	SP	S1
	Shoot tips (Refer to Figure 7)					
27.	Anthocyanin: Absent or present	Present	Present	Present	Present	Absent

No.	Morphological characteristic	Cultivars				
		NYE	IND	LDL	SAR	YP
	Shoot tips (Refer to Figure 7)					
27.	Anthocyanin: Absent or present	Present	Present	Present	Present	Present

Note. SA - 'Semongok Aman'; KCH - 'Kuching'; SE - 'Semongok Emas'; SP - 'Semongok Perak'; S1 - 'Semongok 1'; NYE - 'Nyerigai'; IND - 'India'; LDL - 'Lampung Daun Lebar'; SAR - 'Sarikei', and YP - 'Yong Petai'. Means followed by the different superscript letter within the same row are significantly different at $P \leq 0.05$

The dichotomous key for cultivar diagnosis was constructed by considering both the qualitative and quantitative traits of the ten black peppers.

Identification key to black pepper cultivars in Malaysia:

1a	Leaf area <80 cm² (fully developed leaf from matured and vigorous vine); Number of flowers per inflorescence less than 90	2
1b	Leaf area >80 cm² (fully developed leaf from matured and vigorous vine); Number of flowers per inflorescence more than 90	8
2a	Blade length less than 11 cm long; Blade width-length ratio (Lw^{-1}) ranged from 2.0 to 2.3	3
2b	Blade length more than 12 cm long; Blade width-length ratio (Lw^{-1}) ranged from 1.7 to 2.0 or >2.3	4
3a	Pericarp thickness 2.0-2.2 mm thick; Seed weight 5.0-5.2 (x 10^{-2}) g	'Kuching'
3b	Pericarp thickness 1.6-1.8 mm thick; Seed weight <4.8 (x 10^{-2}) g	'Sarikei'
4a	Leaf base acute; Percent fruit set >70%	'Semongok Aman'
4b	Leaf base rounded; Percent fruit set 60-70%	5
5a	Inflorescence thickness 2.8-3.2 mm thick; Internode 9-10 cm long	6
5b	Inflorescence thickness 3.2-4.0 mm thick; Internode 10-11 cm long	7
6a	Leaf shape lanceolate; Leaf apex acuminate; Plagiotropic branching horizontal type	'India'
6b	Leaf shape elliptical; Leaf apex acute; Plagiotropic branching erect type	'Nyerigai'
7a	Mature leaf, blueish-green colour (RHS colour code: Green group137 series); Mature unripe fruit, pale green (RHS colour code: Green group 141 series)	'Semongok Emas'
7b	Mature leaf, greyish green colour (RHS colour code: Green group NN137); Mature unripe fruit, dark green (RHS colour code: Green group NN137 series)	'Semongok Perak'
8a	Anthocyanin absent in shoot tip (green whitish colour); Seed weight, >5.4(x 10^{-2}) g; internode length, 8-9 cm	'Semongok 1'

8b	Anthocyanin present in shoot tip (Purplish green colour); Seed weight, <4.8(x 10^{-2}) g or 4.8-5.0(x 10^{-2}) g; internode length, >11 cm	9
9a	Fruit spike length, 7-9 cm; Fruit size in diameter, 6-7 mm	'Lampung Daun Lebar'
9b	Fruit spike length, >11 cm; Fruit size in diameter, >7 mm	'Yong Petai'

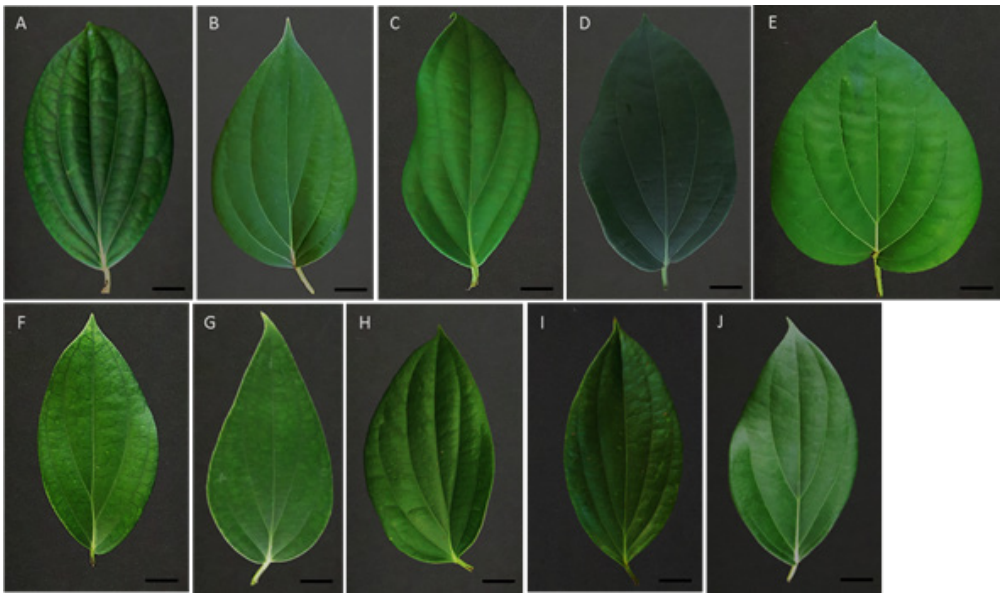

Figure 3. Cultivar designation and leaf shape. A. cv. 'Semongok Aman'- Elliptical; B. cv. 'Kuching'- Ovate; C. cv. 'Semongok Emas'- Elliptical; D. cv. 'Semongok Perak'- Elliptical; E. cv. 'Semongok 1'- Cordate; F. cv. 'Nyerigai'- Elliptical; G. cv. 'India'- Lanceolate; H. cv. 'Lampung Daun Lebar'- Ovate; I. cv. 'Sarikei'- Elliptical; J. cv. 'Yong Petai'- Elliptical. Scale bar: 1cm

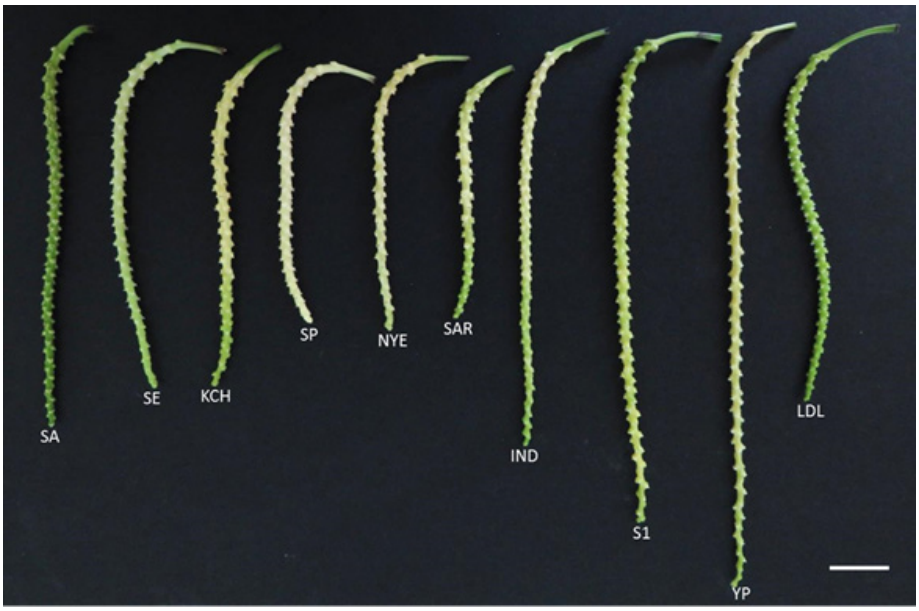

Figure 4. Inflorescence. SA - 'Semongok Aman'; SE - 'Semongok Emas'; KCH - 'Kuching'; SP - 'Semongok Perak'; NYE - 'Nyerigai'; SAR - 'Sarikei'; IND - 'India'; S1 - 'Semongok 1'; YP - 'Yong Petai', and LDL - 'Lampung Daun Lebar'. Scale bar: 2cm

Figure 5. Fruit spike. SA - 'Semongok Aman'; KCH - 'Kuching'; SE - 'Semongok Emas'; SP - 'Semongok Perak'; S1 - 'Semongok 1'; NYE - 'Nyerigai; IND - 'India'; LDL - 'Lampung Daun Lebar'; SAR - 'Sarikei', and YP - 'Yong Petai'. Scale bar: 2cm

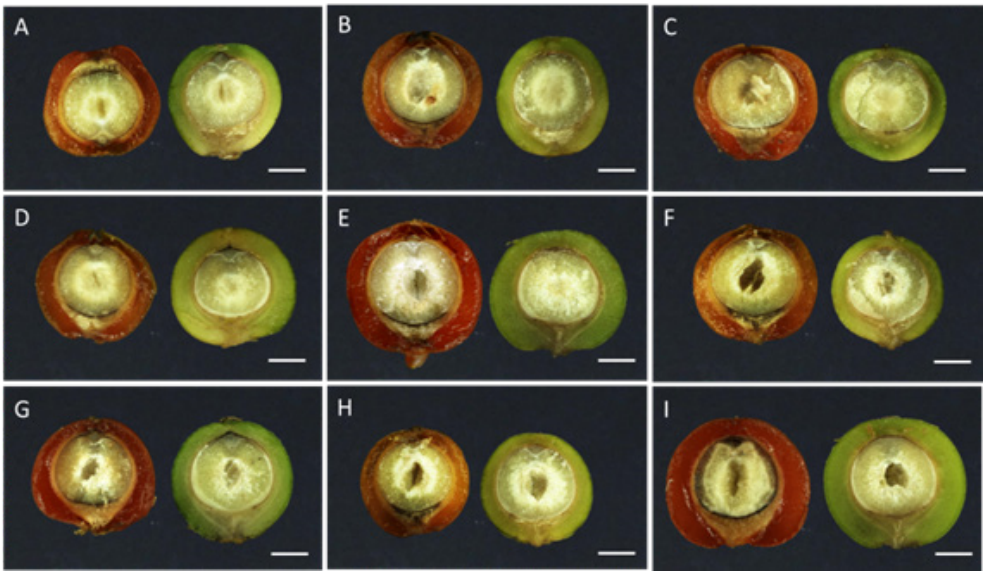

Figure 6. Cross section of ripe and mature fruit. A. 'Semongok Aman'; B. 'Kuching'; C. 'Semongok Emas'; D. 'Semongok Perak'; E. 'Semongok 1'; F. 'Nyerigai; G. 'Lampung Daun Lebar'; H. 'Sarikei', and I. 'Yong Petai'. Scale bar: 2mm

Figure 7. Shoot tips. SA - 'Semongok Aman'; KCH - 'Kuching'; SE - 'Semongok Emas'; SP - 'Semongok Perak'; S1 - 'Semongok 1'; NYE - 'Nyerigai; IND - 'India'; LDL - 'Lampung Daun Lebar'; SAR - 'Sarikei', and YP - 'Yong Petai'. Scale bar: 1cm

Qualitative trait analysis showed leaf shape, leaf apex, leaf base, branch column types (branching behaviour), and anthocyanin colouration at shoot tip were crucial for diagnosing morphological differences among cultivars. Most of the cultivars exhibited elliptical shaped leaf; however, cultivar 'India' had a lanceolate shaped leaf and the cultivar 'Semongok 1' exhibited cordate-shaped leaf. Thus, leaf shape distinctness is an important key diagnosis for the two cultivars. Leaf apex observation showed cultivar 'Semongok Aman' was substantially distinct, with a mucronate-shaped leaf apex; thus, the cultivar could be identified through this trait easily. The only cultivar 'Lampung Daun Lebar' showed ovate leaf shape and oblique leaf base at the same time, another important key diagnosis. In branch column analysis, most cultivars showed a horizontal type of branching, but the cultivar 'Nyerigai' exhibited an erect type of branching, while both cultivar 'Semongok Emas' and cultivar 'Lampung Daun Lebar' exhibited a drooping type. Another important key diagnosis is to shoot tip colouration analysis, where the arrival of anthocyanin at the shoot tip will lead to an exhibition of a purplish colour. Among the ten cultivars, the only cultivar 'Semongok 1' was anthocyanin-free at the shoot tip, thus exhibiting a whitish-green shoot tip. However, qualitative traits, like leaf colour and inflorescence colour, were less substantial for the diagnosis, because the colour intensity is influenced greatly by biotic and abiotic factors (Anita & Anna, 2012; Szakiel et al., 2011). Analyses in this study showed qualitative traits are more influential compared to quantitative traits. This is supported by Khan et al. (2015), Olakojo and Adetula (2014), and Stephan et al. (2016). However, qualitative traits alone with limited variability are insufficient for the diagnosis of certain black pepper cultivars. In this study, the ANOVA test proved the variability for quantitative traits was more substantial compare to qualitative traits. The analysis showed seed weight is among the most important quantitative trait, exhibiting seven significantly different groups among the ten cultivars. Next are traits like blade length-width ratio, fruit size in diameter, conversion rate (%) (fresh to black pepper), inflorescence thickness, number of inflorescence per branch per node, number of flower per inflorescence, per cent fruit set (%), number of node/feet of the stem, and internode length that exhibited six significant groups in the ANOVA test, respectively. The quantitative traits of blade width, blade length, pericarp thickness, seed diameter, and conversion rate (fresh to white pepper) showed five significant different groups, also an important diagnosis key for the ten black pepper cultivars. Thus, the quantitative plays a pivotal role as an additional indicator when the qualitative traits are unable to assist the identification.

The phenetic analysis was done by Chen et al. (2018) revealing that cultivars 'Semongok Aman' and 'Semongok 1' had high distinctive values for identification, thus varietal diagnostic could be very easy. Cultivars 'Nyerigai', 'India', 'Semongok Perak', and 'Semongok Emas' were grouped

in the most diverse cluster among all clusters. The four cultivars had a similarity index as high as 92%; however, investigation on leaf width, leaf width-length ratio, seed weight, and conversion rate (fresh to black pepper) could determine the characteristic differences. Cultivar 'Lampung Daun Lebar' and the cultivar 'Yong Petai' had a similarity of 96%; however, the two showed distinctive differences on leaf width, leaf length-width ratio, spike thickness, and spike length characteristics. The study also reported cultivars 'Kuching' and 'Sarikei' showed the highest similarity index, thus were among the most difficult cultivars to diagnose morphological differences. This finding proved the importance of both qualitative analysis and quantitative analysis in varietal identification of black pepper cultivars.

CONCLUSIONS

Qualitative trait analysis has assisted the diagnosis of ten important cultivars of black pepper in Malaysia as mentioned above while the quantitative traits are crucial as an additional indicator for the diagnosis beside played the role as an indicator of the potential agronomic performance of the cultivar. This study showed cv. 'Semongok 1' exhibited two distinct qualitative traits, a cordate shaped leaf and anthocyanin free shoot tip, and was among the easiest cultivar to identify. Another cultivar with two distinct qualitative traits is cv. 'Semongok Aman', with mucronate shaped leaf apex. The identification for this cultivar can be further verified by quantitative traits, counting the per cent of fruit set. This cultivar exhibited per cent fruit set as high as 76%, averagely. Qualitative trait analysis also discovered the morphological distinctness of cultivar 'Lampung Daun Lebar'. This cultivar showed ovate leaf shape and oblique leaf base at the same time, unique among all the cultivars. The identification for this cultivar was further supported by the quantitative trait of blade length-width ratio (Lw^{-1}), where the cultivar showed the lowest ratio among all cultivars. Cultivar 'Nyerigai' exhibited a unique branching behaviour (branch column type) of an erect type, while others exhibited horizontal or drooping behaviour. The only cultivar that showed a distinctness in leaf and fruit colouration was cv. 'Semongok Emas', with the leaf colour of green group 137 series (RHS code) and fruit colour of green group 141 series (RHS). Cultivar 'India' exhibited a lanceolate shaped leaf, an important diagnosis key for this cultivar. Quantitative trait uniqueness for this cultivar was seed weight; it was the lightest seed among all. Cv. 'Kuching', cv. 'Sarikei', cv. 'Semongok Perak', and cv. 'Yong Petai' did not show qualitative trait distinctness; however, quantitative trait analysis had assisted the diagnosis. Cv. 'Sarikei' had a great distinctness in quantitative traits, including the smallest leaf area, shortest inflorescence and fruit spike, smallest fruit and seed size, and thinnest pericarp. Cv. 'Kuching' showed the highest number of inflorescence (spike) per branch per node and the greatest number of node/feet of the stem, while cv. 'Yong Petai' had the longest inflorescence and

fruit spike, but the thinnest fruit spike. Cv. 'Semongok Perak' only showed significant variability in the conversion rate (from fresh to dried berry), with the lowest rate in both conversions to black (pericarp remained) and white (pericarp removed) peppercorns. The findings of this study enable efficient identification of black pepper cultivar in Malaysia. This is prerequisite toward implementation of the varietal regulation act in the country, at the same time serve as conservation information for the crop.

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Chitosan as a Biopesticide against Rice (*Oryza sativa*) Fungal Pathogens, *Pyricularia oryzae* and *Rhizoctonia solani*

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ABSTRACT

The antifungal potential of chitosan obtained from shellfish was studied in both *in vitro* and *in vivo* conditions against *Pyricularia oryzae* and *Rhizoctonia solani*, causal agents of the blast and sheath blight diseases in rice, respectively. A total of 100% inhibition of mycelial growth was observed on both *P. oryzae* and *R. solani* when a 4% concentration of chitosan was used in this study. A significant reduction in both disease incidence and disease severity was observed between the treated and untreated rice plants. The disease controlling efficacy of chitosan was concentration-dependent with a negative correlation. The disease reduction (DR) capacity of chitosan in this study ranged between 47-95%. Chitosan was able to reduce disease severity (DS) of blast by 85% and sheath blight by 95% while disease incidence (DI) of blast by 77% and sheath blight by 89%. The results demonstrated that chitosan extracted from shellfish has the potential to be developed as a biopesticide for sustainable control of both blast and sheath blight diseases in rice and has broad-spectrum capacity in controlling both diseases.

Keywords: Biopesticide, chitosan, rice blast, sheath blight

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INTRODUCTION

Rice is the world's most important food crop and serves as the main source of carbohydrate for many people around the world (Dorairaj et al., 2017). Food security is jeopardized by the increasing world population. Global rice production was estimated to increase by 30% to meet the global food demand in 2030 (Wang

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et al., 2009). However, rice production is threatened by diseases caused by various pathogens. Two (2) most economically important fungal diseases of rice are blast and sheath blight (Yin et al., 2010).

Both blast and sheath blight diseases are caused by fungal pathogens, *Pyricularia oryzae* and *Rhizoctonia solani*, respectively. Depending on the environmental factors, both diseases can cause yield loss of up to 85% (Singh et al., 2015). Several fungicides have been successfully employed to control these diseases. However, the emergence of resistant fungal populations, the increasing public awareness on the negative effects caused by excessive application of fungicides and their residues on human health, and the rising demands for chemical-free food have led to a search for safer and more sustainable disease management strategies (Zahid et al., 2014). Apart from chemical control (propiconazole), several cultural practices, namely, field sanitation, crop rotation, and maintaining low seeding rate can control these diseases to a certain extent but their efficacy is inconsistent (Bag, 2009). Hence, there is a need to explore new and ecological-friendly approaches to minimize the application of chemical fungicides such as the use of chitosan as an alternative.

Chitosan [poly-(1-4)-β-D-glucosamine], also known as deacetylated chitin is a marine-based (shellfish) biopolymer comprise of high molecular weight cationic polysaccharide (Yin et al., 2010). Chitosan is found to be one of the few cationic polymers found in nature. This polycationic nature and the length of the polymer play a key role in the fungicidal property of chitosan. Chitosan which is positively charged by the protonated NH^{3+} groups interacts with the negatively charged microbial cells creating electrostatic forces that inhibit the growth of fungi (Lawrie et al., 2007) by developing internal osmotic imbalance and hydrolyzing the peptidoglycans in the cell membrane leading to the leakage of internal electrolytes such as potassium ions and low molecular weight proteinaceous constituents such as protein, nucleic acid, and glucose (Bautista-Baños et al., 2006).

Chitosan is known to induce various defense responses including the production of pathogenesis-related proteins as well as phytoalexin (Hassan & Chang, 2017). Chitosan was demonstrated as non-toxic, biodegradable, biocompatible, and possessed antimicrobial properties as well as used to produce an edible coating on fruits to increasing its shelf-life (Zahid et al., 2014). The edible nature of chitosan presents the most desirable aspect to be utilized for disease management in rice. Apart from these, nano/micro-sized chitosan has been used to protect various plants from pathogen infection such as dragon fruit, maize, bell pepper, and cucumber (Elsoud & El Kady, 2019). Liu et al. (2012) had also conducted studies to evaluate the effect of various chitosan to control sheath blight disease. In this study, we have assessed the efficacy of chitosan against both blast and sheath blight diseases in rice (*Oryza sativa*).

MATERIALS AND METHODS

Fungal Strains, Culture Conditions, and Plant Materials

Stock cultures of *P. oryzae* and *R. solani* were obtained from the Culture Collection Unit, Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. *P. oryzae* and *R. solani* were sub-cultured and maintained on Potato Dextrose Agar (PDA) with a pH of 6, at 27±1°C, and alternating light and dark cycle.

Rice seeds of MR219 variety were obtained from the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. The experiment was conducted in the Laboratory of Mycology, Department of Plant Protection and greenhouse, Faculty of Agriculture, Universiti Putra Malaysia.

Preparation of Chitosan Solution

Chitosan powder from shellfish obtained from Pro Advance Technologies Sdn. Bhd. was used as a stock solution by mixing thoroughly 5 g of chitosan powder into 95 g acetic acid. The chitosan powder was made from shellfish. Chitosan solutions with concentrations of 1, 2, and 4% were prepared by diluting the stock solution with sterile distilled water.

In vitro Screening of Chitosan against *Pyricularia oryzae* and *Rhizoctonia solani*

Using Poison Agar Assay as described by Bautista-Baños et al. (2004), the preliminary screening was tested using three different chitosan concentrations and control with six replications for each treatment. PDA was poured into 90 mm diameter Petri plates. Then, 200 µL of each concentration: 1% (T1), 2% (T2), and 4% (T3) was spread over the PDA medium with a sterilized L-shaped glass rod. Control plates contained PDA added with 200 µL of acetic acid (5 mL water mixed with 95 mL acetic acid). A fungal plug of 7 mm diameter from a pure culture of 10 days old *P. oryzae* and *R. solani* were inoculated on the center of the plates, respectively. Petri plates were incubated at 28±2°C for 5 days (Bautista Baños et al., 2004).

Percent inhibition of radial growth (PIRG) was calculated according to Hayman et al. (2017):

$$\mathrm{PIRG}(\%) = [(\mathrm{C} - \mathrm{T})/\mathrm{C}] \times 100$$

where C - mycelium average growth on the control plate (cm); T - mycelium average growth on the treated plate (cm).

In vivo Screening of Chitosan against *Pyricularia oryzae* and *Rhizoctonia solani*

Seed Preparation. Rice seeds were surface sterilized with benomyl fungicide for 18 h to prevent any microbial infection. The seeds were soaked in distilled water and dried for 24 h. Germinated seeds were selected and sowed on trays until they produced true leaves (on the 14th day). On Day 15, the plants were transplanted into pots containing 5 kg soil (3: 2: 1 - topsoil: sand: compost). The water level was maintained at 1–2 cm above the soil surface during the early growth stage and was further raised to 5–7

cm at the later growth stage (Hashim et al., 2015). Each treatment was replicated five times and each replication consisted of four plants in a pot.

Chitosan Application on Rice Plants. Only the best two concentrations tested *in vitro* were selected for the greenhouse study. Leaves sprayed with 2% (T1) and 4% (T2) chitosan until run-off was performed as described by Liu et al. (2012) at 20 days after transplant with the aid of a hand-held sprayer. Control plants were sprayed with distilled water until run-off (T3).

Inoculum Preparation. *Pyricularia oryzae* inoculum was prepared as described by Tuhina-Khatun et al. (2015). *P. oryzae* was maintained on PDA and incubated in the growth chamber at 28±2°C. Conidia spores were harvested at 21 days. Spore density was adjusted to 2×10^5 spores/mL using haemacytometer and 0.05% Tween 20 was added to the spore suspension as an adjuvant before inoculation.

Rhizoctonia solani was maintained on PDA and incubated in the growth chamber at 28±2°C for five days. The mycelium was cut into plugs of 5 mm diameter using sterilized cork borer and used as inoculum (Tuhina-Khatun et al., 2015).

Inoculation of MR219 Rice with *Pyricularia oryzae* and *Rhizoctonia solani*. Both *P. oryzae* and *R. solani* were inoculated on the 21st day after planting. *P. oryzae* was inoculated by spraying 25 mL spore suspension of 2×10^5 spores/mL onto the whole plant. In the case of *R. solani*, mycelial plugs were placed on the stems at one cm below the axial of fully mature leaf and wrapped with parafilm (Khaing et al., 2015; Park et al., 2008). After pathogen inoculation, the plants were covered with plastic bags for 12 h to stimulate infection.

Experimental Design. A completely randomized design (CRD) was implemented in a pot experiment with five replications for each treatment. The same experimental design was used for both the fungi. Three (3) treatments were conducted: T1 (2% chitosan + pathogens), T2 (4% chitosan + pathogens), and T3 (0% chitosan + pathogens) as control.

Disease Assessment. Each disease was assessed on the seventh day after inoculation using a disease rating scale as shown in Table 1 (Khaing et al., 2015).

Disease incidence (DI) was calculated based on the following equation (1) (Maclean et al., 2002) as follows:

$$\text{Disease incidence (\%)} = \frac{\text{Total number of infected tillers}}{\text{Total number of tillers per hill}} \times 100\% \quad (1)$$

Disease severity (DS) was calculated based on the following equation (2) (Lim & Heong, 1984):

$$\text{Disease Severity (\%)} = \frac{\sum \text{N umber of seedlings in the rating X rating number}}{\text{Total number of seedling assessed X highest rating}} \times 100 \quad (2)$$

Disease reduction (DR) was calculated based on the following equation (3):

$$\text{Disease Reduction (\%)} = \frac{\text{DSc} - \text{DSt}}{\text{DSc}} \times 100 \quad (3)$$

where DSc - disease severity of control plants; DSt – disease severity of treated plants.

Table 1
Blast and sheath blight disease rating scale used in this study

Points	Description	
	[a]Blast disease	[b]Sheath blight disease
0	No symptoms	
1	Small brown specks of pinpoint size	Restricted dark brown oval lesions at waterline or infection points
2	Small roundish to slightly elongated, necrotic grey spots, about 1-2 mm in diameter, with a distinct brown margin. Lesions are mostly found on the lower leaves	Few oval or coalesced lesions with broad borders on lower sheaths or at infection points, 5% or less of tissue affected
3	Lesion type same as in 2, but a significant number of lesions on the upper leaves	Lesions on lower leaf sheaths or at infection points, lesions coalescing, less than 10% of tissues affected.
4	Typical susceptible blast lesions, 3 mm or longer infecting less than 4% of leaf area	Lesions mainly restricted to sheaths on the lower third of plant, lowest leaves, or other infection points, lesions discrete or coalescing with narrow red-brown border, 10 to 15% of leaf and sheath tissues affected
5	Typical susceptible blast lesions of 3 mm or longer infecting 4-10% of the leaf area	Lesions coalescing with large necrotic centers and narrow red-brown borders, 15 to 25% of tissues affected
6	Typical susceptible blast lesions of 3 mm or longer infecting 11-25% of the leaf area	Lesions extending to blades of lower leaves or lower leaves killed by injury to the sheath, 25 to 40% of tissues affected
7	Typical susceptible blast lesions of 3 mm or longer infecting 26-50% of the leaf area	Lesions extending to leaf blades of lower two-thirds of plant, 40 to 60% of tissues affected
8	Typical susceptible blast lesions of 3 mm or longer infecting 51-75% of the leaf area many leaves are dead	Lower and middle leaves dead or dying, 60 to 80% of tissues affected
9	Typical susceptible blast lesions of 3 mm or longer infecting more than 75% leaf area affected	Lesions reaching to flag leaf, lower leaves mostly dead, sheath dried, culms brown, collapsing, most tillers lodged, over 80% of tissues affected

Note. [a]Disease assessment score for blast disease (Lim & Heong, 1984); [b]Disease assessment score for Sheath blight disease (Khaing et al., 2015)

Statistical Analyses

All data were subjected to analysis of variance (ANOVA) (SAS, Cary, USA) according to the experimental design used in this study and the least significant difference (LSD) was utilized to compare

the different means of treatment. The correlation analysis was performed using Microsoft Excel 2010.

RESULTS

In vitro Screening of Chitosan against *Pyricularia oryzae* and *Rhizoctonia solani*

Mycelial growth of *P. oryzae* and *R. solani* was inhibited by chitosan of different concentrations (Figure 1) and the efficacy of chitosan to inhibit the fungal growth was concentration-dependent. For *P. oryzae*, no significant mycelial inhibition was observed in T1 (1% chitosan) with only 0.4% of PIRG. As the concentration of chitosan was increased to 2% (T2), the PIRG value increased to 26.5%. An absolute inhibition of *P. oryzae* mycelial growth was observed on plates with 4% chitosan (T3).

A similar trend was observed in the inhibition of *R. solani*. Chitosan of 1% (T1) inhibited slightly the mycelial growth of *R. solani* with PIRG 3.5% while chitosan of 2% (T2) was able to increase PIRG up to 30% and chitosan of 4% (T3) had achieved total inhibition of mycelial growth with PIRG 100%.

In vivo Screening of Chitosan against *Pyricularia oryzae* and *Rhizoctonia solani*

Disease Incidence and Disease Severity. Disease incidence (DI) and disease severity (DS) of rice plants infected with *P. oryzae* and *R. solani* in separate trials were tabulated in Table 2. Disease development in rice plants occurred between 7 to 12 days after the inoculation of both fungi separately. For *P. oryzae*, a pinpoint-sized brown color speck appeared on the seventh day and developed into diamond-shaped lesions with brown borders (Figure 2a) in five plants infected with *P. oryzae* in the control treatment (T3, 0% chitosan). There was a significant ($p \leq 0.005$) difference in both DI and DS between the treated (T1-2% chitosan, T2-

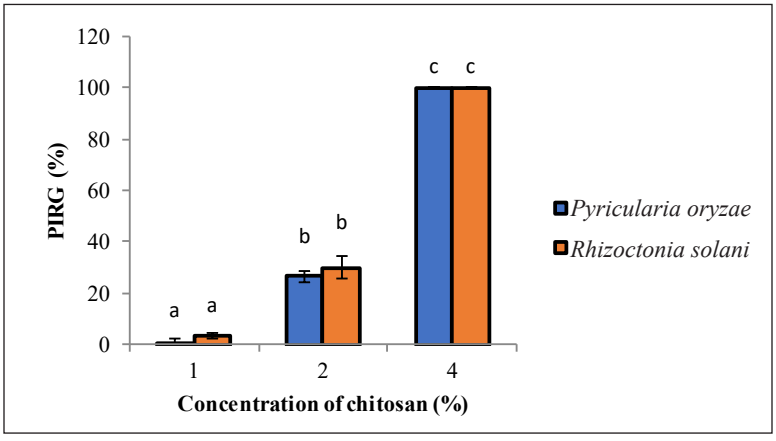

Figure 1. Percent inhibition of radial growth (PIRG) of *Pyricularia oryzae* and *Rhizoctonia solani* at 5 days after plating using poison agar assay with different concentrations of chitosan on PDA medium. Vertical bars indicate standard error of six replicates. Bars with the same alphabets are not significantly different. The standard error for concentration 4% was zero

Table 2
Disease incidence and disease severity for blast and sheath blight diseases on rice plants with or without treatment of chitosan at 7 days after pathogen inoculation

	Blast disease			Sheath blight disease		
Treatment	T1	T2	T3	T1	T2	T3
Disease Incidence (%)	44.7±1.6^{b}	19.4±1.5^{c}	84.1±5.3^{a}	28.8±5.5^{b}	6.1±5.6^{c}	56.4±7.1^{a}
Disease Severity (%)	22.8±1.6^{b}	10.9±0.6^{c}	71.1±5.8^{a}	7.8±1.7^{b}	1.0±0.8^{b}	22.9±9.9^{a}

Note. T1 = 2% chitosan; T2 = 4% chitosan; T3 = 0% chitosan. Values are mean of five replications. Values with the same alphabets in the same row for a respective disease are not significantly different

(a) (b)

Figure 2. Symptoms of rice blast (a) and sheath blight (b) diseases at 12 days after inoculation

4% chitosan) and the control (T3) plants. The control plants (T3) exhibited two folds higher DI (84.1%) and DS (71.1%) compared to the 2% chitosan treated (T1) plants (44.7% and 22.8%, respectively). The lowest DI (19.4%) and DS (10.9%) were observed in plants treated with 4% chitosan (T2).

A similar trend was observed for sheath blight disease. However, the intensity of sheath blight disease was slightly lower than the blast disease. The appearance of a very small dark brown lesion that was oval-shaped was observed in the control (T3, 0% chitosan) plants on the seventh day and developed into irregular lesions with white-gray centers and brown margins (Figure 2b). The result also revealed a significant difference in the values of DI and DS between the treated (T1, T2) and control (T3) plants. Rice plants treated with 4% chitosan (T2) were shown to be effective in controlling sheath blight disease with the minimum DI (7.8%) and DS (1.0%). However, no significant difference was observed in DI and DS between plants treated with 2% (T1) and 4% (T2) chitosan.

Disease Reduction (DR). Chitosan was shown to be effective in controlling both blast and sheath blight diseases in MR219 plants. For disease incidence (DI), there was no significant difference in DR of plants treated with 2% chitosan between blast and sheath blight diseases (46.8 and 48.9%, respectively) (Figure 3). The highest DR was observed in plants pre-treated with 4% chitosan and challenged with *R. solani* (89.1%) followed by those challenged with *P. oryzae* (76.9%) and there was also no significant difference in DR between the two diseases. However, there was a significant difference in DR between the concentrations used (2% vs 4%) for both blast and sheath blight diseases. Chitosan at 4% demonstrated significantly higher DR for DI compared to chitosan at 2% in both diseases.

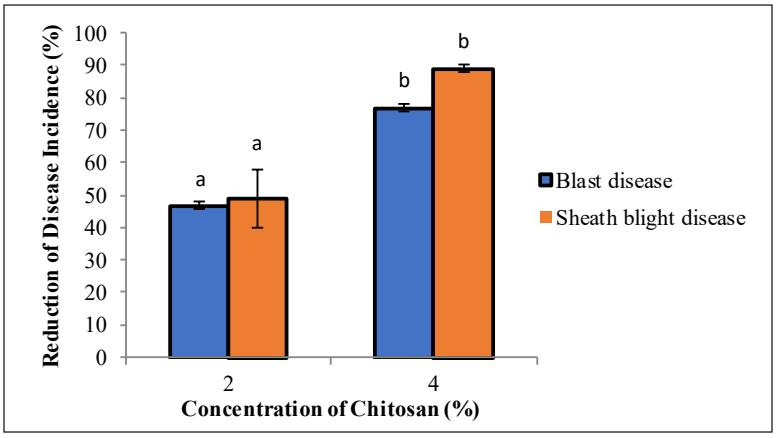

Figure 3. Disease reduction (DR) in disease incidence (DI) of the blast and sheath blight diseases in greenhouse condition with two different concentrations of chitosan as treatments on rice plants. Vertical bars indicate standard error of five replicates. Bars with the same alphabets are not significantly different

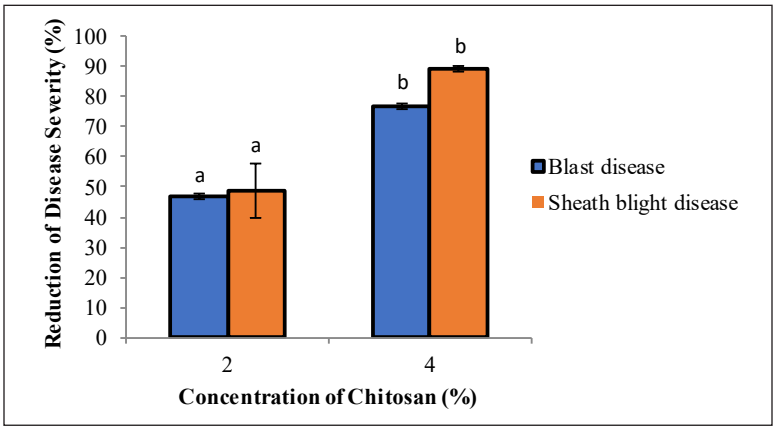

Figure 4. Disease reduction (DR) in disease severity (DS) of the blast and sheath blight diseases in greenhouse condition with two different concentrations of chitosan as treatments on rice plants. Vertical bars indicate standard error of five replicates. Bars with the same alphabets are not significantly different

A similar trend was observed for disease severity (DS) (Figure 4). At 2%, there was no significant difference in DR between plants challenged with *P. oryzae* (67%) and *R. solani* (61%), and at 4%, plants challenged with *P. oryzae* (84.5%) and *R. solani* (95.0%). However, for both diseases, there was a significant difference in DR between the concentrations used (2% vs 4%). Chitosan at 4% demonstrated significantly higher DR for DS compared to chitosan at 2% in both diseases.

Correlation Analysis

Table 3 shows the correlation analysis (*r* values). For blast disease, the *r*-value (-0.6) between the treatment and DI was weak and negatively correlated but the *r*-value (-0.9)

Table 3
Correlation analysis between the treatment, disease incidence and disease severity in rice plants

Blast disease			
	Treatments	Disease Incidence	Disease severity
Treatments	**1**		
Disease incidence	-0.60	**1**	
Disease severity	-0.94	0.83	**1**
Sheath blight disease			
Treatments	**1**		
Disease incidence	-0.99	**1**	
Disease severity	-0.98	0.99	**1**

between the treatment and DS was strong and negatively correlated. The negative *r* value indicates that the higher chitosan concentration used a higher reduction in DI and DS values in rice plants. This indicates that chitosan demonstrated a stronger effect in the reduction of DS than DI. In the case of sheath blight disease, the interaction between treatment, DI and DS was strong and negatively correlated (r = -0.9). In conclusion, chitosan has the potential to control blast and sheath blight diseases in the field.

DISCUSSION

The positive effects of chitosan have been documented in various pathosystems involved in a wide range of plants including both monocotyledon (rice) and dicotyledon (bell pepper, cucumber, dragon fruit), and a diverse range of pathogens including fungi, bacteria, and viruses (Zahid et al., 2014). In this study, we studied the effects of chitosan on rice against the infection of two important fungal pathogens, namely *P. oryzae* and *R. solani*.

In this study, the *in vitro* antifungal test revealed that chitosan was effective against both *P. oryzae* and *R. solani*, which inhibited the mycelial growth of both fungi at the highest concentration used (4%). The rate of inhibition of radial growth by chitosan was concentration-dependent similar to the biostimulants (Surendran et al., 2017). Other studies reported a similar effect when chitosan was used against various plant pathogenic fungi *in vitro*. When the concentration of chitosan was increased from 0.75 to 6.0 mg mL^{-1} in the PDA medium, decrement in the radial growth of *Alternaria alternata*, *Botrytis cinerea*, *Rhizopus stolonifer*, and *Colletrotichum gloeosporioides* was observed (El Ghaouth et al., 1992). A similar effect was also reported in *Sclerotinia sclerotiorum* when chitosan concentration was increased from 1 to 4% (W/V) (Junior et al., 2016). Complete inhibition of the fungi *R. stolonifer*, *Fusarium oxysporum*, *Penicillium digitatum*, and *C. gloeosporioides* was obtained at a concentration of 3% (w/v) (Bautista-Baños et al., 2003, 2004).

Similarly, in this study, the *in vivo* trial results demonstrated that chitosan at 4% concentration was effective to control both blast and sheath blight diseases with more than 80% disease reduction. Chitosan was used to control various *Fusarium* spp. in various economically important hosts including *Fusarium oxysporum, Fusarium graminearum*, and *Fusarium solani* in tomato, wheat, and peas, respectively by reducing DI more than 50% (Al-Hetar et al., 2011; Prapagdee et al., 2007; Sharp, 2013). It was also found that chitosan (0.2 mg/mL) had induced a delayed disease appearance in rice plants (three weeks old) and thus, reduced the disease symptoms in plants (Liu et al., 2012). Boonreung and Boonlertnirun (2013) reported that chitosan sprayed at a concentration of 40 mg/L for four times throughout the crop season before the inoculation of *Helminthosporium oryzae*, *Curvularia lunata*, and *Fusarium moniliformae* reduced 12% of dirty panicle diseases in rice. However, in this study, 4% chitosan spray once throughout the crop season before inoculation of *P. oryzae* and *R. solani* was able to reduce disease severity by 85 and 95%, respectively.

Apart from disturbing the cell wall of the pathogens, chitosan by itself can be a physical barrier to pathogen attack by creating a barrier film or chelating the minerals and make them inaccessible to the pathogens. Chitosan is capable of eliminating the necrotrophic pathogens by neutralizing the mycotoxin produced by these pathogens (Sudarshan et al., 1992). Hence, we speculate that the inhibitory effect of chitosan against *P. oryzae* (a hemibiotroph) in this study was by creating a barrier film or chelating minerals and against *R. solani* (a necrotroph) by neutralizing the mycotoxins produced. These phenomena indicate that chitosan may use a double mechanism of actions to control both types of pathogens.

The nano-sized chitosan was able to control blast disease caused by *Pyricularia grisea* with 100% disease reduction by inducing systemic acquired resistance (SAR) in rice (Xing et al., 2015). Most of the chitosan used in the above-mentioned studies were modified into different forms including nano-sized chitosan because some evidence stated that the negative effect of chitosan on plant growth, shoot length when used in higher concentration in the natural form has been reported (Sandford, 2003). However, the natural chitosan obtained from shellfish used in our study at 4% had revealed a superior disease reduction capacity of 95.0% against sheath blight and 84.5% against blast disease. Chitosan has been produced from various sources ranging from fungi to plants. However, chitosan extracted from shellfish will incur lower costs because it is produced from waste products of the seafood industry (Peniche et al., 2008).

Chitosan and its derivatives have emerged as the best eco-friendly bio-pesticides in the last few decades (Peniche et al., 2008). Other than controlling various plant diseases, chitosan also increases the population of nitrogen-fixing bacteria and vesicular-arbuscular mycorrhizal fungi

(VAM) (Bautista-Baños et al., 2006). US Environmental Protection Agency in 2015 has concluded that chitosan produces no negative impact on the environment (Hassan & Chang, 2017). Due to its eco-friendly and low production cost, chitosan has a huge potential to be used as a biocontrol strategy for sustainable disease management, not only in rice but in other crops as well.

CONCLUSION

To the best knowledge of the authors, this is the first report on the high efficacy and broad-spectrum capacity of chitosan at low concentrations for the control of both blast and sheath blight diseases of rice. Chitosan at 4% concentration had shown a disease severity reduction capacity of 85 and 95% in blast and sheath blight diseases, respectively. To validate this result, field study is required to test the consistency of chitosan in controlling these two fungal diseases in rice. Due to the high demand of rice around the world, these findings could potentially bridge the yield gap in the near future as well as contribute to a sustainable crop production system.

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Screening and Evaluation of Biopesticide Compounds from *Mirabilis jalapa* L. (Caryophyllales: Nyctaginaceae) and Its Combination with *Bacillus thuringiensis* against *Spodoptera litura* F. (Lepidoptera: Noctudiae)

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ABSTRACT

Spodoptera litura is classified as a plant-disturbing organism. Efforts to control *S. litura* by using chemical insecticides have a detrimental impact on the environment and the potential to harm non-target organisms. Bioinsecticides provide a safe alternative for reducing the agricultural pest problem. The purpose of this study was to investigate the specific amino acid from *Mirabilis jalapa* extract using high performance-liquid chromatography (HP-LC) analysis and to identify their potency as a biopesticide. *Mirabilis jalapa* extract in combination with *Bacillus thuringiensis* influences the weakening of the *S. litura* immune system to explain the cause of *S. litura* death. The results showed that the *M. jalapa* extract had seven sequences of the highest amino acid compounds from *M. jalapa*, namely: Glu, Asp, Lys, Val, Leu, Arg, and Ala. Alanine compound has the potential as a biopesticide that breaks down the muscle and nervous system and blocks its receptors. The combination of *B. thuringiensis* in LC_{50} concentration also caused the death of *S. litura*. Finally, the combination of

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0.2% concentration of *M. jalapa* and *B. thuringiensis* at a sublethal concentration (1.07%) applied in 12-hour intervals within 24 hours showed the optimum mortality of *S. litura* (p<0.05): the death of larvae was characterized by damage to the midgut organs in the digestive tract observed by the histological microanatomy.

Keywords: *Bacillus thuringiensis*, biopesticides, *Mirabilis jalapa*, mortality, *Spodoptera litura*

INTRODUCTION

The ever-increasing human demand for agricultural produce has an impact on the polluted environment. One of the causes of environmental damage is the use of chemical insecticides in agriculture. Agricultural pests are an obstacle to food production worldwide, and they have become increasingly resistant to a variety of insecticides. Insecticide synthesis has been widely used to kill various insect pests in agriculture farming, plantation, and even in residential areas because the pest is harmful and even acts as a vector in the spread of diseases (Isman, 2006). The accumulative impact of excessive use of insecticides has given rise to the resistance of insect pests. Insects' resistance as a result of the increasing use of insecticidal doses has caused an urgent problem. Pest control becomes the world's attention to obtain agricultural produce in good quantity and quality. Because of this problem, it is considered necessary to find solutions for the problem of pest resistance and the use of synthetic insecticide (Romeis et al., 2008). Integrated pest management (IPM) offers a solution in controlling insect pests using physical, biological, and chemical combination techniques (Metcalf, 1989; Pedigo, 1999). The success of the IPM program is indispensable to the reduction of the insecticides' purchasing funds and the protection of the environment from further pollution. Additionally, the sustainable nature-based IPM principles support the continuity of the food network that results in a lasting balance because no species are dominant in numbers. In other words, IPM is a program that increases agricultural production and environmentally friendly plantation (Yusof & Kueh, 2013).

The use of botanical insecticides is an offer as well as an effort to control pests (Kumar & Singh, 2015). Botanical insecticides are bioinsecticides made from plants that are known to be environmentally friendly to control insects (Gokce et al., 2010; Kumar et al., 2010). *Mirabilis jalapa* extract is one of the bioinsecticides that can be used to kill pests (Maulina et al., 2018b). The previous studies indicated that *Mirabilis jalapa* L. (Caryophyllales: Nyctaginaceae) as a botanical insecticide inflicted physiological damage on the insects by reducing the number of first and second offspring and refusing to lay eggs on plants that were being banned by botanical insecticides. *Mirabilis jalapa* can produce secondary metabolites in the form of specific compounds indicated as a biopesticide and antifeedant for *Spodoptera litura* F. (Lepidoptera: Noctuidae) (Maulina et al., 2018a, 2018c). *Mirabilis jalapa*

extracts do not kill the insect directly but can influence its physiology. *Spodoptera litura* is the most dangerous pest in crops. *Spodoptera litura* is a polyphagous insect pest that can defoliate up to 80% of the crops' leaves. The resistance of *S. litura* to various chemical compounds needs to be the concern for controlling. Their distribution is very wide across Asia and the South Pacific (Schreiner, 2000; Sparks & Nauen, 2015). Therefore, there is an urgent reason for controlling *Spodoptera* pests with alternative efforts using biopesticides (Kandagal & Khetagoudar, 2013).

The previous study referring to the resistance cases reveals that the molecular mechanisms and physiology are important in preventing the occurrence of resistance in the use of bioinsecticides (Zhu et al., 2016). The mechanism of immune weakening as a physiologic response can be determined by testing the target pest precisely. Immune response indicators are seen in humoral and cellular activity. The use of sublethal biopesticide *M. jalapa* extract causes a low impact on the mortality of *S. litura* (Maulina & Anggraeni, 2014; Suryani & Anggraeni, 2014). Therefore, to improve the work of biopesticides in pest control it is necessary to combine the two types of biological agents in low dosage. Biopesticide *M. jalapa* extracts are applied in combination with the *Bacillus thuringiensis* as an entomopathogenic microbe. *Bacillus thuringiensis* is a bacterium producing delta-endotoxin compounds that damage the digestive system of the pests. The endotoxin crystalline proteins produced by *B. thuringiensis* will work on specific target pests without affecting mortality on non-target organisms. Delta-endotoxins in *B. thuringiensis* are easily biodegradable resulting in no build-up of toxins that pollute the environment (Hansberger, 2000). The preventive response to the occurrence of resistance to larvae of *S. litura* larvae is by applying the combination of the sub-lethal concentration of *M. jalapa* extract with LC_{50} of *B. thuringiensis*.

Sub-lethal concentrations of *M. jalapa* and *B. thuringiensis* were selected as the best optimum concentration to be applied as an appropriate form of pest control. This information is the basis for the development of environmentally friendly IPM that involves a combination of physical, biological, and chemical controls that have never been done before. Thus, pest control that is carried out by measuring the mortality rate of *S. litura* pests is safe for the environment. The purpose of this study was to obtain an optimal formulation in attenuating the immune system of *S. litura* pest after exposure to the combination of insecticides from *M. jalapa* extract with sublethal concentration (0.1%, 0.2%, 0.4%, 0.8% (w/v) and *B. thuringiensis*.

MATERIALS AND METHODS

Insect Larvae Culture

Sample of *Spodoptera litura* larvae was obtained from the Indonesia Sweetener and Fiber Crop Research Institute (ISFCRI/ BALITTAS), Malang, East Java, Indonesia. The fourth instar larvae of *S. litura* were used as a sample in this study. *Spodoptera*

litura larvae were reared at 23°C in the rearing jars, with each jar containing 50 *S. litura* larvae. During the rearing process, the larvae were given a feed of green mustard leave.

Extract of *Mirabilis jalapa*

Mirabilis jalapa leaves were obtained from the field in the Lampung Province. *M. jalapa* leaves were dried (without being exposed to light) and dampened using 96% ethanol. The maceration process was carried out for three days until a crude extract was obtained from the whole *M. jalapa* leaf. The extract was concentrated using an evaporation process to obtain a paste (assumed to have 100% concentration).

The acquired sub-lethal concentration was obtained through multilevel dilution. The used of sub-lethal concentrations were 0.1%, 0.2%, 0.4%, 0.8% (w/v), and the control. In this study, % (w/v) is defined as the percent of the weight of *M. jalapa* extract (in gram) in the total volume of solution (100 ml ethanol). *Mirabilis jalapa* extract having a certain concentration was sprayed throughout the surface of the green mustard feed.

Analysis of Leaf Extract using High Performance-Liquid Chromatography (HP-LC)

The sample in the HP-LC analysis was *M. jalapa* leaf extract. The analysis has obtained a sequence of specific amino acid compounds from *M. jalapa*. This process was performed with test equipment with reverse-phase liquid chromatography **(**LC) system, with a motion phase of trisodium citrate pH 3.25. The technique of splitting the LC pump gradient system (LC-20 AT) and vacuum pump flow rate was 1 ml/min. The detector was a RF 20-A Fluorescence detector, λ 450, OPA, and with wavelength detector at 450 nm. The LC was equipped with ASVP CTO 10 column volume of Shimadzu shim-pack VP ODS 5µm in column dimensions: 150 mm × 4.6 mm with column temperature 40°C. The injection volume was 100 µl of test material using a nitrogen generator at a gas temperature of 250°C. Mass spectrometry detector (SCL -10 AVP) with a positive ionization electro-spray ionization (ESI) technique with a period of 30 minutes. HP-LC analysis was conducted at the Natural Chemistry Laboratory, University of Muhammadiyah Malang.

Dose-response Bioassay of *Bacillus thuringiensis*

Bioinsecticide used in this study was delta-endotoxin of *B. thuringiensis* var. Aizawai strain GC-91: 3.8%. Endospores of *Bacillus thuringiensis* were diluted using distilled water. The fourth instar of *S. litura* larvae was used in these bioassays. The concentration of 0.2% was used as the highest concentration of 100% mortality (sense). Testing to find sublethal dose was conducted using 5 different concentrations; 0.2%, 0.15%, 0.1%, 0.05%, and 0% (w/v). The acquired sub-lethal concentration was obtained through multilevel dilution. Mortality was observed every 24 hours.

Treatments

This study employed a complete randomized design with five concentrations of *M. jalapa* extract: 0.2%, 0.15%, 0.1%, 0.05%, and 0% (w/v). The *S. litura* fourth instar larvas were placed in a petri dish, each containing five larvae. A test plot consisted of five Petri dishes. Every concentration condition hereinafter referred to as the test plot, consists of five plots. The experiments (exposure and measurement) were replicated on five different larvae for each plot condition. The treatment was implemented individually on each larva and was replicated on five different larvae for each condition.

The Combinations of the Two Biological Control Agents Analysis

Spodoptera litura fourth instar larvae were exposed to *Mirabilis jalapa* with different concentrations, and then after 3 and 12 hours (according to the plot experiment), the larvae food was replaced with food containing *B. thuringiensis*. After 24 hours, the number of larval mortalities was recorded. In this assay, bioinsecticides concentrations used were LC_{50} 0.107%.

The combinations of the two biological control agent's data analyses were performed with analysis of variance (ANOVA). Arcsine transformation was used to normalize the cumulative mortality percentage and subjected it to ANOVA using SPSS 17.0 software. Means were separated using the Tukey-Duncan significant difference test at $p<0.05$.

Histological Preparations of Digestive *Spodoptera litura*

Histological preparations were made with the paraffin method. This method began with the process of fixing the *S. litura* sample which had been given combination treatment of *M. jalapa* and *B. thuringiensis* with a different time interval. The sample was immersed in Bouin's solution for 24-hour, then washed twice with 50% alcohol, and 70% ethanol soaked for two days. The next step processing was made with 85%, 95% absolute, and xylene glow alcohols each for two hours. The sample went into the clearing process using xylene absolute then xylene saturated with paraffin cuts by heating at a temperature of 60°C.

The samples were immersed in paraffin-xylene and replaced with pure paraffin. The embedding process used liquid paraffin and cooled to solidify. The trimming stage was done by putting the sample into a paraffin mold, then the sample was sliced using a microtome into an incision band. The result of the incision tape was glued on the glass of the object, to be further dyed using 0.5% eosin dye in 95% alcohol.

RESULTS

The results of HP-LC analysis of *M. jalapa* extract showed that there were 20 types of amino acid content shown in Figure 1. There were seven highest compounds of *M. jalapa*, namely: Glu, Asp, Lys, Val, Leu, Arg, and Ala. The representation of the content of amino acid compounds is shown in Table 1. Based on the magnitude of the content

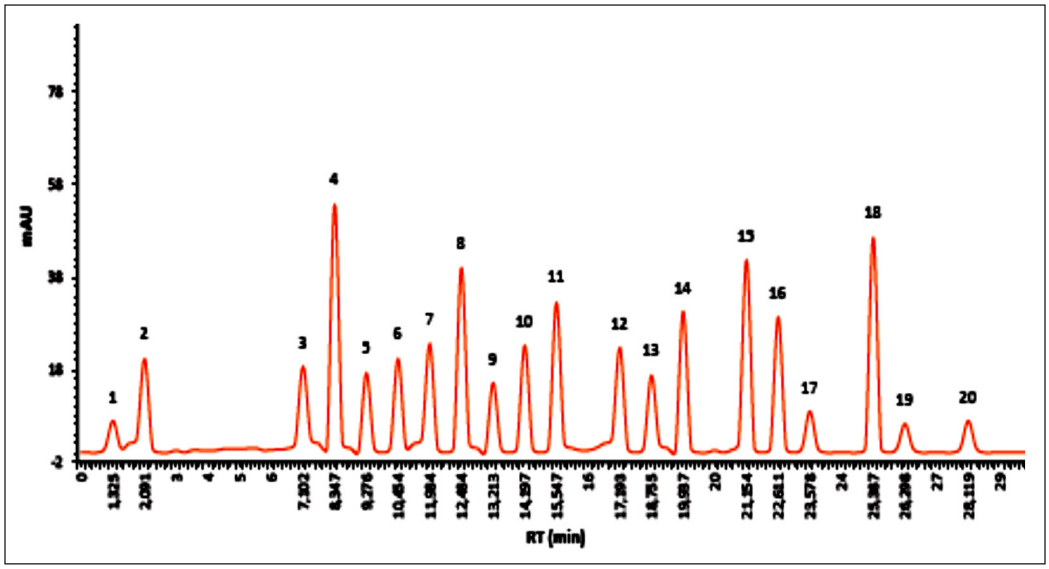

Figure 1. Chromatogram of amino acid compounds from *Mirabilis jalapa* extract

Table 1
Amino acid compounds of Mirabilis jalapa *extract*

Curve peak number	Real-time (min)	Area curve	Result (mg/g)	Result of compound analysis
1	1.33	6.90	0.38	Asn
2	2.10	20.36	1.99	Thr
3	7.10	18.73	1.80	Ser
4	8.35	53.75	6.00	Glu
5	9.27	17.32	1.59	Pro
6	10.45	20.46	1.99	Gly
7	11.98	23.62	2.37	Ala
8	12.48	40.29	4.27	Val
9	13.21	15.07	1.34	Met
10	14.20	23.18	2.28	Ile
11	15.55	32.48	3.31	Leu
12	17.19	22.82	2.24	Tyr
13	18.75	16.76	1.52	Phe
14	19.94	30.62	3.15	His
15	21.15	41.72	4.47	Lys
16	22.61	29.31	2.98	Arg
17	23.58	8.92	0.63	Trp
18	25.38	46.59	4.89	Asp
19	26.30	6.26	0.31	Gln
20	28.12	6.8	0.40	Cys

Note. The grey columns show alanine compounds that identified as biopesticides

of the amino acid compounds, these seven compounds were selected and matched based on the high larvicidal properties. In line with the results of *in silico* analysis which showed that the alanine compound had the highest potential for larvicidal properties, it could be inferred that it had the potential as a biopesticide (Maulina, 2018a).

Alanine is a phenol compound group that has an important role in controlling herbivorous pest insects because these bioactive compounds from alanine peptides act as natural toxins. The compounds produced by secondary metabolites provide good benefits of acting as natural pesticides (Daniel et al., 1999).

Spodoptera litura Mortality with a Combination of Two Biological Agents *Mirabilis jalapa* and *Bacillus thuringiensis*

Both combinations of biological agents were used at sub-lethal concentrations and LC_{50}. The results showed that there was an increase in mortality for a combination of biological agents with a different application of different agendas. Table 2 is the result of mortality test analysis with 3 hours and 12 hours interval of a biological agent after treatment of extract of *M. jalapa* then combined with delta-endotoxin from *B. thuringiensis*.

Table 2 shows that the combination of biological agents with an interval of 3 hours resulted in significantly higher mortality of *S. litura* compared with control (p<0.05). However, an increase in each sub-lethal concentration of *M. jalapa* was not accompanied by an increase in larval mortality. This suggests that an interval of 3 hours of biological agent application causes the performance of both toxins in the larval body not to run synergistically.

Mortality of *Spodoptera litura* Larvae Based on Histological Analysis

The combination of biological agents *M. jalapa* and *B. thuringiensis* causes the death of larvae identified by characteristic changes in cadaver: the larvae's body structure becomes soft, size becomes small, body condition becomes brittle

Table 2
The effect of a combination of Mirabilis jalapa *extract and* Bacillus thuringiensis on *mortality of* Spodoptera litura

Concentration (%)	Mortality	
	3 hours after application of *Mirabilis jalapa*	12 hours after application of *Mirabilis jalapa*
Control	3 + 0.55[a]	1 + 0.45[a]
0.1 Mj + LC_{50}Bt	10 + 2.71[b]	12 + 3.89[b]
0.2 Mj + LC_{50}Bt	10 + 4.23[b]	15 + 3.71[bc]
0.4 Mj + LC_{50}Bt	10 + 2.71[b]	19 + 4.84[c]
0.8 Mj + LC_{50}Bt	11 + 3.84[b]	21 + 5.84[c]

Note. The numbers followed by different alphabet in the same column show a significant difference p<0.05 (Mj: *Mirabilis jalapa*; Bt: *Bacillus thuringiensis*)

accompanied by changes in body color that turns blackish and smelly. The results showed that the cross-sections of the midgut *S. litura* were composed of a composite columnar epithelial layer. Hemocoel was the cavity of a midgut pad where the food circulation was coated by the peritrophic membrane. *Bacillus thuringiensis* plays a role in destroying the epithelial midgut of *S. litura* epithelium. Delta-endotoxins can perforate the epithelial cell membrane of the midgut epithelial cells resulting in lysis due to osmotic events (Figure 2). The results showed that there was a difference in midgut *S. litura* treated with a combination of *M. jalapa* with *B. thuringiensis* at a 3-hour interval after being infected with *M. jalapa* (Figure 2). The concentration of 0.1% has been able to damage epithelial plot to make a hole in the midgut of *S. litura*. The application of 0.2% concentration of *M. jalapa* extract causes epithelial cells in the midgut lysis and detaches from the basement membrane. Midgut damage is getting worse, this condition leads to the death of the organism. Concentrations of 0.4% and 0.8% result in acute tissue damage. Midgut organs become destroyed and organs functionally cannot work anymore.

DISCUSSIONS

The application of *M. jalapa* biopesticide did not not kill *S. litura* directly. However, it was able to induce an immune system reaction by decreasing physiological function. Sub-lethal concentration was aimed at preventing ongoing resistance to target pests (Leng et al., 2011). Resistance prevention

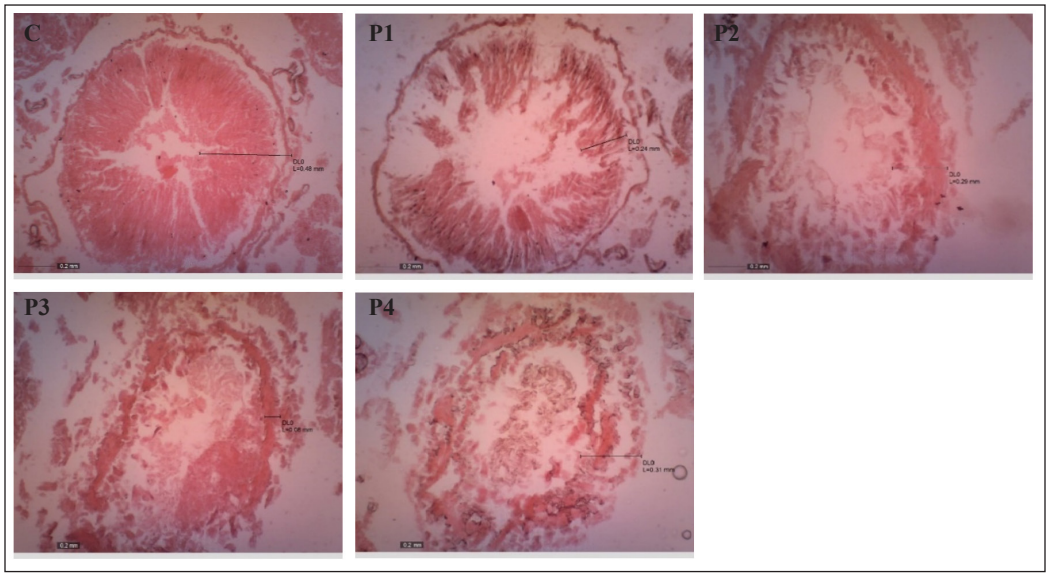

Figure 2. The microanatomy of *Spodoptera litura* midgut treated by the combination of *Mirabilis jalapa* with *Bacillus thuringiensis* at 12 hours interval time

Note. Each microanatomy image has a same size with DL_0; L = 0.48mm and DL_0; L = 0.26mm

C : Control; P1: 0.1% concentration of *M. jalapa* and *B. thuringiensis*; P2: 0.2% concentration of *M. jalapa* and *B. thuringiensis*; P3: 0.4% concentration of *M. jalapa* and *B. thuringiensis*; P4: 0.8% concentration of *M. jalapa* and *B. thuringiensis*

is necessary for easy control of these pests through the application of botanical insecticides (Zibaee, 2011). In the event of resistance, a resurgence is confirmed by the multiplication of insecticidal dosage (Dutcher, 2007; Sparks & Nauen, 2015). Therefore, the use of biopesticide *M. jalapa* prevents these two things from happening following the principle of integrated pest management (IPM).

Alanine compound was detected in *M. jalapa* extract as a biopesticide that could act as an inhibitor of glutamate in the insect's body by changing the conformation of the structure of glutamate in the neurotransmitter signaling (Maulina, 2018a). Glutamate blocking on the original receptors in the insect's body results in a disturbance in response to the stimulating pathways of muscles and smell in insects (Kolodziejczyk et al., 2008; Missbach et al., 2014). Disruption of signaling from the pathway mechanism in neuromuscular has resulted in disruption of the mechanism of muscle movement throughout the insect's body. It does not make body movements dysfunctional but there is a disruption in the muscle mechanism in the respiratory, digestive, and circulatory systems. Coordination of nerves and muscle disruption results in a decrease in motion activity that leads to insect paralysis.

The antenna is an important organ for insects. It is the center of the olfactory. The olfactory nerve cell disruption disrupts the recognition response to the environment. Besides, it fails potential membrane transduction which results in failed action potentials (Stengl et al., 1999). The effect of this series is the failure to receive and continue stimuli originating in the olfactory regulation center, which leads to metabolic disorders.

The *M. jalapa* extract causes disorders of the olfactory and muscle nerve response (neuromuscular). The weakening of various responses to the entry of foreign substances is an indication of the weakening of the insect defense system. The biopesticide toxin of *M. jalapa* causes the defense mechanism in the insect body to become weak. Exposure of *M. jalapa* to *S. litura* causes an increasingly weakened state of immunity. The application of natural compounds from *M. jalapa* as biopesticide compounds to control the amount of *S. litura* could be used safely in nature. Efforts to control *S. litura* pests by measuring mortality rates were performed by combining *M. jalapa* extract and *B. thuringiensis* endotoxin. The two types of biological agents were combined to enhance the action of natural compounds from *M. jalapa* since the subaudible concentrations of *M. jalapa* and *B. thuringiensis* are used to prevent the possibility of resistance to the larvae. The results showed that there was an increase in mortality for the combination of biological agents with a different application of different agendas. Table 2 is the result of mortality analysis with 3 hours and 12 hours' time interval of a biological agent after treatment of extract of *M. jalapa* then combined with delta-endotoxin from *B. thuringiensis*.

The combination of biological agents with an interval of 3 hours resulted in significantly increased mortality of *S. litura* compared with control ($p<0.05$). However,

an increase in each sub-lethal concentration of *M. jalapa* was not accompanied by an increase in larval mortality. This suggests that an interval of 3 hours of biological agent application causes the performance of both toxins in the larval body not to run synergistically. Hillyer stated that the response of *Aedes aegypti* phagocytosis ran 5 minutes after being induced by bacteria (Hillyer et al., 2003). This suggests that an interval of 3 hours of biological agent application causes the performance of both toxins in the larval body not to run synergistically. When the larvae are re-infected with *B. thuringiensis* the larvae can eliminate the endotoxin that enters the body so that the mortality of the larvae becomes low. The average mortality at the time of application of larvae test of *S. litura* with a combination of the concentration of *M. jalapa* equal to 0.8% and delta-endotoxin with an application of time interval of 3 hours was increased by 20%.

The data showed that there was a difference when giving a combination of biological agents with an interval of 12 hours. It is shown in Table 2 that the mortality of *S. litura* differed significantly from controls in the exposure of the four sub-lethal concentrations of *M. jalapa* (p<0.05). The highest mortality occurred in the combination of 0.8% concentration of *M. jalapa* extract with *B. thuringiensis* sub-lethal concentration. The 12-hour physiological impairment performed by toxic compounds in *M. jalapa* can significantly weaken the *S. litura* larvae and the ability of the immune system decreases significantly. The additional combination with delta-endotoxin *B. thuringiensis* results in *S. litura* larvae being unable to activate the immune defense mechanism and death. The *M. jalapa* extract infections given to *S. litura* larvae will affect the immunity characterized by enzyme PO and decrease in average. Giving a concentration of sub-lethal infections of *M. jalapa* is not lethal to *S. litura* pests. It only effectively weakens the larvae's physiology. Therefore, to improve its pest control efficiency biological agents *M. jalapa* and *B. thuringiensis* need to be used (Maulina et al., 2018b).

A toxic substance produced by *M. Jalapa* weakens the insect immunity, while delta-endotoxins from *B. thuringiensis* invades *S. litura* by damaging intestinal epithelial cells along the gastrointestinal tract. When the insect's body's defenses weaken due to *M. jalapa*, the combination of delta-endotoxin *B. thuringiensis* entering the midgut will easily damage the entire tissue and organs. In an unstable condition due to *M. jalapa*'s invasion into the body, the insects will become very weak to repair their damaged ones. Damage to *S. litura* bodies occurs very easily when the immune defenses of *S. litura* larvae have been weakened, tissue and organ damage will occur thoroughly in insect bodies resulting in insect mortality.

The provision of toxins with a 12-hour time interval in the application of *M. jalapa* exposure with a concentration of 0.8% has an impact on increasing the mortality of *S. litura*. *Bacillus thuringiensis* application within 12 hours resulted in a 42% increase in

total mortality compared to controls. Thus, the time interval of combined exposure of biological agents in pest control applications such as combining the exposure of toxic substances greatly affects the increase in total lactational mortality of *S. litura*.

Bacillus thuringiensis has become a biocontrol of insects in many agricultural countries (Weinzierl et al., 1997). The results of the study using the combination of *M. jalapa* and *B. thuringiensis* were focused on the mortality of *S. litura*. The cause of death was found to be the damage to the gastrointestinal tract. Therefore, the results of this study in histopathology form in the damage of the tissue are caused by a combination of biological insecticides used. The combination was performed with a time interval, i.e. 24 hours given *M. jalapa* afterward 3 hours and 6 hours given *B. thuringiensis*. Both biological insecticides are administered simultaneously with oral feed with a synergistic mechanism of action (Agrebi et al., 2010).

The digestive tract of *S. litura* consists of foregut, midgut, and hindgut. Midgut plays a role in the process of food absorption which is a major part of the digestive tract and is an important organ for an insect. The midgut is a hemocoel consisting of a layer of epithelium. Functionally this section holds control of the nutrient traffic (Chapman, 2009). This organ plays an important role in other physiological regulations such as metabolism, immune response, electrolyte homeostasis, osmotic pressure, circulation, and more. Therefore, impaired function and tissue damage in midgut can result in the mortality of insects.

The results showed that the cross-section of the midgut *S. litura* was composed of a compact columnar epithelial layer (Figure 2). This research proved that *M. jalapa* played a role in weakening the immune system and *B. thuringiensis* played a role in damaging the digestive tissue in the body of *S. litura* (Castro et al., 2019). The level of tissue damage that occurred in the midgut depended on the level of concentration of *M. jalapa* and *B. thuringiensis* extracts. The higher treatment concentration of *M. jalapa* and *B. thuringiensis* caused the worse and more acute the tissue damage in the midgut of *S. litura* is. Hemocoel is the cavity of a midgut pad where food is circulated by the peritrophic membrane (Pigott et al., 2007). *Bacillus thuringiensis* plays a role in destroying the epithelial midgut of *S. litura* epithelium. Delta endotoxin can perforate the epithelial membrane of the midgut epithelium that causes the cell to become less because of the osmosis event (Pandey et al., 2009). The concentration of 0.1% had been able to damage epithelial plot to make a hole in the midgut of *S. litura*. The application of 0.2% concentration of *M. jalapa* extract caused epithelial cells to lyse and became detached from one another. Midgut damage got worse, this condition leading to death of the organism. Concentrations of 0.4% and 0.8% resulted in acute tissue damage. The midgut organs become destroyed and organs can not functionally work anymore. The midgut of *S. litura* treated with a combination of *M. jalapa* and *B. thuringiensis* at 6-hour time combination interval suffered severe organ

damage. Each concentration resulted in severe tissue damage. The damage occurred throughout the midgut to the epithelial tissue and even the midgut organs were destroyed.

In the early stages of bacterial infection, insects exhibit a decrease in feeding activity and tend to seek shelter in a hidden place (under the leaves). Furthermore, the larvae experience diarrhea, secrete a fluid from their mouth, paralyzed on the food channel; resulting in decreased movement activity, and ending with death. Bacteria infect through the mouth and gastrointestinal tract, very little through eggs, integuments, and trachea. Bacteria enter as parasitoids and predators. By infecting through the gastrointestinal tract, the bacteria will produce enzymes (lecithinase, proteinase, and chitinase) that will attack the intestines before entering the hemocoel. The toxins will then damage the walls of the intestinal tract. When the intestinal tissue is damaged, the bacteria enter the insect hemocoel (Vega & Kaya, 2012). Here the poison of the bacteria will decompose (hydrolysis). These toxic substances will be released from their endotoxins, which will poison the epithelial cells of the food duct until they are destroyed.

CONCLUSION

Mirabilis jalapa extract had seven sequences of the highest amino acid compounds from *M. jalapa* namely: Glu, Asp, Lys, Val, Leu, Arg, and Ala. Alanine compound has the highest potential for larvicidal properties that have the potential as a biopesticide. The combination of 0.2% concentration of *M. jalapa* and *Bacillus thuringiensis* at the sublethal concentration in 12-hour intervals within 24 hours showed the optimum mortality of *Spodoptera litura* as much as $15+3.7^{bc}$ ($p<0.05$). The death of larvae cadaver was characterized by damage to the midgut organs in the digestive tract observed by the histological microanatomy. Therefore, the combination of *M. jalapa* and *B.thuringiensis* with low concentrations could be considered as integrated pest management.

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Bioactivity Evaluation of *Melaleuca cajuputi* (Myrtales: Myrtaceae) Crude Extracts against *Aedes* Mosquito

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ABSTRACT

Melaleuca cajuputi crude extract in four different solvents viz dichloromethane, ethyl acetate, hexane, and methanol were evaluated for their insecticidal properties against *Aedes aegypti* and *Aedes albopictus* mosquito. Bioassay against larva and adult mosquito was done following World Health Organization's guidelines. Late 3rd and/or early 4th instar of *Aedes* larvae were assayed for different concentrations ranging from 10 to 120 mg/L of *M. cajuputi* crude extract. Larvicidal effects were observed in dichloromethane, hexane, and methanol. Dichloromethane gave the highest of mean mortality, against *Ae. aegypti* (12.6 ± 0.98) and *Ae. albopictus* (10.2 ± 0.37) with LC_{50} of 104.8 mg/L and 106 mg/L, respectively. The adulticidal bioassay was tested against 3 - 5 days old of female mosquitoes with the range concentrations from 0.04 to 6.21 mg/cm^2. Amongst solvents used, extracts of dichloromethane and hexane showed effects against the adult mosquito. Extract in hexane gave 100% mortality against both *Aedes* with LC_{50} of 0.015 mg/cm^2 (*Ae. aegypti*) and 0.022 mg/cm^2 (*Ae. albopictus*). In conclusion, the extract of *M. cajuputi* could be exploited in the development of potential plant-based products in controlling dengue *Aedes* vectors, particularly in the adult mosquito.

Keywords: *Aedes* sp., bioactivity, crude extracts, *Melaleuca cajuputi*, solvents polarity

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INTRODUCTION

In Asia, the mosquito *Aedes aegypti* is a primary vector for dengue and chikungunya (Sam & Abu Bakar, 2006). Meanwhile, its related taxon *Aedes albopictus* has been recognized as a secondary vector for dengue and serves as a vector competence in the maintenance of dengue, and *chikungunya virus* in areas where *Ae. aegypti* is less abundant or absent (Effler et al., 2005; Grard et al., 2014; Li et al., 2012; Wong et al., 2013). The global widespread of vector mosquitoes such as *Aedes* in certain tropical and sub-tropical areas causes a major outbreak of dengue and other mosquito-borne disease-related illness (Rezza, 2014). Utilization of insecticides in the strategy for disease outbreak control is undeniably effective, due to cost-effective, immediate action, and high efficiency against a broad range of vectors. Unfortunately, the effectiveness is threatened by negative and harmful side effects on human, non-target animals, and the environment have become apparent. Nevertheless, the development of resistance among vector populations (Hamid et al., 2018) is the biggest threat to the program's efficacy. Thus, the urge and interest in searching less hazardous alternatives of vector/pest control from plant resources are therefore being renewed and continues today.

To date, many potential plant species with known insecticidal properties and phytochemicals which are rich with biodegradable active compounds are being screened and evaluated (Sharma et al., 2006). For instance, a study on the bioactivity screening of various plant extracts in Malaysia, had shown that *Melaleuca* was the most effective when tested against *Aedes* spp. larva in the laboratory (Bakar et al., 2018). Nevertheless, evaluation of the essential oils of the same plant had also shown its potential insecticidal effects as well (Abu Bakar et al., 2012; Bakar et al., 2019). According to Lowe's report (as cited in De Monte et al., 2014, p. 63), there are many different extractive techniques and approaches working together with various methodologies and solvents to improve the recovery and, the pharmacological profile of their extract products. However, as a result of the differences among the extractive processes and methods, there is a discrepancy in the qualitative and quantitative composition of the extracts obtained from the same plant.

It is known that various solvents of different polarities would extract different phenolic compounds from plants with a high degree of accuracy (Wong & Kitts, 2006). Furthermore, previous studies have shown that solvents with a high polarity such as methanol displayed high effectiveness as antioxidants (Altemimi et al., 2017). The objective of this present study was to evaluate the *in vitro* bioactivity of *Melaleuca cajuputi* plant extracts derived from four different solvents polarity viz. hexane (non-polar), dichloromethane (moderately polar), ethyl acetate (polar), and methanol (polar) against larvae and adult of dengue vector mosquitoes, *Ae. aegypti* and *Ae. albopictus*.

MATERIALS AND METHODS

Collection of Plant Specimens

The leaves of *M. cajuputi* were collected from Port Dickson, Negeri Sembilan area, 2° 31′ 21.1440″ N, 101° 47′ 46.6620″ E in Malaysia. Port Dickson is located 120 km towards the south from Kuala Lumpur 3° 8' 27.0708'' N and 101° 41' 35.5452'' E. The voucher specimen was sent to Forest Research Institute of Malaysia (FRIM) in Kepong, Selangor for species confirmation and specimen deposited at the herbarium.

Preparation of Mosquito

Laboratory strain mosquito was obtained from Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang in the form of eggs. The mosquito colony was cultured and reared in the laboratory under the optimized condition: relative humidity (RH) 80% ± 5% and room temperature 28.5 °C ± 2 °C. During the maintenance period, mosquitoes' larvae were provided with prepared powdered food which contains cat's biscuit, powder milk, grounded (dried) cow liver, yeast, and vitamin B complex. The mosquito colony was continuously maintained throughout the study period.

Preparation of the Crude Extracts

The freshly collected leaves of *M. cajuputi* were dried at room temperature (29 - 31°C) for 5 - 7 days. The dried leaves were grounded mechanically using a household blender. The grounded leaves were extracted with solvents viz: hexane, ethyl acetate, dichloromethane, and methanol with the ratio of 1g sample to 10 mL solvent in a 10L plastic container. The samples were shaken and mixed vigorously and left to sit for 72 hrs. The extracts were filtered through glass funnel with filter paper Whatman No.1. The extract was concentrated using rotary evaporator type EYELA (N-1001S-WD, Japan) at 45°C for eight hrs. The residue obtained was kept in an amber glass vial to be used for subsequent bioassay testing.

Larval Bioassay

The larval bioassay was following the standard guidelines (World Health Organization [WHO], 2005). Five (5) different concentrations of extracts were prepared at 10, 50, 80, 100, and 120 mg/L. A 10 mL stock solution was prepared at a concentration of 100,000 mg/L (100,000 ppm) and kept in a refrigerator at 4 - 5 °C. Five replicates of 20 late third instar larvae were used in each bioassay. The numbers of dead larvae were counted after 24 hrs. of exposure. Positive and negative control solutions were prepared by mixing 1mL solvent in 199mL of distilled water and 2 mL of acetone in 198 mL of distilled water respectively. During the observation, food was not supplied to the larvae. The lethal concentrations (LC_{50} and LC_{90}) were calculated by probit analysis (Finney, 1971).

Adulticidal Bioassay

Bioassay of adulticide was performed as described in the WHO (2016) guideline. Five (5) different concentrations of 2.0 mL plant extracts of 0.04, 0.08, 0.12, 2.48, and

6.21 mg/cm^2 were applied homogeneously at the filter papers Whatman No 1 (12 x 15 cm). The control paper was treated with 2.0 mL acetone. The impregnated papers were dried at room temperature for 24 hrs and kept (4 - 5°C) in an aluminum foil. Four replicates of twenty-five female (3 - 5 days old, blood starved) mosquitoes were aspirated from the mosquito cage into a plastic holding tube. The mosquitoes were allowed to acclimatize in the tube for 1 hr. and later were exposed to the treated impregnated filter paper for 1 hr. At the end of the 1 hr. exposure period, the mosquitoes were transferred back to the holding tube and kept for mortality observation for 24 hrs. A pad of cotton wool soaked in 10% sugar water was placed on the mesh-screen. The number of moribund and dead mosquitoes was recorded at intervals of 1, 5, 10, 15, 20, 25, 30, 45-, and 60-minutes post-exposure. Any knocked down mosquitoes, were considered moribund and counted as dead. A mosquito was classified as dead or knocked down if it is immobile or unable to stand or take off.

Statistical Analysis

Percentage mortality that lies between 5% to 20% will be corrected using Abbott's formula (Abbot, 1925). Larvicidal and adulticidal effects were reported in median lethal concentration (LC_{50}) with a 95% confidence interval subjected to a log probit analysis test. The comparative effectiveness of crude extracts among different types of solvents and *Aedes* mean mortality were analyzed using paired t-test and one-way ANOVA. Results with the value of $p \leq 0.05$ were reported to be statistically significant. All data were analyzed and calculated using SPSS statistics software.

RESULTS

The insecticidal bioefficacy of the *M. cajuputi* crude extracts of dichloromethane, ethyl acetate, hexane, and methanol were tested at 10 mg/L, 50 mg/L, 80 mg/L, 100 mg/L, and 120 mg/L against dengue vectors, *Ae. aegypti* and *Ae. albopictus*. Table 1 summarizes the bioactivity against larvae and adults' stage of *Aedes* vectors. From the results obtained, dichloromethane, hexane, and methanol showed some larvicidal effects when tested against *Aedes* larvae. Meanwhile, adulticidal effects were only observed in dichloromethane and methanol. The bioassay test against larvae and adults *Aedes* mosquito showed a significant increase in the mortality percentage (%) with the increase of concentration.

In Table 2, the paired t-test and the one-way analysis of variance (ANOVA) were analyzed in mean mortality of *Aedes* sp. larvae, and solvents used. Statistical analysis of one-way ANOVA revealed no significant difference between and within groups among solvents and *Aedes* sp. Meanwhile paired t-test between solvents and *Aedes* sp. showed a significant difference ($p \leq 0.05$) in hexane ($p = 0.04$) and methanol (p=0.003) solvent respectively. The highest larvicidal activity was observed in dichloromethane against *Aedes* sp. with the LC_{50} values of 104.8 mg/L and 106.0 mg/L for *Ae. aegypti* and *Ae. albopictus* at 24 hrs. respectively

Table 1

Bioactivity of Melaleuca cajuputi *crude extracts against* Ae. aegypti *and* Ae. albopictus

Solvents	Larvicidal	Adulticidal
Dichloromethane	√	√
Ethyl acetate	-	-
Hexane	√	√
Methanol	√	-

Note. √ toxic effects

Table 2

Mean mortality (± SE) of M. cajuputi *crude extracts against larvae of* Ae. aegypti *and* Ae. albopictus

Aedes sp.	Dose	*Mean mortality ± SE		
	(mg/L)	Dichloromethane[1]	Hexane[2]	Methanol[3]
[a]*Ae. aegypti*	10	1.20 ± 0.20	0	1.40 ± 0.25
	50	3.40 ± 0.25	1.20 ± 0.20	1.60 ± 0.25
	80	6.80 ± 0.37	4.20 ± 0.37	2.40 ± 0.40
	100	9.80 ± 0.66	5.40 ± 0.51	2.80 ± 0.20
	120	12.6 ± 0.98	6.60 ± 0.51	3.00 ± 0.32
	Control**	0	0	0
[b]*Ae. albopictus*	10	0	0	0
	50	0	0	0
	80	7.40 ± 0.25	0.60 ± 0.25	0
	100	10.0 ± 0.32	1.00 ± 0.32	1.60 ± 0.25
	120	10.2 ± 0.37	2.00 ± 0.32	2.00 ± 0.00
	Control**	0	0	0

Note. *Mean value of five replicates
Control** = acetone 0.1%
[1, a,b] No significant difference
[1, 2, 3, a] No significant difference
[2, a, b] Significant difference $p < 0.05$ ($p = 0.04$)
[1, 2, 3, b] No significant difference

(Table 3). Figures 1 and 2 show the highest percentage mortality values of 63% in *Ae. aegypti* and 51% in *Ae. albopictus* at 120 mg/L of dichloromethane extract in *M. cajuputi*.

The results of the adulticidal activity of hexane and dichloromethane extracts of *M. cajuputi* against *Ae. aegypti* and *Ae. albopictus* are presented in Table 4. There was a significant difference between hexane and dichloromethane in mean mortality of *Aedes* spp. with the *p*-values of 0.007 for *Ae. aegypti* and 0.003 for *Ae. albopictus*. However, no significant difference was observed in mean mortality between *Aedes* spp. in each solvent used, hexane and

Table 3

LC values of M. cajuputi *crude extracts against* Aedes *sp. larvae*

Solvents	*Ae. aegypti*					*Ae. albopictus*				
	LC_{50} (mg/L)	95% Confidence Limit		df	χ^2	LC_{50} (mg/L)	95% Confidence Limit		df	χ^2
		LCL	UCL				LCL	UCL		
Dichloromethane	104.8	94.9	117.8	2	0.43	106.0	N/A	N/A	2	15.9
Hexane	164.4	132.1	260.3	2	0.71	429.0	N/A	N/A	2	1.60
Methanol	349.5	N/A	N/A	2	0.19	N/A	N/A	N/A	N/A	N/A

Note. LCL = lower confidence limit; UCL = upper confidence limit; df = degree of freedom

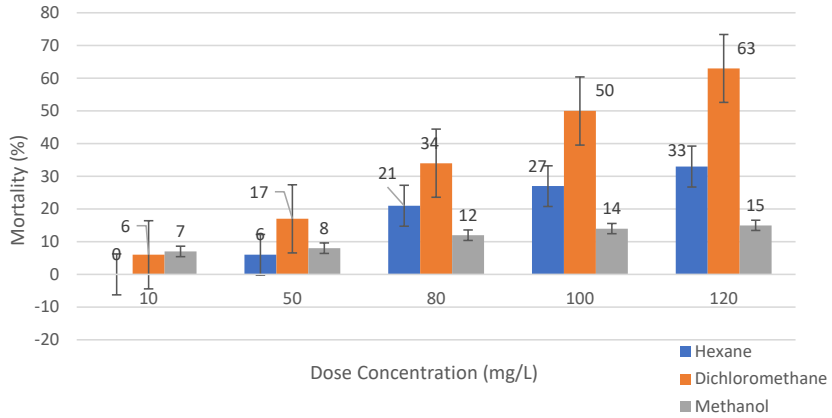

Figure 1. Percentage mortality of *M. cajuputi* crude extracts against *Ae. aegypti* larvae

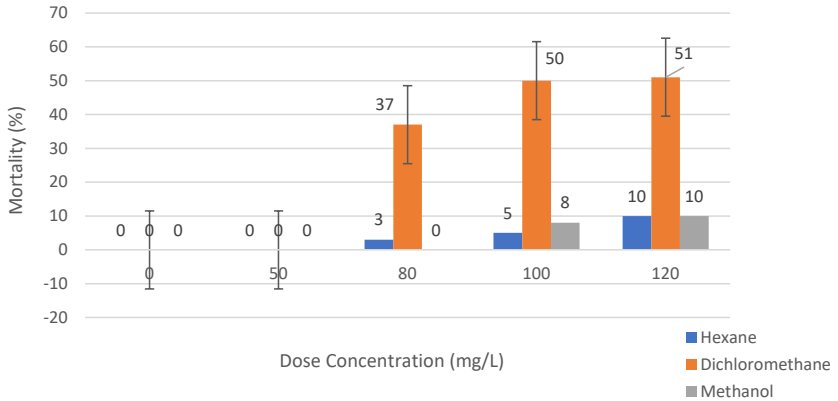

Figure 2. Percentage mortality of *M. cajuputi* crude extracts against *Ae. albopictus* larvae

dichloromethane. The probit analysis of 95% confidence limits LC_{50} (UCL-LCL) was also calculated and presented in Table 5. The chi-square values were not significantly different at $p \leq 0.05$. Among the solvents used, hexane gives lower LC_{50} of 0.015 mg/cm^2 (0.005-0.025) and 0.022 mg/cm^2 (0.009-0.003) in *Ae. aegypti* and *Ae. albopictus*, respectively.

In this study, the results showed that bioactivity of *M. cajuputi* crude extracts against *Aedes* spp. was varied according to the species, stage of life, and solvents used (Tables 1, 2, and 4). The extract of *M. cajuputi* in dichloromethane possessed moderate effects against *Ae. aegypti* larvae. On the other hand, the sensitivity of dichloromethane, hexane, and methanol extract against *Ae. albopictus* showed minimal larvicidal effects after 24 hr. of exposure at various concentrations. Meanwhile, observation in adulticidal assays using dichloromethane, ethyl acetate, hexane, and methanol showed nonconformity with the results of the larvicidal assays. Of these, dichloromethane and hexane extracts of *M. cajuputi* showed adulticidal effects against *Ae. aegypti* and *Ae. albopictus*. However, hexane extract was the most effective against *Ae. aegypti* and *Ae. albopictus* adult's mosquito. From Table 4, more than 50% mortality was observed in *Ae. aegypti* (74%) and *Ae. albopictus* (69%) at lowest concentration of 0.04 mg/cm^2 and 100% mortality when exposed at higher concentrations of 2.48 and 6.21 mg/cm^2.

Table 4

Adulticidal effects of M. cajuputi *crude extracts against* Aedes aegypti *and* Aedes albopictus

Solvents Used	Dose (mg/cm^2)	[1] *Ae. aegypti*		[2] *Ae. albopictus*	
		Mortality (%)	*Mean mortality ± SD	Mortality (%)	*Mean mortality ± SD
[a]Hexane	0.04	74	18.50 ± 1.29	69	17.25 ± 1.26
N=100	0.08	77	19.25 ± 0.96	71	17.75 ± 1.89
	0.12	79	19.75 ± 0.96	89	22.25 ± 3.30
	2.48	100	25.00 ± 0.00	100	25.00 ± 0.00
	6.21	100	25.00 ± 0.00	100	25.00 ± 0.00
	**Control	0	0	0	0
[b]Dichloromethane	0.04	31	7.75 ± 1.15	54	13.50 ± 3.00
N=100	0.08	50	12.50 ± 3.00	56	14.00 ± 2.16
	0.12	55	13.75 ± 0.96	57	16.75 ± 2.50
	2.48	80	20.00 ± 1.83	80	20.00 ± 0.00
	6.21	87	19.25 ± 6.29	81	20.20 ± 0.5
	**Control	0	0	0	0

Note. *Mean value of four replicates **Control=acetone 0.1%

[1, 2, a] No significant difference $p > 0.05$ [1, a, b] Significant difference $p \leq 0.05$ ($p = 0.007$)

[1, 2, b] No significant difference $p > 0.05$ [2, a, b] Significant difference $p \leq 0.05$ ($p = 0.003$)

Table 5

LC values of M. cajuputi *crude extracts against* Aedes *sp. adults*

Solvents	*Ae. aegypti*					*Ae. albopictus*				
	LC_{50} (mg/L)	95% Confidence Limit		df	χ^2	LC_{50} (mg/L)	95% Confidence Limit		df	χ^2
		LCL	UCL				LCL	UCL		
Dichloromethane	0.116	0.071	0.176	3	3.73	0.030	0.007	0.069	3	0.83
Hexane	0.015	0.005	0.025	3	4.57	0.022	0.009	0.033	3	5.86

Note. LCL = lower confidence limit; UCL = upper confidence limit; df = degree of freedom

DISCUSSION

Many researchers have reported the potential of plant extracts for controlling mosquito-borne diseases (Ghosh et al., 2012; Kamaraj et al., 2010; Rehimi et al., 2011). Up to date, there are now more than 2,000 potential plant species that have been evaluated for their insecticidal properties worldwide (Maiza et al., 2013; Roark, 1947; Shaalan et al., 2005; Sukumar et al., 1991). In some developing countries, pesticidal plants offer unique and challenging opportunities for the exploration and development of their botanical sources. Furthermore, one of the most important factors affecting the benefits and efficiency of bioactivity from plant materials and their consequent health is the extraction solvents used (Ngo et al., 2017). Thus, the selection of solvents used depends on the purpose either as of choice for yielding high content or for specific extraction of phytochemical compounds that would be useful for its medicinal and/or insecticidal properties.

The polarity effect depends on the reactivity and selectivity of radical chemistry that has been identified over 50 years ago (Walling, 1957). Most chemical reactions that are carried out in laboratories or the industry are in the form of solutions. Hence the proper and appropriate solvent selection as one of the reaction parameters is important for a good and successful reaction (Reichardt, 2005). According to Rawani et al. (2010), phytochemicals found in plants may play an important role (bioactivities) in vector control if applied appropriately. The phytochemicals in plants can be obtained from the whole plant or specific parts of the plant with different solvents such as petroleum ether, benzene, chlorophyll, methanol, and acetone.

Ghosh et al. (2012) showed that the extraction of active biochemical from plants depended upon the polarity of the solvents. Polar solvents will extract polar molecules and non-polar solvents extract non-polar molecules. In this study, hexane (polarity index of 0.1), dichloromethane (polarity index of 3.1), ethyl acetate (polarity index of 4.4), and methanol (polarity index of 5.1) (Corradini et al., 1998; Harris, 2015) had been used to investigate the insecticidal properties of *M. cajuputi* extract

against *Ae. aegypti* and *Ae. albopictus*. From the results obtained, hexane and dichloromethane solvents that had lower to moderate polarity index were observed to give moderate effects against *Aedes* mosquito. These findings agree with the previous study by Ghosh et al. (2012), which described the efficacy of solvents polarity in the bioassays. Biochemical extracted using moderate polarity index solvents showed good results in a few bioassays. This study revealed the bioactivity variance of *M. cajuputi* crude extracts when tested against larvae and adults of *Aedes* sp. Even though larvicidal effects were observed in dichloromethane, hexane, and methanol extracts of *M. cajuputi*, the effectiveness was slightly weak. However, the adulticidal activity showed good effects against *Ae. aegypti* and *Ae. albopictus*.

CONCLUSION

The bioactivity of crude plant extracts is characterized by a mixture of complex active compounds. Thus, plants containing beneficial phytochemicals may supplement and would be useful plant-based insecticides for future development. Variations of an insecticidal potential of *M. cajuputi* crude extract varied with the different solvents used in the extraction process. Due to the variation's efficacy in the larvicidal and adulticidal effects against *Ae. aegypti* and *Ae. albopictus* mosquitoes. More work is still needed to confirm its effectiveness, especially in the field. A study on the phytochemicals of an active compound of the *M. cajuputi* crude extract in different solvents used can be carried out to characterize the insecticidal properties in different research settings. It can be used as a solution of variants efficacy in a situation of chemical instability of whole or unprocessed plant products.

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Tree Community Structure and Diversity of *Shorea lumutensis* (Balau Putih) Dominated Forest at Segari Melintang Forest Reserve, Perak

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ABSTRACT

Monodominant forests are often dominated by a single tree species at the canopy layer of the forest. At Segari Melintang Forest Reserve, Perak, where *Shorea lumutensis* dominates the forest, a study was conducted to understand the floristic composition and the soil properties that drove the abundance of *S. lumutensis* in the study area. To achieve the objectives, all trees with a diameter at breast height (DBH) of 5 cm and above and soil samples were collected within eight random subplots of 25 m × 50 m each. A total of 1,207 trees were enumerated, which comprised 117 species, 70 genera, and 35 families. The most speciose family and family with the highest density were Euphorbiaceae (12 species) and Dipterocarpaceae (201 individuals/ha), respectively. The total basal area for all trees in the study plots was 32.63 m^2/ha, with Dipterocarpaceae and *S. lumutensis* showing the highest basal area of 10.64 and 2.9 m^2/ha, respectively. For the diversity indices, the Shannon diversity index showed a value of 3.92, whilst the Shannon evenness index was 0.82. The redundancy analysis (RDA) ordination diagram showed that *S. lumutensis* is associated with magnesium (Mg) and calcium (Ca). The distribution pattern of tree communities is associated with the soil characteristics of the study site.

Keywords: Forest dynamic, *Shorea lumutensis*, tree association

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INTRODUCTION

Shorea lumutensis is one of the hyper-endemic species from 160 species of the Dipterocarpaceae family found in Peninsular Malaysia. This species is known by the local community in Malaysia as

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Balau Putih, *Balau Bukit*, and *Damar Laut*, and is easy to be identified based on the white color present underneath its leaves (Ashton, 1982; Smith & Kochummen, 1979; Symington, 1974; Turner, 1995). This species has a limited distribution (Lee et al., 2006) and only grows at altitudes of 300 to 500 m above sea level (a. s. l.) (Evans, 1995) with specific soil conditions and topography (Ashton, 1976, 1982; Plotkin et al., 2000; Rivers & Barstow, 2019; Whitmore, 1973).

Recently, *S. lumutensis* has been classified as one of the critically endangered species (Chua et al., 2010; International Union for Conservation of Nature [IUCN], 2012; Rivers & Barstow, 2019). Because of having recalcitrant seeds (Boshier, 2011; Dudash & Carr, 1998; Keller & Waller, 2002; Lee et al., 2006; Symington, 1974; Tompsett, 1992) and demanding conditions for growth. Ghollasimood et al. (2011) demonstrated that *S. lumutensis* was closely associated with two species of palms, *Eugeissona tristis* and *Calamus castaneus*. Abiotic and biotic factors are crucial in facilitating the distribution of tree species (Nik Norafida et al., 2018; Nizam et al., 2009); thus, an intensive study was conducted to assess the soil properties associated with *S. lumutensis*.

This study aimed to identify the ecological features of the forest dominated by *S. lumutensis* and the association of the tree community with soil factors. To achieve the main objectives, the ecological parameters, namely forest structure, species diversity, tree biomass, and soil characteristics, and their relationships between tree distribution and soil component at Segari Melintang Forest Reserve, Perak were determined. The Segari Melintang Forest Reserve has an area of approximately 4,566 ha and is classified as a coastal lowland dipterocarp forest. It is a unique forest because it is a combination of forests, beaches, and sea, and has been designated as a high conservation value forest (HCVF) by the Perak State Forestry Department. The effort to conserve this species should be followed by a broad understanding of the biodiversity around it (Mohd. Zaki et al., 2013).

MATERIALS AND METHODS

Study Site

Eight subplots of 50 × 25 m each were established at random in compartments 40 and 42 at Segari Melintang Forest Reserve, Perak located between altitude 100°33′30″T to 100°38′00″T and longitude 04°17′30″U to 04°26′00″U.

Experimental Design and Sampling

All trees with a diameter at breast height (DBH) of 5 cm and above were marked, measured, and identified based on the taxonomic textbook (Ashton, 1982; Corner, 1978; Ng, 1978, 1989; Symington, 1943; Whitmore, 1972, 1973). Three (3) soil cores at depths 0–20 cm were taken from each subplot, air-dried, and pooled before analysis. The soil's physical characteristics were determined accordingly, such as moisture content, organic matter (Avery & Bascomb, 1982), and particle size distribution (Abdulla, 1966). The soil's

chemical characteristics such as pH (Avery & Bascomb, 1982; Metson, 1957), available macronutrient (phosphorus [P], potassium [K], and magnesium [Mg]) (Murphy & Riley, 1962), cation exchange capabilities [CEC] (McLean, 1965), and inorganic nitrogen (ammonium–N [NH_4–N] and nitrate–N [NO_3–N]) were determined using a spectrophotometer based on calorimetry principles.

Data Analysis

The basic community structure in the study plot was further explained by floristic composition, dominance, and abundance parameters calculated based on Brower et al. (1997). The total biomass estimation comprised aboveground (Kato et al., 1978) and belowground (Niiyama et al., 2010) biomass estimations. Species diversity and richness were determined by using the Shannon diversity index (Spellerberg & Fedor, 2003) and Margalef index (R′) (Brower et al., 1997), respectively. The relationships between tree communities and soil properties were analyzed using CANOCO program version 4.5 (Lepš & Šmilauer, 2003; ter Braak & Šmilauer, 1998) and the diagram was generated using CANODRAW 4.12. Before analysis, tree species in the data matrix having frequencies of one and two were omitted to improve the accuracy of the analysis, and soil data were $\log_{10}$ transformed. The significance of the degree of the relationship was assessed through the Monte Carlo permutation test based on 499 randomized tests at a significance of 0.05 (ter Braak, 1990).

RESULTS AND DISCUSSION

Floristic Composition and Species Diversity

In this study, 1,207 individuals were identified, which belong to 35 families of 70 genera and 117 species. The largest family and genus, represented by the greatest number of species, were the Euphorbiaceae (12 species) and *Syzygium* (10 species), respectively (Table 1). Table 2 shows *Gluta elegans*, *Canarium littorale*, *Dacryodes costata*, *Santiria rubiginosa*, *Shorea multiflora*, *Vatica maingayi*, and *Palaquium herveyi* were the species with the highest frequency at 100% and present in all eight subplots. *Shorea lumutensis* was only present in seven of the eight subplots. To determine the dominance of a family or species, the importance index (IVi) was calculated and the finding shows *Gluta elegans* (Anacardiaceae) (5.05%), *Drypetes kikir* (Euphorbiaceae) (4.24%), and *Shorea lumutensis* (Dipterocarpaceae) (4.04%) as the most dominant species in the study area. The total basal area of the 1,207 individuals was 32.63 m^2/ha with *S. lumutensis* (Dipterocarpaceae) as the largest-basal species of 2.9 m^2/ha. Two (2) individuals of *S. lumutensis* in the plots recorded the highest diameter of 90.1 cm and 80.2 cm, which contradicts Symington's (1943) finding where the diameter of this species rarely exceeds 50 cm. A similar finding was also reported in Sungai Pinang Forest Reserve and Lumut Forest Reserve, where the tree diameter recorded was over 100 cm (Lee et al., 2006). Shannon diversity index (H′) revealed a value of 3.92 and the

maximum value of the Shannon index, H'_{max}, was 4.76. This indicates that the forest is highly diverse as the index value exceeds 3.5. Such a finding is commonly reported in tropical rainforests (Magurran, 1988). The well-known high species richness of the tropical rainforest is displayed in a rather small study area at the study site (Phillips et al., 1994). The Margalef richness index (R′) is used to assess species richness in a given area and, in this study, the R′ was 37.64. From biomass estimation, we can predict the productivity of the vegetation. In this study, the total biomass was 462.41 tonnes/ha, contributed by the aboveground biomass (AGB) of 397.36 tonnes/ha and belowground biomass (BGB) of 62.18 tonnes/ha. Dipterocarpaceae was the largest family, contributing 169.68 tonnes/ha (37.08%), followed by Sapotaceae and Anacardiaceae with 63.89 tonnes/ha (13.96%) and 41.07 tonnes/ha (8.88%), respectively. At the species level, *S. lumutensis* (Dipterocarpaceae) was the species with the highest biomass value of 51.99 tonnes/ha, followed by *Payena*

Table 1
Five leading families and genera based on the number of species in the Segari Melintang Forest Reserve, Perak

Family	No. Species	Genus	No. Species
Euphorbiaceae	12	*Syzygium*	10
Dipterocarpaceae	11	*Shorea*	5
Myrtaceae	11	*Diospyros*	5
Anacardiaceae	9	*Santiria*	4
Sapotaceae	8	*Gluta*	3

Table 2
Summary of abundance parameters of the five leading species in all subplot at Segari Melintang Forest Reserve, Perak

Species	No. Individual	BA (m^2/ha)	Frequency (%)	IV_i (%)
Gluta elegans	93	1.69	100	5.05
Drypetes kikir	94	0.96	88	4.24
Shorea lumutensis	15	2.90	88	4.04
Shorea multiflora	54	1.68	100	3.96
Canarium littorale	67	1.19	100	3.82

Table 3
Five leading families and genera based on the number of species in the Segari Melintang Forest Reserve, Perak

Family	Total Biomass (tonnes/ha)	Species	Total Biomass (tonnes/ha)
Dipterocarpaceae	169.68	*Shorea lumutensis*	51.99
Sapotaceae	63.89	*Payena lucida*	29.04
Anacardiaceae	41.07	*Shorea laevis*	24.30
Euphorbiaceae	31.43	*Shorea multiflora*	24.09
Burseraceae	28.11	*Shorea curtisii*	20.11

lucida (Sapotaceae) at 29.04 tonnes/ha and *Shorea laevis* (Dipterocarpaceae) at 24.30 tonnes/ha (Table 3). According to Zani et al. (2018), the estimation of biomass within three different forests, which are lowland dipterocarp forest, riparian forest, and hill dipterocarp forest, does not greatly differ with mean total tree biomass values of 415.11, 323.33, and 579.05 tonnes/ha, respectively.

Soil Properties

The soil texture at the study site was dominated by a sandy clay texture with high sand content, as summarized in Table 4. The soil was acidic with an average pH of 4.39 and low in organic matter content (1.03 ± 0.09%). This is a common feature of tropical soil, which is acidic due to a high amount of organic matter and cationic substances, which consist of H^+ and Al^{3+} (Neina, 2019; Othman & Shamshuddin, 1982). According to Longman and Jenik (1987) and Othman and Shamsuddin (1982), tropical soil has low organic matter because the rate of decomposition, temperature, and moisture is high in the tropics (Longman & Jenik, 1987; Othman & Shamsuddin, 1982). The mean values of available macronutrients such as phosphorus (P), magnesium (Mg), and potassium (K) were 2.92 ± 0.19, 7.60 ± 0.64, and 75.99 ± 7.93 µg/g, respectively. The contributors to these three macronutrients are from the decomposition of leaves, bark, and seedlings; animal bones and feces; and weathering process (Parzych & Trojanowski, 2006; Samuel & Werner, 1975). Available phosphorus (P) in the soil was the least as it easily dissolves and the bond with organic matter is weak (Khan et al., 2009). The P concentration is also closely related to pH, which decreases as the soil acidity increases (Barrow & Debnath, 2014; Penn & Camberto, 2019). The available K is categorized as high as it exceeded 10.63 µg/g (Landon, 1991). The available Mg content is also categorized as low and its content is inversely correlated with soil pH and organic matter, where low soil pH and organic matter decrease its content in the soil (Choudhury & Khanif,

Table 4
Summary of soil parameters in all subplots at Segari Melintang Forest Reserve, Perak

Soil parameter	Mean ± Standard error (s.e.)
pH	4.39 ± 0.04
Organic matter content (OM) (%)	1.03 ± 0.09
Available magnesium (Mg) (µg/g)	7.60 ± 0.64
Available phosphorus (P) (µg/g)	2.92 ± 0.19
Available potassium (K) (µg/g)	75.99 ± 7.93
Nitrate–N (NO_3–N) (µg/g)	2.91 ± 0.36
Ammonium–N (NH_4–N) (µg/g)	5.61 ± 0.15
Silt (%)	11.54 ± 1.75
Clay (%)	35.93 ± 1.77
Sand (%)	52.52 ± 2.16

2003; Nizam et al., 2006). Furthermore, the contents of ammonia–nitrogen (NH_4–N) and nitrate-nitrogen (NO_3–N) at the study site were 5.61 ± 0.15 and 2.91 ± 0.36 µg/g, respectively. This inorganic nitrogen was crucial for the production of chlorophyll and plant growth.

Relationship between Tree Communities and Soil Properties

Before analysis, the tree community data were tested using detrended correspondence analysis (DCA) to confirm that the data were unimodal and constrained linear ordination, with the length of the gradient of 1.696, which was greater than 4.0 SD (standard deviation). Hence, the use of Redundancy detrended analysis (RDA) is appropriate (ter Braak & Prentice, 1988; ter Braak & Šmilauer, 2002; Svenning et al., 2004). Based on the RDA, the correlation of species-environment is low at 1.000 with the first and second axis eigenvalues of 0.360 and 0.193 (Table 5). The results of the Monte Carlo permutation test also show no significant difference between the eigenvalues for the three ordinal axes (p = 1.000). This p-value explains that the distribution of tree species is independent and does not depend on soil characteristics.

The RDA diagram shows the relationship between the tree species and the soil properties, indicated by the direction and magnitude of the arrow that exits the center of the ordering (Figure 1) in which each number in the figure represents a different species as listed in Table 6. This analysis shows that although the study was only conducted in a single hectare area, a variation of soil conditions might have influenced the distribution and abundance of species between subplots. Species *Payena lucida* (63), *Gluta curtisii* (1), *Vatica cuspidata* (21), and *Stemonurus malaccensis* (44) show a close association with phosphorus (P) factor, while *Ixonanthes reticulata* (45) and *Botryophora geniculata* (30) with nitrate–N (NO_3–N) factor.

The species of interest, *S. lumutensis*, shows a close association with Ca^{2+} and Mg. *Kapur* trees (*Dryobalanops aromatica*) are highly associated with Mg and this is also supported by experiments in the glasshouse (Nik Norafida, 2018). The

Table 5
Summary of redundancy detrended analysis (RDA) on the vegetation and soil data in all subplots at Segari Melintang Forest Reserve, Perak

Axes	1	2	3	4	Total inertia
Eigenvalues	0.360	0.193	0.130	0.106	1.000
Species–environment correlations	1.000	1.000	1.000	1.000	
Cumulative percentage variance of species data	36.0	55.3	68.3	78.9	
Cumulative percentage variance of species-environment relation	36.0	55.3	68.3	78.9	
Sum of all eigenvalues					1.000
Sum of all canonical eigenvalues					1.000

species has also been found to be closely related to its distribution with *Drypetes kikir* (32), *Calophyllum canum* (38), *Canarium littorale* (9), and *Shorea laevis* (18). Most other species of Dipterocarpaceae such as *Dipterocarpus kerrii* (15), *Vatica cuspidata* (21), and *Dipterocarpus costatus* (14) show a close association with P. According to Sukri et al. (2012) stated that dipterocarp species in Borneo forests are closely associated with particular nutrients such as exchangeable and total calcium (Ca), magnesium (Mg) and potassium (K), total carbon (C), total nitrogen (N), total phosphorus (P). Overall, the distribution patterns of tree species in relation to the soil factors in this study illustrate that habitat variations influence the distribution of tree species communities in the study area. This statement is also supported by other studies conducted in various ecosystems (Nizam et al., 2012; Walthert & Meier, 2017).

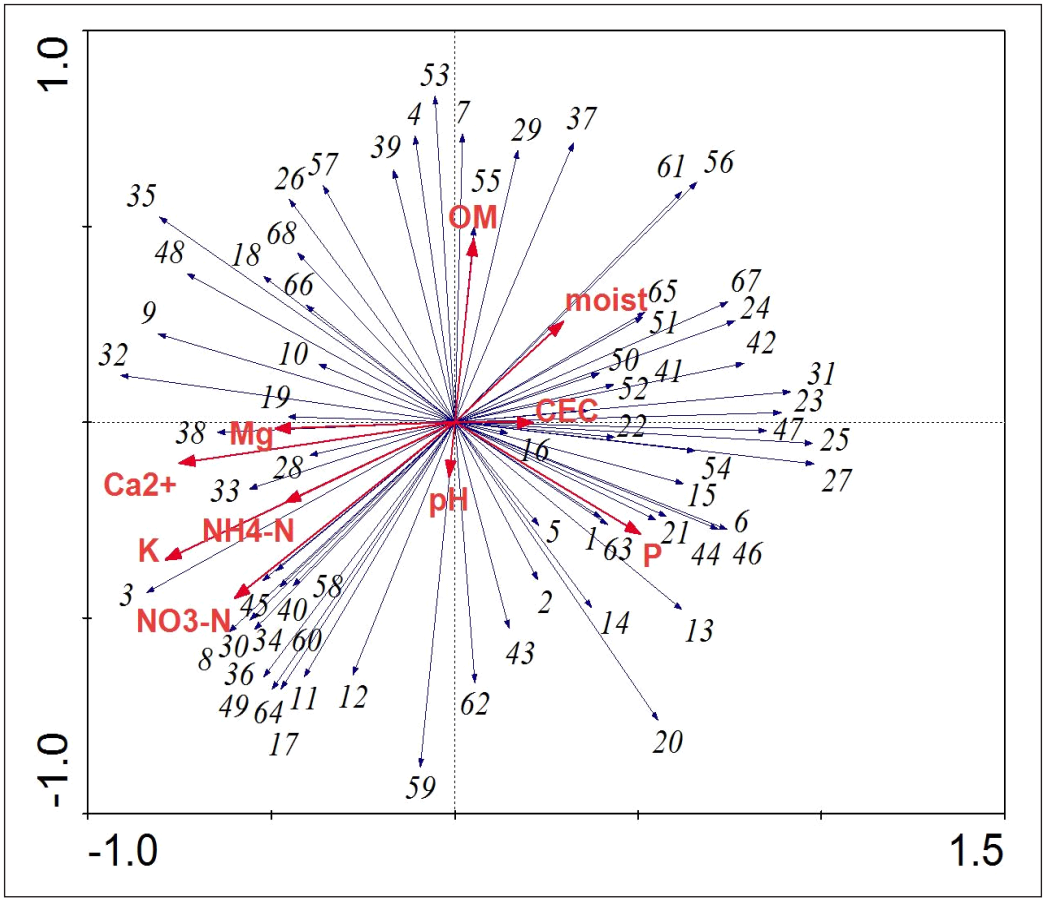

Figure 1. Redundancy detrended analysis (RDA) biplot of species and soil variables showing the species occurrence in relation to edaphic variables. The lengths and directions of vectors indicate the strengths and directions of gradients. *Note*. pH = soil pH; moist = soil moisture; Mg = available magnesium; P = available phosphorus; OM = organic matter content; CEC = total cation exchange capacity; K = available potassium; NH_4–N = ammonia–nitrogen; NO_3–N = nitrate–nitrogen. List of species as in Table 6.

Table 6
List of species number in ordination diagram of Figure 1

No.	Species	No.	Species
1	*Gluta curtisii*	35	*Mallotus lackeyi*
2	*Gluta elegans*	36	*Castanopsis* sp
3	*Gluta wallichii*	37	*Lithocarpus elegans*
4	*Mangifera quadrifida*	38	*Calophyllum canum*
5	*Melanochyla angustifolia*	39	*Calophyllum* sp.1
6	*Parishia insignis*	40	*Garcinia parvifolia*
7	*Swintonia floribunda*	41	*Garcinia urophylla*
8	*Mezzettia parviflora*	42	*Mesua kunstleri*
9	*Canarium littorale*	43	*Mesua racemosa*
10	*Dacryodes costata*	44	*Stemonurus malaccensis*
11	*Santiria apiculata*	45	*Ixonanthes reticulata*
12	*Santiria rubiginosa*	46	*Cinnamomum sintoc*
13	*Crypteronia griffithii*	47	*Barringtonia macrostachya*
14	*Dipterocarpus costatus*	48	*Lijndenia laurina*
15	*Dipterocarpus kerrii*	49	*Memecylon campanulatum*
16	*Shorea balanocarpoides*	50	*Artocarpus lanceifolius*
17	*Shorea curtisii*	51	*Horsfieldia brachiata*
18	*Shorea laevis*	52	*Knema communis*
19	*Shorea lumutensis*	53	*Syzygium* sp.1
20	*Shorea multiflora*	54	*Brackenridgea hookeri*
21	*Vatica cuspidata*	55	*Xanthophyllum monticolum*
22	*Vatica maingayi*	56	*Diplospora malaccensis*
23	*Diospyros buxifolia*	57	*Porterandia anisophyllea*
24	*Diospyros maingayi*	58	*Xerospermum laevigatum*
25	*Elaeocarpus floribundus*	59	*Madhuca laurifolia*
26	*Elaeocarpus pedunculatus*	60	*Madhuca* sp.1
27	*Agrostistachys gaudichaudii*	61	*Madhuca* sp.2
28	*Baccaurea maingayi*	62	*Palaquium herveyi*
29	*Baccaurea minor*	63	*Payena lucida*
30	*Botryophora geniculata*	64	*Payena* sp.1
31	*Cleistanthus lanuginosus*	65	*Eurycoma longifolia*
32	*Drypetes kikir*	66	*Gordonia multinervis*
33	*Drypetes pendula*	67	*Pentace curtisii*
34	*Endospermum diadenum*	68	*Gironniera parvifolia*

CONCLUSION

The flora composition and soil nutrient data obtained can serve as supporting evidence for conservation purposes, specifically for *S. lumutensis* and its habitat in general. Further study in greenhouses should be conducted to increase the information accuracy relating to plant-soil relationships.

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Associations of Growth and Phenology Cycles with Environmental Variables on the Population Dynamics of Non-climbing Rattan *Calamus castaneus* Griff.

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ABSTRACT

In Malaysian forests, the population dynamics of rattan has not been fully documented. Hence, this study was aimed to investigate the population dynamics pattern of the *Calamus castaneus*, a non-climbing rattan. The association of growth and phenology cycle to environmental variables on the population dynamics were studied for one year. Three forest reserves were selected as the study sites: Segari Melintang Forest Reserve (SMFR) in Perak, Teluk Bahang Forest Reserve (TBFR), and Bukit Mertajam Forest Eco-Park (BMFEP) in Penang. The fieldwork was conducted from March 2017 until March 2018. With the plot size of 10 m × 10 m (100 m²), five plots were established in each study site. All the *C. castaneus* individuals inside the plots were marked with numbered plastic tags. A total of 180 individuals were evaluated: TBFR (52 individuals), SMFR (86 individuals), and BMFEP (42 individuals). The findings have revealed that the *C. castaneus* abundancy is comparatively similar in all sites, as shown by the Kruskal-Wallis test with no significant difference ($p > 0.05$) between all life stages. The canonical correspondence analysis (CCA) for all sites has displayed that the rattan growth parameters are closely associated with the microclimate parameters and soil properties. Overall, SMFR showed the fastest growth increment and the largest population size of *C. castaneus* among all three sites.

Keywords: Arecaceae, *Calamus castaneus*, non-climbing rattan, palm, population dynamics

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INTRODUCTION

Rattan (derived from the Malay word "rotan") is a type of a spiny climbing palm from the Arecaceae family under the subfamily of Calamoideae (Dransfield, 2001). Rattan is mostly used to make furniture (Sastry, 2001). Thirty (30) out of 106 species from 8 genera of rattan found in Peninsular Malaysia are harvested and utilized by the country's rattan industry. Rattan from the genus *Calamus* mostly has high economic value with international rattan trade over USD 6.5 billion per year (Ali & Barizan, 2001; Dransfield, 1979; Wan Ariffin et al., 2018). However, in this study, the species monitored is the non-climbing rattan, *Calamus castaneus* (Figure 1). This species has no flagellum or cirrus which usually are the climbing parts of rattan. The *C. castaneus* is easily recognized by its striking scaly chestnut-colored fruits and dull dirty grey indumentose under the leaflet. Primates such as macaques devour the sweet yet acidic taste of *C. castaneus* (Dransfield, 1979; Ruppert et al., 2016). This species prefers a watercourse area with a shady canopy to grow (Dransfield, 1979). Aside from roofing material, this species also possesses medicinal value. The fruit is utilized by the aboriginal people to treat cough (Dransfield, 1979; Sunderland & Dransfield, 2002).

Calamus castaneus is selected as the model rattan species to be studied is due to the availability of this species. It is a very familiar rattan in Peninsular Malaysia and this species can be easily found. According to Ruppert et al. (2012), this rattan species possesses broad lush green leaves which creates shade for growth of plants with low light tolerant. Dransfield (1979) stated that this genus was also known for its symbiosis with the ants. The leaves act as litter-collector which provide building materials

Figure 1. Calamus castaneus with yellowish-based spines

for ants to build a nest and in return provide nutrient for the host. As a result of low yield of long canes and large diameter stem, this species is rarely exploited by humans making its natural populations unaffected (Kidyoo & McKey, 2012). Hence, by knowing the importance of this species in maintaining forest ecology, further studies on its population dynamics were conducted. *Calamus* is spiny climbing palms and can climb up to 10 meters. To avoid these difficulties, *C. castaneus*, an acaulescent palm, were chosen.

In this study, the phenology of the *C. castaneus* plant was monitored for a year in three forest reserves in the northern region of Peninsular Malaysia. The focus of this article is to observe the life cycle of *C. castaneus* population for a year and to associate with its environmental requirement. Renuka and Rugmini (2007) stated that the population dynamics and population structure differed according to habitat confines. Thus, it was expected that there would be a difference in regeneration status, population size, and the relationship between plant phenology and environmental variables (e.g. climate and habitat factors) throughout the year.

MATERIALS AND METHODS

Description of the Study Sites

The study was conducted at three dipterocarp forests in the northern region of Peninsular Malaysia which were Teluk Bahang Forest Reserve (TBFR; 05° 26' 34" N, 100° 13' 14" E) and Bukit Mertajam Forest Eco Park (BMFEP; 05° 21' 57" N, 100° 28' 58" E) in Pulau Pinang as well as Segari Melintang Forest Reserve (SMFR; 04° 19' 34" N, 100° 34' 57" E), which is located in Perak as shown in Figure 2. The total area of the TBFR is 117 ha. This lowland dipterocarp forest is strictly protected as a Virgin Jungle Reserve (VJR). The BMFEP covers 37 ha and were well known to the locals as a

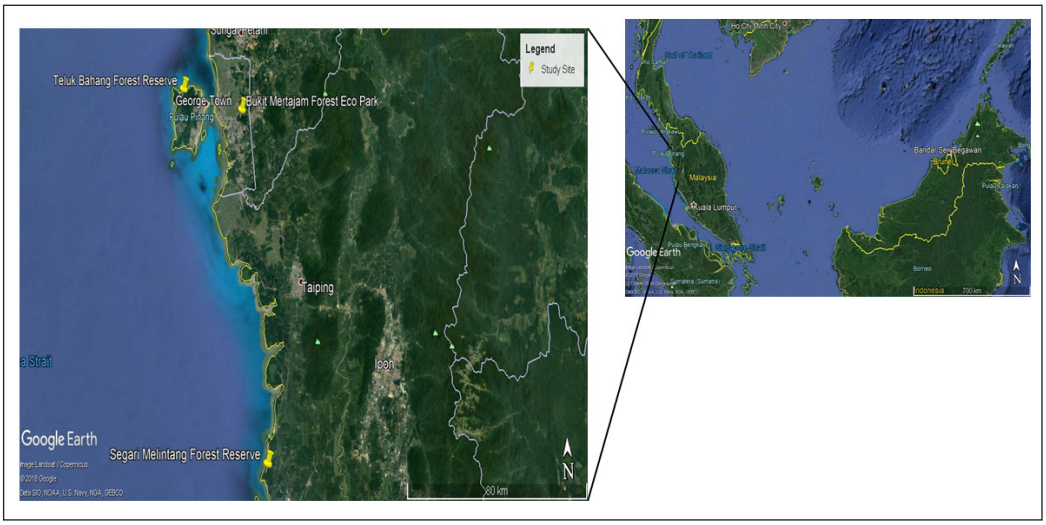

Figure 2. Map of the three study sites, which were extracted from Google Earth

famous hiking spot as it comprises of a hilly area. Both sites act as the water catchment area of which Teluk Bahang Dam is located near TBFR while Mengkuang Dam is close to BMFEP. The SMFR is located along the coastal area of the Manjung district and is mainly composed of primary coastal forest and alluvial freshwater swamp vegetation (Rusdi, 2019). There is a watercourse area inside the plot on this study site. Only 408 ha from 2741 ha of the SMFR is protected as a VJR while the rest was logged before and regenerated as Permanent Forest Reserve (PFR). This study was conducted for a year, between March 2017 to March 2018.

Plots Preparation and Rattan Assessment

Using the square plot design, five plots of 10 m × 10 m (100 m²) were established within each of the study sites. Forest trail was used as the main reference line where plots were established 500 m from the trail entrance. The cluster of rattan within the sampling plots was randomly distributed. To facilitate the monitoring of the whole population in a day, the distance between plots was fixed at 50 meters. In these plots, all individuals of the *C. castaneus* were identified and marked with numbered plastic tags (Bøgh, 1996). The identification of the specimens was based on the keys in "A Manual of the Rattans of the Malay Peninsula" (Dransfield, 1979). A complete sample of the *C. castaneus* stem with fresh sheath, inflorescences, and/or infructescences was collected from the field and deposited at the USM Herbarium. To analyze the influence of climate on the recurrence of such annual phenomena of *C. castaneus*, parameters such as plant sexes, life stages, leaf sheath number, presence of flower, fruit, and length of petiole and rachis were recorded monthly for 12 months. Only one leaf with complete petiole and rachis were tagged and recorded throughout the studies. However, three readings were taken each time to get the average length. The life stages of the studied rattan species such as its seedling, young, or adult were noted. Life stages were classified according to height since this species in an acaulescent rattan: 1) seedling - ≤ 1 meter, 2) young plant - ≥ 1 meter but without signs of flowering and fruiting, and 3) adult - ≥ a 1-meter plant that bears flower or fruit. The sexes of each plant were identified by observing the flowers or fruits from the previous seasons (Kidyoo & McKey, 2012). Meanwhile, the length of petiole and rachis were measured using a measuring tape.

Microclimate Sampling and Soil Analysis

Microclimate readings (i.e. relative humidity, air temperature, soil temperature, light intensity, percentage of gap opening, and level of disturbance) were taken *in situ* every month between 10 a.m. until 12 p.m. time range (Hardwick et al., 2015). A portable thermo-hygrometer (Hanna Instrument model HI 9564) was used to measure the air temperature and relative humidity while a portable luxmeter (Hanna Instrument model HI 97500) was used in measuring light intensity. The readings were taken 1.5 meters from the ground near the cluster of

C. castaneus inside the plots. Using a soil thermometer, the soil temperature was taken in a cleared ground that was not covered by any rocks or leaf debris to avoid damaging the soil temperature's probe. The readings were taken three times each when the meter reading was already stable. For soil analysis which includes soil bulk density, soil moisture content, soil pH, and soil texture analysis, the topsoil samples from 0 - 15 cm depth were collected using polyvinyl chloride (PVC) tube. Three soil samples were taken near the cluster of studied species. The soil samples were sealed in a zip-lock storage bag for soil analysis purposes. The soil pH was measured using a pH meter kit with a water ratio of 1: 5. The determination of soil texture was made using the hydrometer method. The percentage of sand, silt, and clay were referred to as the USDA textural triangle in determining the texture of the soil.

Canopy Gap Opening

The percentage of gap opening status and disturbance level (Table 1) were referred to as Mansor (2001).

Level of Disturbance

Table 1
Percentage range and gap opening status

Percentage range (%)	Gap opening status
0 - 25	Closed area
26 - 40	Partly closed area
41 - 60	Slightly opened area
61 - 80	Partly opened area
81 - 100	Highly opened area

The level of disturbance was modified from Mansor (2001) by Rozali (2014) using disturbance index (DI) considering certain factors such as ecology, socioeconomic, and infrastructure. The index was scaled from 1 until 5 with 1 represented as the lowest disturbed and 5 represented as the highest disturbed (Table 2).

Based on the following disturbance index (DI) calculation, the percentage of disturbance were calculated:

$$DI = g_1 + g_2 + g_3 + g_4 + g_5$$

where:
g_1 = Trails
g_2 = Number of visitors
g_3 = Land use
g_4 = Water supply
g_5 = Gap forest cover

$$\text{Percentage of disturbance} = [(g_1 + g_2 + g_3 + g_4 + g_5) / (25)] \times 100$$

Based on the total disturbance index (DI) from each plot, the percentage of disturbance were ranged as in Table 3.

Data Analysis

All *C. castaneus* individuals inside the plots were counted and the growth parameters were observed and recorded every month. To calculate the mean abundance of the *C. castaneus*, statistical package for social sciences, SPSS software version 21 was used to perform the normality test and Kruskal-Wallis test. Non-parametric of the Kruskal-Wallis test were applied as the

Table 2
Characterization for disturbance criteria

Feature	Disturbance scale
	Trails (g1)
Infrastructure:	
Unexplored forest overgrown by big trees	1
Unexplored forest and filled with bushes	2
Explored forest with small trails < 50 cm width (rarely used by visitors)	3
Explored forest with bigger trails > 50 cm width (always used by visitors)	4
Paved roads	5
	Visitors per day (g2)
Socioeconomics:	
1 - 15 visitors	1
16 - 25 visitors	2
26 - 35 visitors	3
36 - 45 visitors	4
> 45 visitors	5
	Land use (g3)
Ecology:	
Virgin forest	1
Secondary forest	2
Reserve forest	3
Agriculture	4
Clearings forest	5
	Water resource (g4)
Big streams or waterfalls	1
Watercourse	2
Recreational ponds	3
Damp	4
Dried area, no watercourse in the forest	5
	Canopy opening (g5)
0 - 25 %	1
26 - 40 %	2
41 - 60 %	3
61 - 80 %	4
81 - 100 %	5

data for this analysis violates the parametric assumptions. Microclimate data were analyzed using the parametric test: one-way ANOVA with post-hoc Tukey test.

Table 3
Percentage range and disturbance status

Percentage range (%)	Disturbance status
0 - 25	Low disturbed area
25 - 50	The moderately low disturbed area
50 - 75	The moderately high disturbed area
75 - 100	Highly disturbed area

The association between the environmental parameter on the population dynamics of the *C. castaneus* were analyzed using the canonical correspondence analysis (CCA) and was performed with the CANOCO version 4.5. CCA was used as it performs quite well with highly intercorrelated environmental variables and with a situation where not all the factors determining species composition are known (Palmer, 1993).

RESULTS

Life Stages and Population Size

A total of 180 *C. castaneus* individuals were observed during the one-year sampling period. From Table 4, Segari Melintang Forest Reserve (SMFR) displayed the most abundant of seedling (40 individuals), adult plant (41 individuals), and the fewest amount of young plant (5 individuals) compared to the other two sites. This site also showed the highest abundance (86 individuals) of *C. castaneus* followed by Teluk Bahang Forest Reserve (TBFR) (52 individuals) and lastly Bukit Mertajam Forest Eco-Park (BMFEP) (42 individuals). TBFR displayed the smallest number of seedlings individual (10 individuals), adult plant (6 individuals) but with the highest

amount of young plant (36 individuals). Canopy opening and vegetation may have affected the distribution of the *C. castaneus* on the site since this species prefers shady and watercourse area.

Leaf-sheath number per individual in TBFR and SMFR showed an increment but BMFEP showed otherwise (Table 5). SMFR recorded a rapid growth on petiole and rachis length per month in all life stages. On the other hand, TBFR and BMFEP displayed a similar rate for average petiole and rachis length per month. SMFR recorded the fastest rate for an average of rachis length (adult & young) and young petiole. Generally, the rate of average adult petiole length was the same for all sites. Overall, the average height of seedling per month in SMFR was the fastest with 11.6 cm/month followed by BMFEP with 1.7 cm/month and TBFR with 0.9 cm/month. Generally, all sites showed an increasing rate of mortality with TBFR the highest followed by SMFR and BMFEP. The example of causes of mortality was a landslide, tree fall, cut by a human, scorching (leaf tip burning), predator, heavy rain, and strong wind as shown in Table 5. Recreational activities such as hiking were done by direct observation at the site and informal interviews with the locals. Based on observation, *C. castaneus* were not infected by the disease and mostly die due to natural disaster, human activities (e.g. hiking) and predation (e.g. wild boar). BMFEP (0.084/m²) recorded the smallest population density meanwhile SMFR (0.172/m²) the highest.

Recruitment of *Calamus castaneus*

The Kruskal-Wallis test ($p > 0.05$) in Figure 3 shows that there is no significant difference between the regeneration status of *C. castaneus* seedlings, young plants, and adult plants in all investigated forests.

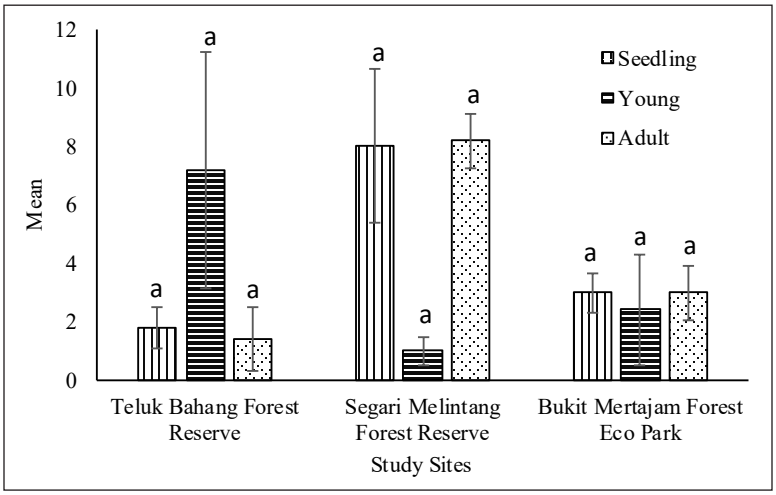

Figure 3. Mean abundance of *Calamus castaneus* in different life stages based on the Kruskal-Wallis test ($p > 0.05$). Bars (mean ± standard error) with letter 'a' showed that there was no significant difference between each site

Table 4
Number of Calamus castaneus *individuals according to different life stages in all study sites*

Life stages	Teluk Bahang Forest Reserve	Segari Melintang Forest Reserve	Bukit Mertajam Forest Eco Park
Seedling	10	40	15
Young	36	5	12
Adult	6	41	15
Total	52	86	42

Table 5
Rate of observed rattan parameters, mortality and population density of Calamus castaneus *individuals for a year*

Site	Life stages	Number of leaf sheath/ individual		Average petiole length (cm)/ month	Average rachis length (cm)/ month	The average height of seedling (cm/month)	Mortality of individuals		Causes of mortality	Population density (per m²)
		t_0	t_1				t_0	t_1		
TBFR	Adult	12	13	0.5 ± 0.1^a	0.6 ± 0.1^a	-	6	2	Landslide, tree fall, cut by human, heavy rain and strong wind	0.104
	young	8	9	0.5 ± 0.0^a	0.6 ± 0.0^a	-	36	16		
	seedling	-	-	-	-	0.9	10	4		
SMFR	Adult	12	13	9.3 ± 2.7^a	15.6 ± 4.0^b	-	41	32	Tree fall, scorching, predator, heavy rain and strong wind	0.172
	young	8	12	4.2 ± 3.1^b	4.2 ± 3.1^b	-	5	2		
	seedling	-	-	-	-	11.6	40	35		
BMFEP	Adult	11	10	0.6 ± 0.0^a	0.6 ± 0.0^a	-	15	14	Landslide, tree fall, cut by human, heavy rain and strong wind	0.084
	young	8	6	0.6 ± 0.0^a	0.6 ± 0.0^a	-	12	12		
	seedling	-	-	-	-	1.7	15	10		

Note. t_0: initial reading (taken in March 2017); t_1: final reading (taken in March 2018). Superscripts a and b indicated the significant differences at $p < 0.05$ by post-hoc Tukey test in each parameter between sites

Relationship of Environmental Variables with the *Calamus castaneus* Phenology

Table 6 shows the mean environmental variables parameter that was recorded from March 2017 until March 2018. The data was collected twice each month. One-way ANOVA was used to analyze the significant difference between each site since microclimate is a parametric data. The significant difference between sites was denoted as in Table 6. From ten variables listed, only four which were air temperature, light intensity, canopy gap opening, and soil bulk density displayed the same on each site.

The CCA of growth and environmental data which are summarized in Tables 7, 8, and 9 indicate that the species-environment correlations were low of which eigenvalues (the score of the maximized dispersion of the species on the ordination axis and the strength of an axis) were less than 0.5 (values over 0.5 often express a good division of the species along the axis). The cumulative variance explained by the first three axes of the species-environment

Table 6
Environmental variables parameter (Mean ± SE) measured from March 2017 until March 2018

Parameter	TBFR	SMFR	BMFEP
Relative humidity (%)	83.70 ± 1.34^{b}	71.59 ± 2.21^{a}	81.14 ± 2.18^{b}
Air temperature (°C)	25.0 ± 2.14^{a}	28.50 ± 1.01^{a}	25.35 ± 0.40^{a}
Light intensity (kLux)	1.00 ± 0.18^{a}	1.11 ± 0.18^{a}	1.10 ± 0.18^{a}
Soil temperature (°C)	26.15 ± 0.10^{b}	26.81 ± 0.15^{c}	24.28 ± 0.14^{a}
Canopy gap opening (%)	25.5 ± 4.77^{a}	46.7 ± 8.47^{a}	36.4 ± 5.41^{a}
Disturbance index (%)	37.6 ± 0.98^{b}	30.8 ± 2.15^{a}	60.8 ± 1.5^{c}
Soil pH	5.74 ± 0.15^{b}	6.09 ± 0.33^{b}	4.82 ± 0.05^{a}
Soil moisture content (%)	24.82 ± 0.65^{a}	32.26 ± 3.30^{a}	45.26 ± 3.70^{b}
Soil bulk density (gcm^{-3})	0.97 ± 0.12^{a}	0.91 ± 0.14^{a}	0.76 ± 0.09^{a}
Soil texture analysis	Sand	Sand	Loamy sand

Note. Superscripts a, b and c indicated the significant differences at $p < 0.05$ by post-hoc Tukey test in each parameter between sites

Table 7
Summary of the CCA of the Calamus castaneus *plant growth and environmental data in Teluk Bahang Forest Reserve*

Axes	1	2	3	4	Total inertia
Eigenvalues	0.213	0.005	0.001	0	0.651
Species-environment correlations	0.744	0.159	0.147	0.226	
Cumulative percentage variance					
of species data	32.8	33.6	33.7	33.7	
of species-environment relation	97.1	99.4	99.8	99.9	
Sum of all eigenvalues					0.651
Sum of all canonical eigenvalues					0.22

Table 8
Summary of the CCA of the Calamus castaneus *plant growth and environmental data in Segari Melintang Forest Reserve*

Axes	1	2	3	4	Total inertia
Eigenvalues	0.119	0.017	0.002	0.001	0.738
Species-environment correlations	0.555	0.236	0.48	0.282	
Cumulative percentage variance					
of species data	16.2	18.5	18.8	19	
of species-environment relation	85.1	97.4	98.9	99.8	
Sum of all eigenvalues					0.738
Sum of all canonical eigenvalues					0.14

Table 9
Summary of the CCA of the Calamus castaneus *plant growth and environmental data in Bukit Mertajam Forest Eco-Park*

Axes	1	2	3	4	Total inertia
Eigenvalues	0.193	0.008	0.002	0.001	0.699
Species-environment correlations	0.697	0.194	0.427	0.224	
Cumulative percentage variance					
of species data	27.6	28.8	29	29.2	
of species-environment relation	94.3	98.2	99.1	99.7	
Sum of all eigenvalues					0.699
Sum of all canonical eigenvalues					0.205

relationship in the CCA in TBFR was 99.8% (Table 7), SMFR is 98.9 % (Table 8), and BMFEP with 99.1 % (Table 9). The CCA ordination plot is as displayed in Figures 4, 5, and 6. The direction and length of the arrows which spread out from the centre of the ordination diagram illustrate the strength and direction accordingly between plant growth and environmental variables.

Figure 4 displayed that three out of five plant growth parameters listed in a study such as a rachis length, petiole length and the number of leaf sheath were increased as the increasing value of soil bulk density, canopy gap opening, air temperature, disturbance index level, relative humidity, soil temperature, soil pH, and percentage of soil moisture content. Light intensity gives no effect on the rattan parameter listed. The number of flowers has no relation to the environmental parameter listed.

From Figure 5, soil pH and soil bulk density give a negative influence on the number of leaf sheath produced, an average of petiole and rachis length. The higher the value of soil pH and soil bulk density will decrease the number of leaf sheath produced, an average of petiole and rachis length. Besides that, a high percentage of soil moisture content, disturbance index level, and canopy gap opening will also reduce the number of flowers produced throughout

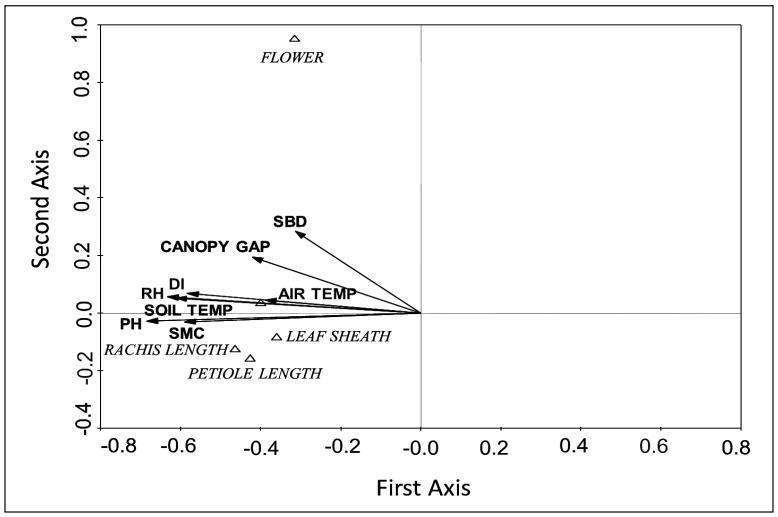

Figure 4. Ordination plot of canonical correspondence analysis (CCA) between environmental variables (arrows) and *Calamus castaneus* plant growth (triangles) in Teluk Bahang Forest Reserve. *Note*. SBD = soil bulk density; DI = disturbance index; RH = relative humidity; SOIL TEMP = soil temperature; AIR TEMP = air temperature; PH = soil pH; SMC = soil moisture content

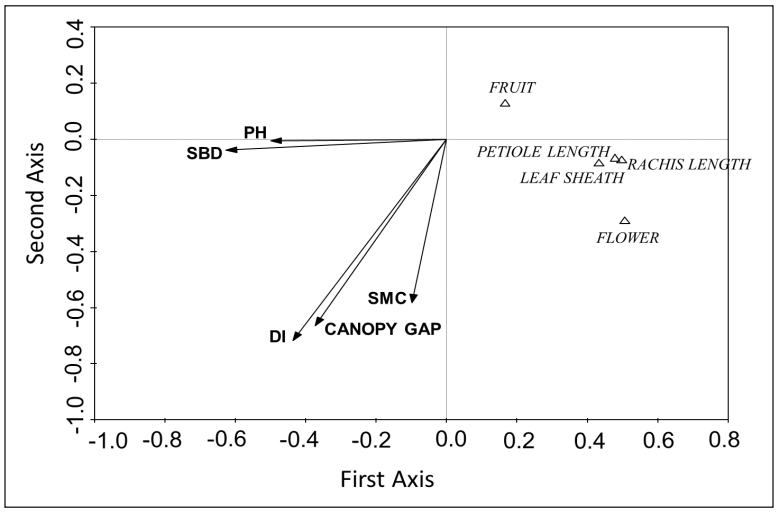

Figure 5. Ordination plot of canonical correspondence analysis (CCA) between environmental variables (arrows) and *Calamus castaneus* plant growth (triangles) in Segari Melintang Forest Reserve. *Note*. SBD = soil bulk density; DI = disturbance index; PH = soil pH; SMC = soil moisture content

the year. Meanwhile, the production of *C. castaneus* fruits in SMFR will increase by lowering the percentage of soil moisture content, percentage of canopy gap opening, disturbance index, soil bulk density, and soil pH.

All nine environmental parameters that were tested in this study had a positive influence on the rattan parameter (Figure 6). In BMFEP site, as the increasing value of soil bulk density, light intensity, percentage of canopy gap opening, disturbance index

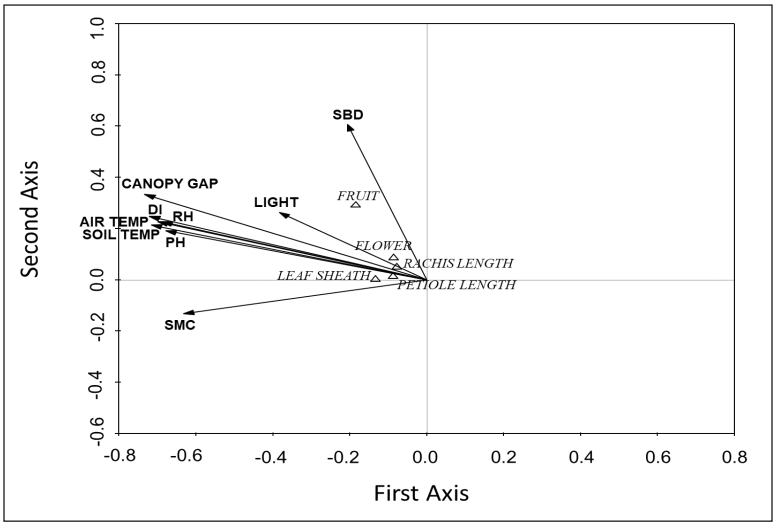

Figure 6. Ordination plot of canonical correspondence analysis (CCA) between environmental variables (arrows) and *Calamus castaneus* plant growth (triangles) in Bukit Mertajam Forest Eco-Park. *Note.* SBD = soil bulk density; DI = disturbance index; RH = relative humidity; SOIL TEMP = soil temperature; AIR TEMP = air temperature; PH = soil pH; SMC = soil moisture content; Light = light intensity

level, relative humidity, air temperature, soil temperature, soil pH, and soil moisture content would increase fruit and flower production, a number of leaf sheath produced, rachis length and also petiole length.

DISCUSSION

Recruitment, Mortality and Population Size

Segari Melintang Forest Reserve (SMFR) displayed a tremendous number of individuals followed by Bukit Mertajam Forest Eco-Park (BMFEP) and Teluk Bahang Forest Reserve (TBFR). From observation, TBFR, and BMFEP plots suffered from landslides, the tree falls from heavy rain and strong winds during monsoon season in November 2017 until January 2018. This had affected the population of *C. castaneus* in both sites. There was no significant difference ($p > 0.05$) shown after performing the Kruskal-Wallis test in all life stages in all sites. This had proven that the abundance of *C. castaneus* is the same for all sites. According to Rozali (2014), the continuation of regeneration in a locality was dependent on the abundance of seedlings in that area. This is proven by looking at the small seedlings number in BMFEP which shows a lower level of a succession of this rattan growing into the adult plants compared to the other two sites.

Although SMFR displayed the greatest number of seedlings, only a few of them would make it to the young stage. The competitiveness between the young plant with the fully established mature plant was high, thus resulting in the lower population of the young *C. castaneus* plants in this site. Throughout this study, SMFR had suffered from large tree falls in July 2017 caused by

strong coastal wind during heavy rain. This finding suggests that natural disasters such as tree falls had altered the regeneration of rattan and dispersion of seedlings in the site. The tree falls had generated gap opening that caused scorching during the seedling stage. Vongkaluang (1985) stated that the high opening of the canopy and direct penetration of light might be the major cause for the non-survival of seedlings in open areas. Strong wind and tree fall had also damaged the leaf sheath of *C. castaneus*. Based on Table 5, BMFEP displayed a decreased number of leaf sheath production throughout the year. It can be concluded that all three sites experienced high mortality due to tree falls. Another cause of mortality was predation. A predator such as wild boar had been noticed eating fallen fruit of *C. castaneus* and digging soil near the clump had crushed the new rattan seedlings which result in mortality.

Besides that, a high rate of recreational activities such as hiking in a site would reduce the rattan population and creating gap opening particularly in TBFR and BMFEP (personal observation). Hikers especially in the large group tend to cut off rattan or any disturbing plants that came across the trail. The low level of rattan formation in the sites are mostly due to these undesirable conditions. A large population size in SMFR might be due to its composition of background vegetation. *C. castaneus* prefers to grow on lower hillslopes and streamsides (Dransfield, 1979). Based on the observation in this study, this species fits more in a partially cleared area with a shady canopy gap. The rattan habit of clumping makes this species to vegetate near one another, creating *C. castaneus* population. Human activities have put stress on rattan regeneration on the site. By avoiding these circumstances, the juveniles' growth might raise (Renuka & Rugmini, 2007; Rusdi, 2019).

Overall, the SMFR displayed a speedy rate for young petiole length, rachis length, and a number of leaves produced per month compared to the other two sites (Table 5). On the other hand, TBFR and BMFEP show a similar rate for rachis and petiole length. A study on a threatened rattan species by Renuka and Rugmini (2007) found that the population structure and population dynamics would differ according to habitat confines. Hence, it is estimated that the SMFR provides the most favorable habitat and the requirement for the succession and growth of the *C. castaneus*, therefore increasing the population density of this species on the site.

In a natural population, the growth rate may vary among the rattan species as it relies upon environmental factors and genetic variations (Rusdi, 2019). In addition, it is common for the Arecaceae family to develop slower during the early stage (Bøgh, 1996; Dransfield & Manokaran, 1994). Moreover, rattan species that grow under larger trees in the forest also showed a slow growth rate (Table 5) (Dransfield, 1979). According to the percentage range and gap opening status by Mansor (2001), all three sites have partly closed and slightly opened areas within a range of 25% to 47%

canopy gap opening. Thus, this might be the reason for the slow growth in the height of the seedlings compared to other parts of the listed rattan growth (Table 5).

Association Between Microclimate Parameters and Soil Properties on Population Dynamics

Most of the environmental variables; relative humidity, air temperature, light intensity, soil temperature, percentage of canopy gap opening, disturbance index, soil pH, soil moisture content and soil bulk density have had a positive influence on the *C. castaneus* plant growth; a number of the leaf sheath, petiole length, rachis length, number of flowers and fruits produced in TBFR (Figure 4) and BMFEP (Figure 6). Soil temperature can influence the community structure of any plant species through the alteration of its anatomical and physiological processes (Rozali, 2014). Warmer soil temperature as in tropical regions probably relates to the high diversity and abundance of plants and rattan species. In addition, certain minerals are needed since growth in stem diameter is possible at a high acidity level. High soil bulk density would help in increasing the rate of nutrient absorbent from the soil (Rusdi, 2019). On the other hand, high relative humidity is caused by the small opening of the forest canopy. This situation is sufficient for the growth of these rattan species as it grows under the low light requirement of a well-aged forest with dense canopies (Powling, 2004). Some rattan species depend upon the soil with adequate moisture and high light intensity to grow (Powling, 2004). Binh (2009) also stated that the increasing growth rate of rattan was dependent upon the amount of light exposure on its crown.

However, from the CCA ordination plot of the SMFR in Figure 4, it is shown that moisture content (32.26%), soil bulk density (0.91 gcm^{-3}), percentage of gap opening (46.7%), soil pH (6.09), and disturbance index (30.8%) have had a negative influence on the *C. castaneus*. Highly acidic soil may affect rattan maturity and would cause retardation in plants (Lilly, 2010). Other than that, a high bulk density of soil restricts the growth of root, inhibits the gaseous exchange in the root zone, and might also decrease the penetration of water (Brady & Weil, 2013; Lilly, 2010). Based on one year of sampling, this species does have a specific environmental condition for its growth since it favored the watercourse and shady area (Dransfield, 1979).

CONCLUSION

The abundance of the *C. castaneus* is the same since there was no significant difference in each study site. Among the three sites, SMFR recorded the fastest increment of young petiole length and rachis length for adult and young plants. Meanwhile, the rate for rachis and petiole length for TBFR and BMFEP were similar. Causes of mortality such as tree fall, landslides, heavy rain, strong winds, scorching, and predation had damaged the leaf sheath and the plants itself hence resulting in death. SMFR showed the largest population size of *C. castaneus* with 0.172 individuals per m^2.

Generally, the population size was strongly dependent on environmental factors. It can thus be concluded that almost all listed environmental parameters will give either a positive or negative influence on the phenology and mortality of the *C. casteneus* in all three sites.

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Effects of Age at Slaughter and Sex on Carcass Characteristics and Meat Quality of Betong Chicken

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ABSTRACT

The purpose of this study was to determine the effects of age at slaughter and sex on carcass characteristics and meat quality of Betong chickens that were raised under a semi-free-range system. A total of 360 chickens from Takbai, Narathiwat Province, Thailand were arranged into 3x2 factorial design with 3 levels of age at slaughter (16, 20, 24 weeks) and sex (male and female) in CRD. The experiment consisted of 6 treatments with 3 replications. Ten chickens of each replicate from each treatment group were randomly sampled for carcass yield and meat quality. Results showed that slaughter and carcass weights were significantly higher when the age at slaughter increased ($P<0.01$). Males had heavier slaughter and carcass weights ($P<0.001$) with lower breast percentage than the female chickens in each particular age. Higher yellowness value was significantly illustrated when the age of chickens increased. This color value was higher in both breast and thigh meat of the female chickens ($P<0.05$). Considering texture analysis, the males had significantly higher shear force value than the females ($P<0.05$). This shear value tended to increase when the age of the chicken increased ($P>0.05$). There was no significant difference observed between age at slaughter on chemical composition ($P>0.05$). Nevertheless, the males had less fat content in breast meat and higher collagen content in thigh meat than the female Betong chickens ($P<0.05$). The results show that males should be slaughtered at 20 weeks, while the females

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should be slaughtered when they reach the age of 24 weeks.

Keywords: Age at slaughter, Betong chicken, meat quality, sex

INTRODUCTION

Betong chicken (*Gallus domesticus*) is a native Thai chicken predominantly found in the three provinces located in the Deep South of Thailand, especially in the Betong District, Yala Province, and in the northern part of Hulu Perak, Perak State, Malaysia. Farmers in the Deep South of Thailand commonly raise this chicken indoors until eight weeks into the growth period. Then they are raised under semi-free-range conditions until reaching the market weight of 1,500 to 2,000 g for females or 2,000 to 2,500 g for males (Chanjula et al., 2004; Chatreewong & Waree, 2006; Nualhnuplong et al., 2019b).

From a literature review, some research work on Betong chicken has been reported, for example, phenotypic characteristics and productive performance of this chicken (Chanjula et al., 2004); optimum age and weight of Betong chicken for the market (Chatreewong & Waree, 2006); dietary protein and energy level on growth performance and reproductive system development in the female during growth to pullet period (Buakeeree & Nualhnuplong, 2018); nitrogen-corrected true and apparent metabolizable energy (TMEn and AMEn) of crude glycerin (CG) for Betong chicken (Sopian et al., 2018); and effect of rearing system on meat quality (Nualhnuplong et al., 2019a; Ritchoo et al., 2019). However, less information about the carcass yield and meat quality of Betong chicken has been reported. Thus, this study aimed to provide basic information about the effects of age at slaughter and sex on the carcass yield and meat quality in terms of physical characteristics and chemical composition. Results from this work will be used as reference information for managing Betong chicken production.

MATERIALS AND METHODS

Sample Preparation

A total of 360 male and female Betong chickens were randomly divided into 6 treatment groups. According to the treatment groups, The Betong chickens were arranged as 3 x 2 factorial in a completely randomized design with 3 levels of age at slaughter (16, 20, 24 weeks) and sex (male and female). Each treatment group consisted of 3 replicates (pens) of 20 Betong chickens. The chickens in each treatment were raised in a semi-free-range system. They were raised in the house (5 birds/m^2) with access to grass paddock or backyard or garden (3 birds/m^2). During week 1 to 3 chickens were kept in a house and fed commercial diet containing 21% of crude protein (CP), while they were allowed to walk outdoors and fed 19% CP from week 8 to 24. Nevertheless, cooked rice and corn were mixed with a commercial diet at a ratio of 0.5:0.5:1. Once they reached the target age, chickens were sampled from the flock and transferred to slaughter at a local abattoir. The slaughtering method used in this study

had followed the regulations of the National Bureau of Agricultural Commodity and Food Standards (ACFS) (2007), Ministry of Agriculture and Cooperatives, Thailand. After slaughter, eviscerated carcasses were weighed and immediately stored at chill temperature for about 24 h for further determination.

Sample Collection and Analyses

After 24 h, the eviscerated carcasses were weighed and separated for retail parts, such as breasts, thighs, drumsticks, wings, feet, head, neck, and skeletal. The percentages of the chilled carcasses were expressed as a percentage of live weight, while the retail parts were determined based on chilled carcass weight. For meat quality determination, breast and thigh muscles were sampled for physical and chemical analysis. Ten (10) Betong chickens of each replicates from each treatment group were randomly sampled for the carcass yield and meat quality included physical characteristics and chemical composition of meat. The variables for analysis were the following:

Physical Properties Determination. The pH of breast and thigh muscles was measured by digital pH meter (Seven2Go, Mettler-Toledo, Switzerland) 45 min after slaughter for pH_0 and 24 h after slaughter for pH_{24}. Meat and skin color were measured on the raw muscle and skin with a Konica Minolta colorimeter (Konica Minolta, Japan) after 24 h chill at 4°C. The result was reported on the complete International Commission on Illumination (CIE) system color profile of lightness (L*), redness (a*), and yellowness (b*). The meat samples for drip loss and cooking loss were trimmed to a dimension of 2.0 x 1.0 x 0.5 cm. Each piece was weighed (weight before storage = W1) and put into a sealed plastic bag, with drip loss stored at a chilled temperature (4°C) for 24 h. The sample was then removed from the sealed plastic bag, blotted, and weighed (W2 is the weight after storage); the cooking loss was measured by heating in a water bath at 80°C for 10 min. After cooking, the sample was cooled to room temperature and weighed (W2 is the weight after cooking). Drip loss and cooking loss were calculated as a percentage of weight loss: ((W1- W2)/ W1) x 100. Shear force was measured on cooked meat samples using a Texture Analyzer (TA-XT plus Stable Micro System Texture Analyzer, UK) with a 2 mm/s crosshead speed and a 50-load cell. The score obtained after a Warner-Bratzler blade cut the sample represented the shear force (Dawson et al., 1991).

Chemical Composition Determination. Moisture, crude protein, crude fat, and ash were analyzed according to the Association of Analytical Chemists (AOAC) (2006). Total collagen was determined after acid hydrolysis as described by Palka (1999). Hydroxyproline contents in the hydrolysate were determined by the procedure of Bergman and Loxley (1963) and converted to collagen contents using the factor 7.25, as described by Wattanachant et al. (2004).

Statistical Analysis. The data on carcass percentages and meat quality were analyzed by two-way Analysis of Variance (ANOVA) in a 3 x 2 factorial completely randomized design (3 age levels and 2 sex levels). The significance between means was analyzed using Duncan's Multiple Range Test (DMRT) in the Statistical Analysis System (SAS) (1998).

RESULTS AND DISCUSSION

Effects of Age at Slaughter and Sex on Carcass Characteristics and Retail Cuts of Betong Chicken

The effects of age at slaughter and sex on carcass yield and retail part percentage of Betong chicken are shown in Tables 1 and 2, with a significant interaction effect between age at slaughter and sex on slaughter weight and breast percentage ($P<0.05$) (Figure 1). Figure 1 clearly shows that the slaughter weight of male Betong chickens tended to increase from the age of 16 weeks to 20 weeks and the weight was stable until reaching the age of 24 weeks, while the female chickens in the 16-20 weeks had no significant difference in weight. But slaughter weight tended to be higher when the chicken reached the age of 24 weeks old. Under this condition, both sexes had higher weight at slaughter at 24 weeks than the chicken aged 16 and 20 weeks. The range of slaughter weight obtained in this work was about 1,896-2,051 g in the male and 1,476-1,562 g in female.

When discussing the retail cuts, slaughter age did not show any significant effect on breast meat percentage of the male ($P>0.05$), but breast meat was significantly lower when the slaughter age increased ($P<0.05$). More breast muscle percentage in chicken aged 16 and 20 weeks old, after which it tended to decrease at the age of 24 weeks. Nevertheless, the male had a lower percent breast (18.39-19.10%) than the female (20.15-20.93%).

Table 1

Effects of age at slaughter and sex on carcass yield (g) and carcass percentage (%) of Betong chicken (mean ± SD)

Items		Slaughter weight	Warm carcass weight	Chill carcass weight	Carcass percentage
Age	16	1,689.17^{b}	1,175.73^{b}	1,158.82^{b}	68.50
	20	1,770.17^{a}	1,236.75^{a}	1,217.30^{a}	68.73
	24	1,787.60^{a}	1,275.88^{a}	1,238.98^{a}	69.09
P-value		0.0005	<0.0001	0.0006	0.3746
Sex	Male	1,993.93^{a}	1,401.79^{a}	1,377.31^{a}	69.24^{a}
	Female	1,505.23^{b}	1,045.99^{b}	1,025.51^{b}	68.25^{b}
P-value		<0.0001	<0.0001	<0.0001	0.0040

Table 1 *(Continued)*

Items		Slaughter weight	Warm carcass weight	Chill carcass weight	Carcass percentage
Age	16	1,689.17[b]	1,175.73[b]	1,158.82[b]	68.50
	20	1,770.17[a]	1,236.75[a]	1,217.30[a]	68.73
	24	1,787.60[a]	1,275.88[a]	1,238.98[a]	69.09
P-value		0.0005	<0.0001	0.0006	0.3746
Sex	Male	1,993.93[a]	1,401.79[a]	1,377.31[a]	69.24[a]
	Female	1,505.23[b]	1,045.99[b]	1,025.51[b]	68.25[b]
P-value		<0.0001	<0.0001	<0.0001	0.0040
Interaction between sex x age					
Male	16	1,896.67±118.07[b]	1,326.87±94.95	1,308.83±86.20	69.02±1.92
	20	2,045.16±154.10[a]	1,433.45±112.10	1,412.58±108.23	69.08±1.57
	24	2,051.74±170.58[a]	1,450.50±151.58	1,414.27±148.13	69.64±2.46
Female	16	1,481.67±122.93[d]	1,024.60±107.17	1,008.80±105.66	67.98±2.12
	20	1,476.21±158.96[d]	1,026.48±126.39	1,008.55±109.50	68.35±2.49
	24	1,562.59±118.08[c]	1,094.28±119.97	1,063.69±116.09	68.45±2.78
P-value		0.0020	0.1282	0.0603	0.9610
SEM		27.136	22.566	21.423	0.408

[a,b] Means within the column with different superscripts differ significantly (P<0.05)

Table 2

Effects of age and sex on the retail cut (%) of Betong chicken (mean ± SD)

Items		Breast	Thigh	Drumstick	Wing	Skeletal
Age	16	19.61	18.49	16.30	12.59[a]	30.25[b]
	20	19.60	18.64	16.27	12.28[b]	32.38[a]
	24	19.70	19.12	16.64	12.23[b]	31.64[a]
P-value		0.8949	0.0884	0.0921	0.0359	0.0004
Sex	Male	18.73[b]	19.37[a]	18.03[a]	12.64[a]	30.32[b]
	Female	20.53[a]	18.13[b]	14.88[b]	12.09[b]	32.54[a]
P-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Interaction between sex x age						
Male	16	18.39±1.42[c]	18.95±1.49	17.97±0.92	12.75±0.81	29.42±3.12
	20	19.10±1.05[c]	19.21±1.76	17.65±1.06	12.58±0.80	31.30±2.79
	24	18.75±1.15[c]	19.98±1.41	18.47±0.94	12.59±0.82	30.19±2.18

Table 2 *(Continued)*

Items		Breast	Thigh	Drumstick	Wing	Skeletal
	20	20.15±1.11[b]	18.04±0.98	14.79±0.94	11.96±0.78	33.53±2.68
	24	20.47±1.50[ab]	18.29±1.69	14.87±0.76	11.83±0.73	33.04±4.05
P-value		0.0108	0.3511	0.0623	0.2348	0.4369
SEM		0.232	0.296	0.185	0.147	0.537

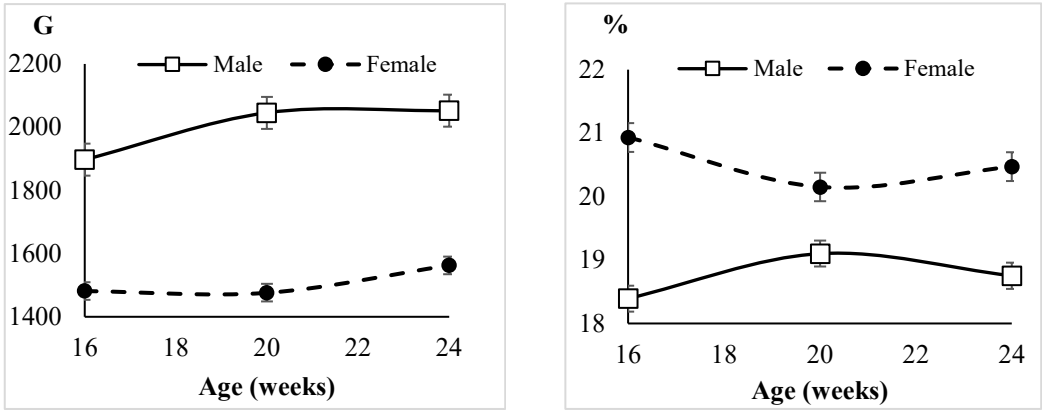

Figure 1. Effect of interaction between age and sex on slaughter weight (left) and breast percentage (right)

Considering the effect of age at slaughter, slaughter weight, warm carcass weight, chilled carcass weight, and skeletal percentage were increased as the age at slaughter increased (P<0.01). The highest slaughter, warm carcass, and chilled carcass weights were observed at 24 weeks of age. According to the finding of Poltowicz and Doktor (2012), carcass percentage of the slow-growing broiler at 84 days was higher than that at 70 and 56 days (P<0.05). However, based on the percent of slaughter weight, chickens at 16 weeks of age had the highest wing percentage, but the lowest skeletal percentage, when compared with chickens at 20 and 24 weeks (P<0.05).

In terms of sex differences, males had significantly higher slaughter, warm carcass, and chilled carcass weights, and higher carcass, thigh, drumstick, and wing percentages than female chickens. Nevertheless, breast and skeletal percentages of the female were significantly higher than those of the male (P<0.01). This result was following Lopez et al. (2011), who reported that male broilers had higher body weight but lower carcass and breast percentages than females (P<0.05).

Effects of Age at Slaughter and Sex on Physical Characteristics of Betong Chicken Meat

The effects of age at slaughter and sex on the physical characteristics of Betong chicken meat are presented in Tables 3, 4, and 5. Age at slaughter had a significant effect on the pH_0 of breast and thigh meat, and pH_{24} of breast meat (P<0.01), with

pH_0 value tending to increase with age at slaughter. However, this work found that sex affected pH_{24} in both breast and thigh meat ($P<0.01$). The males had higher pH_{24} values in breast meat than the females. This was in concordance with the work of Poltowicz and Doktor (2012) who found a lower pH_{24} of broiler meat at 56 days than that of meat from broiler aged 70 and 84 days ($P<0.05$). Similar work was obtained by Atthaporn et al. (2009) who found that high pH_{24} value in Thai native chicken (Kai Nong Dang) when the age of chicken increased. The value of pH in meat was related to the amount of glycogen in the muscle and the stress condition of the animal (Warriss, 2000) which were resulting in the level of lactic acid in the meat. This result was closely associated with the color and water holding capacity of the meat (Warriss, 2000; Wattanachant, 2008).

From the results, significant interaction effects on the shear force value of breast meat, drip loss, and color (particularly lightness (L*) and redness (a*) of thigh meat) were noted among the age at slaughter and sex ($P<0.05$) (Figure 2).

Considering the effect of age at slaughter, this study indicated that the shear force value in male Betong chicken meat tended to be increased when the slaughter age increased. Although this value did not show any significant difference between the age of 16 and 20 weeks, it was significantly higher at the age of 24 weeks old. This work was similar to the work of Atthaporn et al. (2009) who found a higher shear force value of breast and thigh muscle when the age of Thai native chicken increased. A similar result was reported by Poltowicz and Doktor (2012) on a slow-growing broiler. The increasing shear force value when the age of chicken increased is not only related to the increasing of muscle fiber diameter (Atthaporn et al., 2009; Dransfield & Sosnicki, 1999; Wattanachant, 2008) but also associated with a higher intermolecular cross-linking of connective tissue in meat (Castellini et al., 2002; Chen et al., 2013; Dawson et al., 1991; Husak et al., 2008). Sex had a significant effect on the shear force value of both breast and thigh meat ($P<0.05$), with males having higher values than females ($P<0.05$). This work was in agreement with Fanatico et al. (2005), and Chen et al. (2006). This was due to the larger diameter of muscle fiber and more thickness of perimysium (Wattanachant, 2008).

No interaction between age at slaughter and sex on drip and cooking losses of the breast meat (Table 3) was observed, but significantly interaction effect on drip loss of the thigh meat (Table 4). Both sexes had a lower drip loss percentage at the age of 16 weeks, but it significantly increased when the age at slaughter increased ($P<0.01$). This report was in agreement with the work of Li et al. (2020), although earlier researches found no significant differences between male and female chicken meat on water holding capacity (Chen et al., 2006; Fanatico et al., 2005; Kirmizibayrak et al., 2011; Lopez et al., 2011; Uhlirova et al., 2018).

In terms of the color of meat (Table 5), it was found that the female Betong chickens had higher L*, but lower a* than male

Table 3

Effects of age at slaughter and sex on pH value, drip loss, cooking loss and shear force of breast meat Betong chicken (mean ± SD)

Items		pH_0	pH_{24}	Drip loss (%)	Cooking loss (%)	Shear force (kg/cm^3)
Age	16	5.92[b]	5.77[b]	2.09	16.21[b]	3.06
	20	6.10[a]	5.80[ab]	2.09	18.64[a]	3.22
	24	6.10[a]	5.82[a]	1.85	17.47[ab]	3.67
P-value		<0.0001	0.0032	0.3508	0.0010	0.0707
Sex	Male	6.04	5.82[a]	1.88	18.42[a]	3.61[a]
	Female	6.04	5.77[b]	2.14	16.45[b]	3.12[b]
P-value		0.8877	0.0009	0.1000	0.0002	0.0273
Interaction between sex x age						
Male	16	5.94±0.19	5.79±0.06	2.09±0.78	17.61±2.93	2.83±1.13[b]
	20	6.10±0.19	5.82±0.11	1.80±1.04	19.20±3.50	3.44±1.48[ab]
	24	6.10±0.16	5.84±0.08	1.77±1.28	18.41±3.76	4.19±1.74[a]
Female	16	5.91±0.22	5.75±0.08	2.09±0.78	14.81±3.56	2.54±0.35[b]
	20	6.11±0.25	5.77±0.09	2.42±0.58	18.04±3.32	2.99±1.27[b]
	24	6.10±0.29	5.81±0.09	2.33±1.36	16.56±3.86	3.26±1.00[b]
P-value		0.8756	0.6225	0.2356	0.5263	0.0471
SEM		0.040	0.016	0.190	0.639	0.244

[a,b] Means within the column with different superscripts differ significantly (P<0.05)

Table 4

Effects of age at slaughter and sex on pH value, drip loss, cooking loss and shear force of thigh meat Betong chicken (mean ± SD)

Item		pH_0	pH_{24}	Drip loss (%)	Cooking loss (%)	Shear force (kg/cm^3)
Age	16	6.26[b]	6.09	0.71[b]	21.05	3.75
	20	6.42[a]	6.07	1.11[a]	22.30	3.81
	24	6.44[a]	6.05	1.15[a]	20.94	4.18
P-value		<0.0001	0.1184	<0.0001	0.3801	0.0715
Sex	Male	6.37	6.03[b]	0.89[b]	21.22	4.13[a]
	Female	6.37	6.09[a]	1.08[a]	21.65	3.75[b]
P-value		0.8605	0.0066	0.0061	0.6269	0.0209
Interaction between age x sex						
Male	16	6.28±0.24	6.04±0.12	0.77±0.33[c]	21.50±3.92	4.00±0.93
	20	6.38±0.20	6.05±0.06	1.10±0.40[b]	21.60±6.50	3.91±0.94

Table 4 *(Continued)*

Item		pH_0	pH_{24}	Drip loss (%)	Cooking loss (%)	Shear force (kg/cm^3)
	24	6.45±0.25	6.05±0.09	1.00±0.37^{bc}	20.51±7.37	4.42±0.70
Female	16	6.23±0.30	6.14±0.20	0.75±0.38^{c}	20.59±5.80	3.42±1.35
	20	6.47±0.17	6.09±0.06	1.11±0.40^{b}	23.04±6.54	3.69±1.04
	24	6.43±0.19	6.05±0.07	1.40±0.70^{a}	21.35±4.73	3.96±1.01
P-value		0.2785	0.0608	0.0083	0.5202	0.5479
SEM		0.042	0.021	0.085	1.080	0.184

^{a,b} Means within the column with different superscripts differ significantly (P<0.05)

Table 5

Effects of age at slaughter and sex on color meat of breast and thigh meat Betong chicken (mean ± SD)

Items		Breast meat			Thigh meat		
		Lightness (L*)	Redness (a*)	Yellowness (b*)	Lightness (L*)	Redness (a*)	Yellowness (b*)
Age	16	48.52^{a}	1.43	4.90^{b}	49.86^{a}	3.83^{b}	4.82
	20	48.51^{a}	1.54	4.83^{b}	51.30^{a}	4.77^{b}	5.07
	24	45.90^{b}	1.63	5.89^{a}	45.92^{b}	5.89^{a}	5.34
P-value		0.0056	0.3173	0.0112	<0.0001	0.0002	0.7221
Sex	Male	47.43	1.70^{a}	4.51^{b}	46.69^{b}	6.70^{a}	4.52^{b}
	Female	47.96	1.31^{b}	5.87^{a}	51.44^{a}	2.65^{b}	5.67^{a}
P-value		0.4671	0.0005	<0.0001	<0.0001	<0.0001	0.0337
Interaction between age x sex							
Male	16	48.09±4.14	1.53±0.76	4.03±1.59	48.86±6.50^{bc}	5.57±2.91^{b}	4.84±3.56
	20	47.58±6.36	1.74±0.79	4.01±2.38	47.81±5.23^{c}	7.50±3.18^{a}	4.85±3.34
	24	46.43±3.18	1.84±0.87	5.63±2.48	43.01±4.03^{d}	8.02±2.51^{a}	4.81±2.66
Female	16	48.95±4.00	1.33±0.26	5.78±1.71	50.87±4.45^{b}	2.09±2.72^{d}	4.80±1.86
	20	49.43±5.40	1.30±0.26	5.72±2.33	55.03±5.02^{a}	2.85±1.82^{d}	5.28±3.92
	24	45.47±4.32	1.29±0.95	6.12±1.93	48.55±3.45^{bc}	4.01±1.90^{c}	6.52±3.30
P-value		0.2689	0.6728	0.2833	0.0035	0.0235	0.0954
SEM		0.865	0.208	0.382	0.894	0.482	0.590

^{a,b} Means within the column with different superscripts differ significantly (P<0.05)

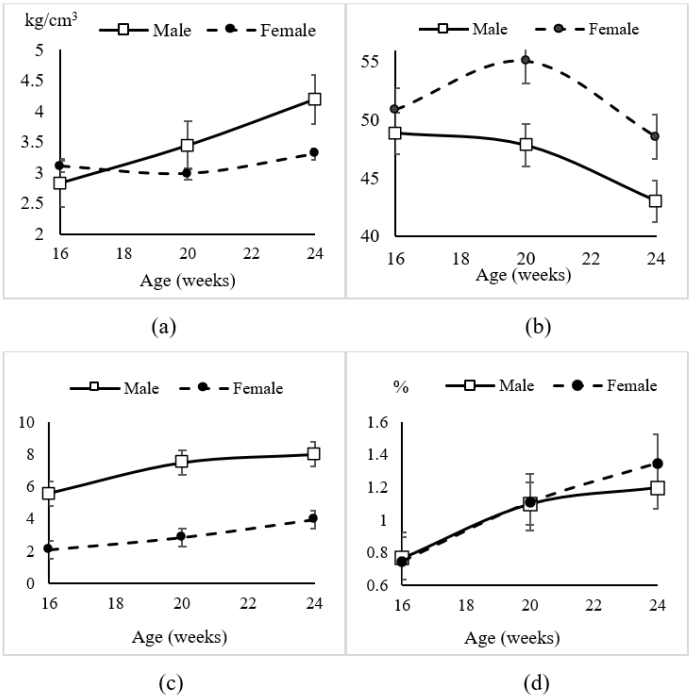

Figure 2. Effect of interaction between age and sex on the shear force of breast meat (kg/cm^3) (a), lightness of thigh meat (b), redness of thigh meat (c), and a drip loss percentage of thigh meat (d)

chickens for 16-24 weeks. The L* of both males and females tended to decrease from the age of 16 weeks to the age of 24 weeks. Nevertheless, redness (a*) of both sexes tended to increase when the age of chicken increased. Although there was no significant effect on the lightness of breast meat of the male and female chickens (P>0.05), males showed higher reddish meat than the female Betong chickens. Likewise, we found that in chicken meat, higher b* was observed in the older Betong chickens. This value could result from xanthophyll in diet and natural carotenoids from plants' free-range. The higher b* value was observed in the breast muscle (P<0.05), while the females had higher b* value than the male chickens. The result of this present study was the following results from Kirmizibayrak et al. (2011) and Uhlirova et al. (2018), that male chicken's meat had a higher a* value than the meat of females. Similar results were reported by Lopez et al. (2011) and Fanatico et al. (2005), who found higher b* values in female chicken meat than in male meat (P<0.05). Higher a* in the male than the female chicken was probably related to the higher activity of the male than the female. However, males had lower L*and b* values than female chickens. Besides, chicken meat color may depend on many factors such as genetic, raising system, type of feed, and age at slaughter (Wattanachant, 2008).

Table 6

Effects of age at slaughter and sex on chemical meat quality of breast and thigh (mean ± SD)

Items		Breast meat				Thigh meat			
		Moisture (%)	Protein (%)	Fat (%)	Collagen (%)	Moisture (%)	Protein (%)	Fat (%)	Collagen (mg/100g)
Age	16	74.34	24.13	0.40	5.34	75.84	20.75	3.12	9.39
	20	74.52	24.09	0.42	6.30	75.90	20.70	3.23	10.18
	24	74.73	24.09	0.44	6.59	75.72	21.09	3.30	12.30
P-value		0.3235	0.9879	0.1376	0.2573	0.6786	0.4564	0.2523	0.1395
Sex	Male	74.96[a]	23.87[b]	0.34[b]	6.86	76.16[a]	20.51[b]	3.15	12.75[a]
	Female	74.12[b]	24.34[a]	0.50[a]	5.52	75.41[b]	21.21[a]	3.32	9.10[b]
P-value		0.0189	0.0347	<0.0001	0.2697	<0.0001	0.0159	0.2126	0.0117
Interaction between age x sex									
Male	16	74.67±0.66	23.82±0.86	0.31±0.03	6.01±0.37	76.33±0.70	20.51±0.91	3.06±0.15	10.57±1.57[b]
	20	74.69±1.02	23.84±0.65	0.35±0.03	6.92±0.80	76.29±0.46	20.36±1.27	3.13±0.00	11.43±1.55[b]
	24	74.54±0.73	23.93±0.90	0.36±0.02	7.39±1.08	75.88±0.64	20.66±0.61	3.25±0.18	16.25±1.70[a]
Female	16	74.03±0.50	24.53±0.92	0.49±0.00	4.90±1.29	75.15±0.68	21.05±0.46	3.16±0.03	8.22±0.38[c]
	20	74.34±1.38	24.32±0.68	0.50±0.03	5.86±1.63	75.47±0.61	21.03±1.48	3.32±0.35	9.34±1.10[b]
	24	73.73±0.70	24.25±0.51	0.52±0.04	5.79±1.14	75.55±0.70	21.53±1.20	3.48±0.37	9.67±1.59[b]
P-value		1.0000	0.74000	0.7051	1.0000	0.1635	0.9455	0.9124	0.0500
SEM		0.167	0.139	0.021	0.219	0.117	0.196	0.219	0.545

[a,b] Means within the column with different superscripts differ significantly (P<0.05)

Effects of Age at Slaughter and Sex on Chemical Composition of Betong Chicken Meat

As seen in Table 6, no significant interaction between age at slaughter and sex on the proximate composition of either breast or thigh meat (P>0.05). However, the highest collagen content was found in thigh meat from 24-week old male Betong chicken while the lowest collagen content was found in 16-week old females. Nevertheless, age at slaughter did not show any significant effect on any parameters (P>0.05), although the collagen content tended to increase as the age at slaughter increased. High collagen content when the age of chicken increased was following shear force value reported in Table 3. In this study, the result of proximate analysis contradicted that of Boni et al. (2010), who found a significantly higher fat content but lower levels of protein, moisture, and ash contents in older quail than in younger quail (P<0.05). However, in this study, the higher collagen content in meat from 24-week-old Betong chicken than in 16-and 20-week old was probably due to the type of collagen content and muscle fiber diameter as described by Wattanachant (2003). This was confirmed by Atthaporn et al. (2009), who found larger fiber diameter and perimysium thickness in Thai native chicken meat (Kai Nok Dang) as the age of the chicken increased. Fat contents tended to increase with age at slaughter in both breast and thigh meats (P>0.05). A high amount of fat deposition when the age of chicken increased was related to the reduction of energy for muscular growth, while high energy feed supplements could lead to more body fat deposition. This was in agreement with the work of Atthaporn et al. (2009), Nikolova et al. (2007), and Zerhdaran et al. (2005).

In terms of sex, the breast and thigh meat of males had higher moisture content but lower protein content than that of females (P<0.05). In addition, the male Betong chickens had lower fat content in breast meat with higher collagen content in thigh meat than the females (P<0.05). This was similar to the work of Corzo et al. (2005) and Nikolova et al. (2007), who studied broilers, and Atthaporn et al. (2009), who studied in Thai native chicken (P<0.05).

CONCLUSION

Based on our findings, a distinctive feature of raising Betong chickens under a semi-free-range system was that the meat was low in fat content. (0.31 to 0.52% in breast and 3.06 to 3.48% in thigh meat). However, because the male Betong chicken grew much faster than the female, raising the male until 24 weeks old would increase the amount of collagen content that results in tougher meat than the female. Also, more reddish meat would be shown when the male was raised until 24 weeks of age. To control the quality of the meat, especially the redness, toughness, and amount of collagen, the male should be raised not more than 20 weeks while the female should be raised until the age of 24 weeks. However, it is recommended that the consumer's preference for Betong chicken meat be explored in further studies.

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Physicochemical Properties of Sodium Alginate Edible Film Incorporated with Mulberry (*Morus australis*) Leaf Extract

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ABSTRACT

In this study, sodium alginate film incorporated with mulberry leaf extract [0-4 % (v/v)] were evaluated in terms of its physicochemical properties. Results showed that with the increase of mulberry leaf extract concentration, the thickness of the film increased (from 0.07 mm to 0.11 mm), while the color of film produced increased in its green and yellow intensity. In terms of mechanical properties, with the increase of mulberry leaf extract concentration, a significant increase in the tensile strength but a significant decrease in the elongation at break of the film were observed, while no significant effect (p>0.05) on the puncture force was observed. Similarly, no significant effect (p>0.05) on moisture content, water solubility, and water activity of sodium alginate films were found, with the increase of mulberry leaf extract. An increase in the total phenolic content from 8.92 mg GAE/100 g to 41.88 mg GAE/100 g was observed when 4 % (v/v) of mulberry leaf extract was added.

Keywords: Mulberry leaf extract, physicochemical properties, sodium alginate, total phenolic content

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INTRODUCTION

The edible film can be defined as the coating of food material which can be consumed together with the food product itself (Pavlath & Orts, 2009). An increase in the demand from consumers towards natural and safe product leads to the work for the development of edible film, where it can serve as alternative coatings for plastic material coatings and packaging (Janjarasskul & Krochta, 2010; Shit & Shah, 2014).

Edible film base materials can be categorized into three groups, namely polysaccharide, protein, or lipid (Vieira et al., 2011). With the application of edible film, the shelf life of food can be extended by minimizing its contact with air, thus reducing prolong lipid oxidation (Cirillo & Spizzirri, 2015). In addition, the edible film can be incorporated with antimicrobial and antioxidant sources such as herbs and spices (Emiroğlu et al., 2010).

Among the different edible film base, alginate is one of the common polysaccharides used for the development of the edible film (Ferreira et al., 2016). It is extracted from seaweed and structured by 1-4b-D-mannuronic acid (M) and a-L-guluronic acid (G) (Kim, 2013). Its fibrous structure can form films, while its hydrophilic properties can reduce lipid oxidation when applied to foods (Pavlath & Orts, 2009; Varela & Fiszman, 2011).

For film formation, glycerol is commonly used as a plasticizer to enhance flexibility, while calcium chloride forms crosslinking between its calcium ions and sodium alginate, improving the film's water resistance (Rhim, 2004; Vieira et al., 2011). In addition, herbs and spices that have an antimicrobial effect such as oregano, rosemary essential oil, thyme essential oil, *Melastoma malabathricum* extract, and 'asam keping' were also incorporated into film (Chan et al., in press; Choong et al., 2019; Jouki et al., 2014a, b; Seydim & Sarikus, 2006; Zaman et al., 2018).

Luís et al. (2019) incorporated licorice essential oil into carboxymethyl xylan film. They found that the film with the presence of licorice essential oil exhibited positive antimicrobial effect against *Enterococcus faecalis* and *Listeria monocytogenes* while Abdollahi et al. (2019) who added summer savory essential oil into carboxymethyl cellulose-agar biocomposite film reported on the high antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus*, *and Listeria monocytogenes* with essential oil at 1.5% (v/v). Also, rosemary and *Aloe vera* essential oil were added into cellulose acetate in which it had high antimicrobial activity against *Escherichia coli* and *Bacillus subtilis* in the study of El Fawal et al. (2019).

Mulberry leaf is a heart shape or mitten-shaped leaf belongs to Moraceae family (Rahman & Khanom, 2013; Srichaikul et al., 2011). It is used as silkworms' foods, papermaking, Chinese medicine, diabetes, and blood pressure treatment (Chen et al., 2007; Vichasilp et al., 2012). Mulberry leaf was reported to have antimicrobial

and antioxidant properties, in which flavonoid such as quercetin-3-glucoside, kaempferol-3-glucoside, and quercetin-3-(6-malonylglucoside) was found (Islam et al., 2008). To the best of our knowledge, the information on mulberry leaf extract based edible film were scarce. Hence, this work aimed to study the effect of mulberry leaf extract on the physical, mechanical, chemical, antimicrobial, and antioxidant properties of alginate edible film.

MATERIALS AND METHODS

Preparation of Mulberry Leaf Extract

Mulberry leaf was obtained from roadside bushes near Puchong, Selangor, Malaysia. It was identified by the red and black mulberry fruit produced from the tree. It was cut and dried overnight at 40 °C and ground using a grinder (Sharp, EM-11, Malaysia).

The extraction process was performed according to Sarbadhikary et al. (2015) with slight modification. The mulberry powder (10 g) was added with ethanol (100 mL) and stirred for 24 hours in a rotary shaker (Infors AG, Switzerland) at 150 rpm before filtration with filter paper (Whatman No. 3, Filters Fiorini, Sweden) and centrifuged (Centrifuge 5810 R, Eppendorf, Germany) for 15 minutes at 9,860 x *g*. The extract was then subjected to the rotary evaporator (R-000, BÜCHI, Switzerland) under vacuum at 40 °C and the extract (50 mg/mL concentration of mulberry leaf extract, with 5 g extract dissolved 100mL solvent) was then kept at 4 °C for further analysis.

Analysis of Mulberry Leaf Extract

Antimicrobial Properties. The disc diffusion method was carried out with *Staphylococcus aureus* and *Escherichia coli* on Mueller-Hinton Agar (Sarbadhikary et al., 2015). Antibiotic chloramphenicol disc (Oxoid, UK, 30 µg) was used as positive control and a paper disc (6 mm diameter) infused with 50 µL of ethanol served as the negative control. The inhibition zone was measured with a micrometer.

Antioxidant Properties of Mulberry Leaf Extract. The antioxidant property of mulberry leaf extract was performed by utilizing DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay (Wong et al., 2014). Mulberry leaf extract (0.1 mL) was added with 3.9 mL of 0.004% ethanolic DPPH solution into a test tube (wrapped with aluminum foil). The mixture was vortexed and left to stand in the dark for 30 minutes before measuring its absorbance value at 517 nm, using a UV-Vis Spectrophotometer (Uviline 9400, Secomam, France). Percentage of DPPH scavenging activity was calculated by using Equation 1:

$$\text{DPPH Scavenging activity (\%)} = \frac{Abs\ of\ DPPH - Abs\ of\ sample}{Abs\ of\ DPPH} \times 100 \quad (1)$$

where Abs of DPPH is the absorbance value of 0.004% ethanolic DPPH solution and the Abs of sample is referring to the absorbance value of the extract at 517 nm.

Total Phenolic Content of Mulberry Leaf Extract. Folin-Ciocalteu's reagent (1.5 mL) was pre-diluted 10 times with distilled water and mixed with mulberry leaf extract (0.3 mL) and sodium carbonate (1.2 mL). The mixture was mixed and left for 30 minutes in dark, before measuring the absorbance at 765 nm with a UV-Vis spectrophotometer (Uviline 9400, Secomam, France). A standard curve was generated by using gallic acid solution (0- 0.1 mg/g), with regression equation of $y = 0.0119x + 0.0124$ and $R^2 = 0.9991$ (Wong et al., 2014).

Production of Edible Film Incorporated with Mulberry Leaf Extract

About 1.5% (w/v) of sodium alginate powder (Synertec, Malaysia) was added with 200 mL of distilled water and stirred for 30 minutes at 70 °C. Glycerol 0.75% (v/v) was added, followed by stirring for another 15 minutes. Mulberry leaf extract with concentration at 0, 1, 2, 3, and 4% (v/v) was added into film solution, respectively. A preliminary study shows with the addition of 5%, the film formed is sticky and not smooth.

The mixture (25 g) was poured on a petri dish (90 mm × 15 mm) and dried at 40 °C for 24 hours in an oven (Memmert, Germany) (Benavides et al., 2012). The sodium alginate film formed was dipped into the calcium chloride solution (45 mL) and dried again for 1 minute. The films formed peeled using forceps and kept in desiccators.

Analysis of Film

Thickness. Micrometer (JY, China) is used to measure the thickness of the film, by placing it at 5 different locations of the film (Garsuch & Breitkreutz, 2009).

Moisture Content and Water Activity. The initial film weight was measured using an analytical balance (XT 220 A, Precisa, Switzerland). After measuring the initial weight, the film was dried in an oven (Memmert, Germany) at 90 °C for 24 hours, before measuring the dried film weight again (Choong et al., 2019). The moisture content was calculated according to Equation 2:

$$\text{Moisture content (\%)} = (M_i - M_0/M_i) \times 100 \quad (2)$$

where M_0 is defined as the initial mass of the film and M_i is defined as the final mass of the film.

On the other hand, the water activity of the film was determined using a water activity meter (AquaLab Pre, METER Group, USA).

Water Solubility. Determination of water solubility was carried out according to the work of Ma et al. (2016) with slight modifications. The film cut into 2 cm × 2 cm and dried in an oven (60 °C) for 24 hours, after which the weight for the dehydrated film (W_1) was measured. The film was then immersed with distilled water (25 mL) for 2 hours at room temperature in a covered condition, after which the film was then dried again for 24 hours at 60 °C). The second weight measured was the final mass (W_2). Water solubility was calculated according to Equation 3:

$$\text{Water solubility} = \frac{W_1 - W_2}{W_1} \times 100\% \quad (3)$$

where W_1 is interpreted as the initial mass of the film and W_2 is interpreted as the final mass of the film.

Color Profile. The color of the film was determined using a colorimeter (ColorFlex Ez, Hunter lab, US) (Zaman et al., 2018). The colorimeter was a spectrophotometer with Pulsed Xenon Lamp as a light source, coupled with a 256-element diode array and a high- resolution concave holographic grating. The colorimeter was calibrated with white and black tile standards before the sample test. The films were then placed against the sample cup to measure the color.

The color was expressed as L* (lightness-darkness), a* (red-green) and b* (yellow-blue) values, while the total color differences (ΔE) for the films was calculated according to the Equation 4:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}, \quad (4)$$

where ΔL*, Δa*, and Δb* represent the difference of readings as the parameter values compared the sodium alginate with mulberry leaf extract film to the pure sodium alginate film.

Tensile Strength and Elongation at Break. The tensile analysis was performed using the tensile testing machine (TA-XT Plus, Surrey, UK) (Remya et al., 2015). Films strips (50 mm x 20 mm) were placed on the grip, with separation of the initial grip fixed at 50 mm. The speed was set at 25 mm/min for the crosshead. Calculations for the tensile strength and elongation at break were proceeded by using Equations 5 and 6, respectively:

$$\text{Tensile strength} = \frac{Peak\ load\ (N)}{\text{Cross} - \text{sectional area (mm}^2\text{)}} \quad (5)$$

$$\text{Elongation at break} = \frac{Final\ length\ of\ film\ ruptured\ (mm)}{\text{Initial grip length (mm)}} \times 100\% \quad (6)$$

Puncture Force. Puncture force was determined using a texture analyzer, with the needle probe (2 mm diameter) moving at a constant rate of 1 mm/s (Muppalla et al., 2014). The puncture force was calculated using Eq. 7:

$$\text{Puncture force} = \frac{Maximum\ force\ at\ break\ (N)}{Thickness\ at\ broken\ area\ (mm)} \quad (7)$$

Fourier Transform Infrared Spectroscopy (FTIR). Fourier transform infrared spectroscopy (FTIR) spectrometer (Nicolet iS5, Thermo Fischer Scientific, USA) equipped with OMNIC Spectra Software in transmission mode was used to measure the spectra absorbance of the film (Pranoto et al., 2005). The resolution was set as 4 cm^{-1} with an average of 20 scans for wavenumbers in between the range of 500 cm^{-1} to 4000 cm^{-1} against a background spectrum from an empty cell.

Antioxidant and Antimicrobial Properties of Film. The antioxidant properties (free scavenging activity and total phenolic content) were performed following the method of Wong et al. (2014), while antimicrobial properties were conducted according to Fernández-Pan et al. (2012).

Statistical Analysis

Data were represented as a mean value ± standard deviation and were analyzed using analysis of variance (one-way ANOVA) and Tukey's post hoc test was used for analyzing the significant difference among the film properties (SPSS version 23). The *p*-value of ≤0.05 was defined as statically significant.

RESULTS AND DISCUSSION

Antimicrobial Properties of Mulberry Leaf Extract

Mulberry leaf extract (50 mg/mL) was found to have an antibacterial effect against *E. coli* with the minimal inhibition zone of 1.46 ± 0.34 mm while having no antimicrobial activity from the mulberry leaf extract against the *S. aureus*. This result is in agreement with the study done by Thabti et al. (2014), who showed that mulberry leaf extract exhibited antibacterial activity against *E. coli*. Besides that, the result of the antimicrobial activity against *S. aureus* is in agreement with the study done by Manjula and Shubha (2011), who reported that there was no antimicrobial activity against the *S. aureus* (concentration of mulberry leaf extract concentration at 25 µL/L).

Antioxidant Properties and Total Phenolic Content of Mulberry Leaf Extract

It was found that the mulberry leaf extract (50 mg/mL) had free radical scavenging activity of 49.88 ± 1.23%. On the other hand, the total phenolic content of mulberry leaf extract was found to be 553.93 mg GAE/100g. These findings are consistent with results shown by the study of Memon et al. (2010) who found similar free radical scavenging activity in mulberry leaf extract (48.13 ± 1.20%). The antioxidant activity and total phenolic content were contributed by flavonoid compounds such as quercetin and rutin (Katsube et al., 2009). According to Katsube et al. (2006), quercetin is the most abundant flavonoid compound present in mulberry leaf thus, contributing to the antioxidant activity of mulberry leaf.

Thickness

Figure 1 (a) shows the physical properties of sodium alginate incorporated with different concentrations of mulberry leaf extract. With the increase of the mulberry leaf extract, it was found that the thickness of the edible films increased after 3% (v/v) incorporation of mulberry leaf extract (from 0.07 mm to 0.10 mm). Further incorporation of the leaf extract (4% v/v) resulted in no significant difference (p>0.05) with those with 3% (v/v) leaf extract. The increase of thickness was also observed in the work of Benavides et al. (2012) in the film added with oregano essential oil, where the thickness increase was due to the increase of total solids in the film-forming solution. As reported by Utami

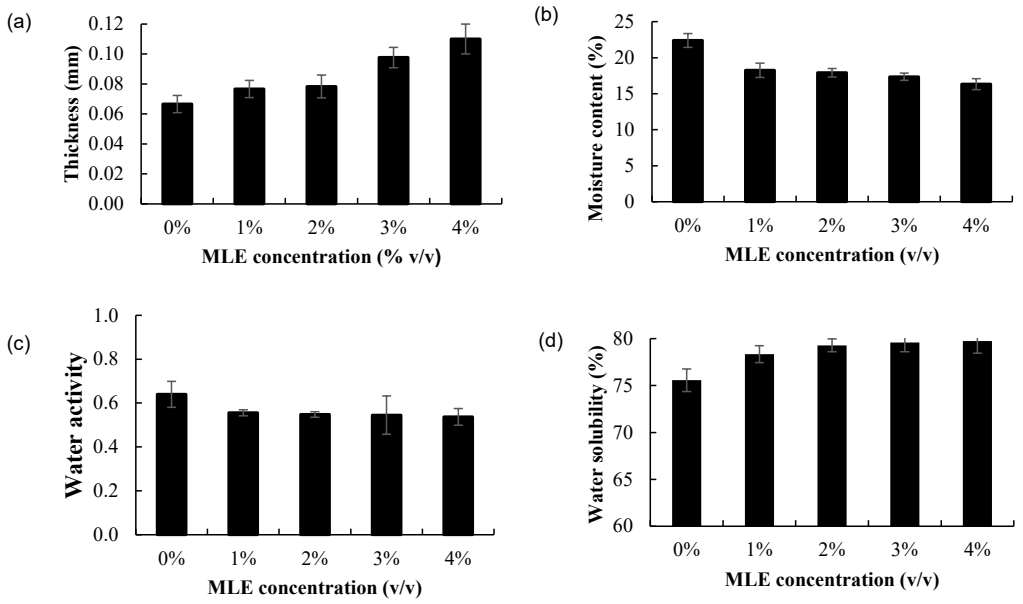

Figure 1. Physical properties including (a) thickness, (b) moisture content, (c) water activity, and (d) water solubility of sodium alginate edible film incorporated with different concentration of mulberry leaf extract (MLE)

et al. (2019), the increase in cinnamon essential oil caused an increase in the total solids of the film-forming solution thus, increasing the film thickness.

Moisture Content and Water Activity

From Figure 1 (b) it was observed the moisture content was decreased with the addition of mulberry leaf extract concentration, with no significance between film added with different extract concentrations (p>0.05). This may be explained by the concentration of mulberry leaf extract at 1% (v/v) that had reached a sufficient level to occupy the microstructural network space of sodium alginate (DeMan et al., 2018). Adding on to that, the decrease in moisture content might be due to the decreased water affinity of films containing more hydrophobic mulberry leaf extract (Han et al., 2018). This is in agreement with the result where an increase of mulberry leaf extract had no significant effect (p>0.05) on the water activity of film produced (Figure 1 c). Low water activity is favored for edible film because it inhibits the growth of organisms and extends shelf life (Ijabadeniyi & Pillay, 2017). Moisture content and water activity of the film influence its moisture absorption behavior, which in turn affects the water solubility (Gennadios & Weller, 1994).

Water Solubility

The result in Figure 1 (d) shows that the sodium alginate without mulberry leaf extract has 75.57% water solubility. Low

water solubility is desirable for edible film, to avoid the complete dissolution of the film (Ozdemir & Floros, 2008). With the addition of mulberry leaf extract [1% - 4% (v/v)], the water solubility of the film was reported to be in the range of 78.35-79.74%. There was no significant difference (p>0.05) between the water solubility of the film when 1% to 4% (v/v) concentration of the mulberry leaf extract was added into the film.

The findings from this analysis are in agreement with the study done by Jutaporn et al. (2011), where the water solubility of the film increased when phayom wood extract was added. The increase in water solubility may be contributed by the decreased polymer network interaction density due to the presence of mulberry leaf extract. Furthermore, alginate film is highly water-soluble, due to its hydrophilic nature (Shit & Shah, 2014).

Color Profile

The color of the film is important as it influences customer acceptance towards a product (Galus & Lenart, 2013). For the films, it is most favorable that the films are light in color or transparent so that it would not affect the food color (Acevedo-Fani et al., 2015). According to Table 1, with an increase in mulberry leaf extract, the sodium alginate film has a decrease in L* value, a* value, while an increase in its b* value and total color change (ΔE). This indicates that the film is darker, greener, and yellower with an increase of extract due to the dark

Table 1

The color profile of sodium alginate edible films with mulberry leaf extract

The concentration of mulberry leaf extract incorporated (%)	Color			
	L*	a*	b*	ΔE
0	25.32 ± 0.77^{c}	-0.26 ± 0.05^{b}	0.27 ± 0.01^{a}	0.00 ± 0.00^{a}
1	21.43 ± 0.38^{b}	0.11 ± 0.04^{c}	2.58 ± 0.13^{b}	4.54 ± 0.25^{a}
2	20.86 ± 0.30^{b}	-0.32 ± 0.03^{b}	4.08 ± 0.16^{c}	5.87 ± 0.21^{a}
3	19.28 ± 1.47^{a}	-0.49 ± 0.03^{a}	4.91 ± 0.17^{d}	7.62 ± 1.33^{b}
4	18.55 ± 0.78^{a}	-0.52 ± 0.05^{a}	5.31 ± 0.15^{e}	8.44 ± 0.74^{c}

Note. $^{a-e}$ Mean ± standard deviations followed by different superscript letters within the same column are significantly different at $p \leq 0.05$ according to Tukey's test

color of mulberry leaf extract. The increase in yellowish and greenish color of the film was from the natural color of the mulberry leaf. Du et al. (2009) reported that the color of the films was influenced by essential oils added, which result in a decrease of L* and a* with the concentration cinnamon essential oil. The result is also in agreement with the study done by Mehdizadeh et al. (2012), who found that there was a decrease in L* and a* with increased *Thymus kotschyanus* essential oil.

Tensile Strength and Elongation at Break

Tensile strength is the maximum amount of stress that a sample can withstand during tensile testing (Benavides et al., 2012). According to Figure 2 (a), the tensile strength increases from 12.91 to 23.45 MPa when the concentration of mulberry leaf extract increased from 1% (v/v) to 3% (v/v). The decrease of tensile strength at high mulberry leaf extract concentrations may be attributed to the formation of discontinuities creating stressed regions in the film matrix, leading to decreased tensile strength (Frank et al., 2018). The decrease of tensile strength at high concentration was also observed by Han et al. (2018) when sodium alginate/carboxymethyl cellulose films were incorporated with cinnamon essential oil.

In terms of elongation at break, the value decreases with the increase of mulberry leaf extract concentration from 0% (v/v) to 4% (v/v), which showed a reduction from 35.60% to 5.76% (Figure 2 b). This result correlates with the findings of Sánchez-González et al. (2010) who found that the addition of tea tree essential oil to chitosan films caused a decrease in elongation at break of the films. This may be due to the increase in compound concentration generating a cross-linking effect through the interaction between polymers with the compound, leading to the reduction of the flexibility for the molecular of the polymer (Ojagh et al., 2010).

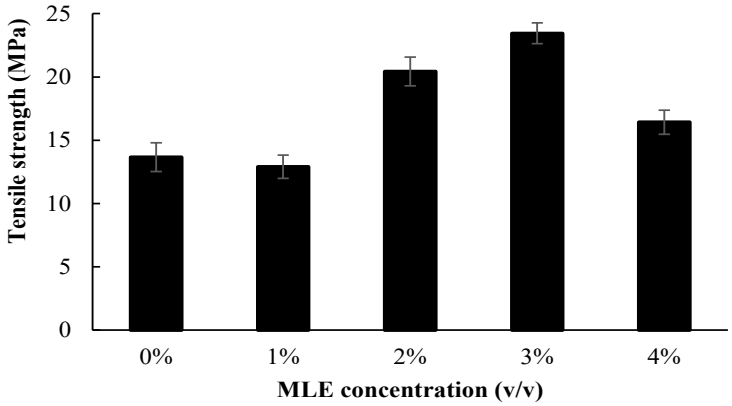

Figure 2. Mechanical properties: (a) tensile strength

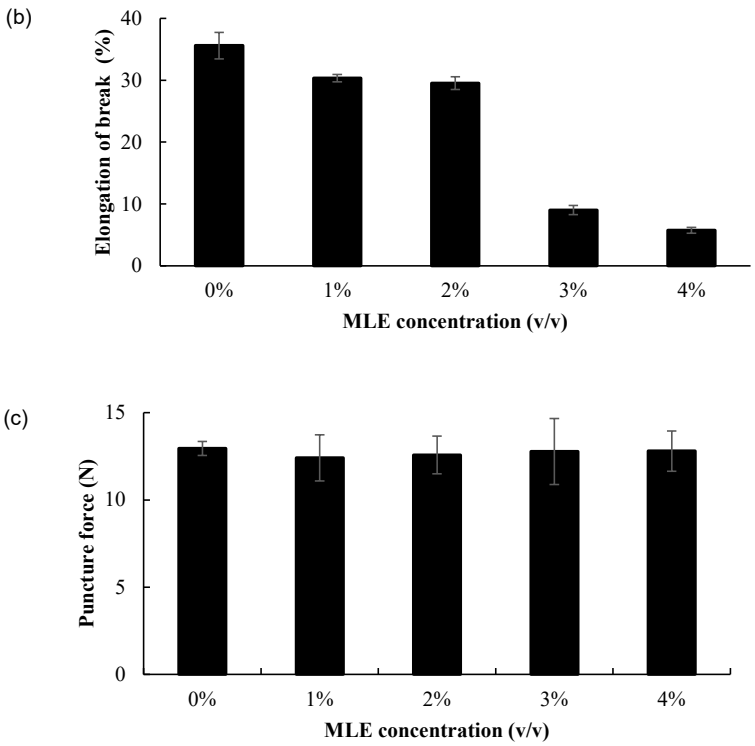

Figure 2 (Continued). Mechanical properties: (b) elongation at break, and (c) puncture force of sodium alginate edible films with mulberry leaf extract

Puncture Force

From Figure 2 (c), it is observed that the puncture force of films ranges from 12.41-12.95 N, with no significant difference (p>0.05) among the films. This result is consistent with the study from Gómez-Estaca et al. (2009), with no significant difference (p>0.05) among the bovine-hide gelatin film incorporated with a low and high concentration of oregano extract. The puncture force did not decrease with an increase in the extract. This is favorable for film properties. Adding on, the same result was observed by Aguirre et al. (2013), who found that there was no significant difference (p>0.05) on the puncture force of triticale protein films incorporated with different oregano essential oil concentrations.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was conducted to identify and analyze the functional group of present and the FTIR spectra can be observed in Figure 3. The film samples had similar broadband around 3279 cm^{-1} to 3286 cm^{-1}, where the absorbance peak was the intensity of O-H stretching (Ba et al., 2010). The appearance of an absorption band for O-H stretching was broad as it was

influenced by the intermolecular hydrogen bonding between the O-H groups. A strong hydrogen bond causes a long O-H bond, and this allows the O-H bond to be stretched easily (Burrows et al., 2017). It can be observed that the amplitude of the band decreased when higher concentrations of mulberry leaf extract were added. This means that the interaction between the bonds was lower at high concentrations. This can explain the lower tensile strength observed when a higher concentration of mulberry leaf extract was added (Tongnuanchan et al., 2013).

The presence of the peak around at 1149 cm^{-1} on the film sample incorporated with 3% (v/v) and 4% (v/v) mulberry leaf extract was associated with the epoxy C-O stretching vibration and glycosidic linkage C-O-C stretching (Čopíková et al., 2013; Hanifah et al., 2015).

The epoxy C-O and glycosidic linkage C-O-C stretching indicate the presence of polysaccharide compound from the mulberry leaf extract. There is also a peak around at 1600 cm^{-1} which indicate the presence of phenolic compound such as chlorogenic acid in the mulberry leaf extract (Liang et al., 2016). However, there is no peak observed around 1600 cm^{-1} for film added with 1% (v/v) and 2% (v/v) mulberry leaf extract, this may be due to the insufficient amount of the phenolic compound from the mulberry leaf extract.

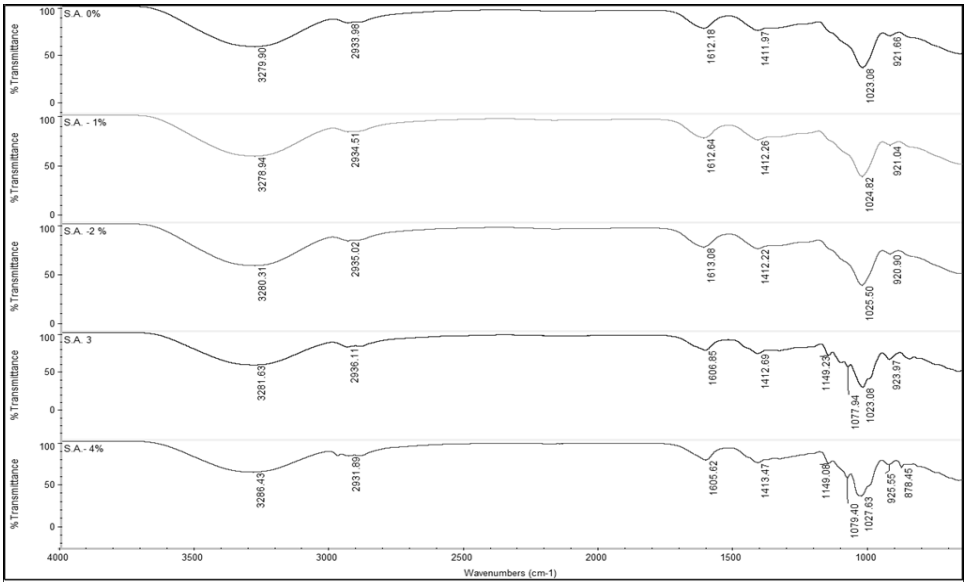

Figure 3. Spectra of Fourier transform infrared spectroscopy (FTIR) of SA-MLE edible film (a) sodium alginate + 0% MLE, (b) sodium alginate + 1% MLE, (c) sodium alginate + 2% MLE, (d) sodium alginate + 3% MLE, and (e) sodium alginate + 4%

Antioxidant Properties and Total Phenolic Content of Sodium Alginate Edible Films with Mulberry Leaf Extract

According to Figure 4 (a), sodium alginate film with no mulberry leaf extract have scavenging activity at 2.48% as the alginate polymer can provide scavenging activities (Ueno & Oda, 2014). With the addition of mulberry leaf extract (from 1%- 4% v/v), the scavenging activity in the film increased from 5.67% to 9.98%. Increasing scavenging activity was due to an increase of mulberry leaf extract concentration (Mehdizadeh et al., 2012).

Similarly, the total phenolic content of the film samples increased from 8.92 mg GAE/100g to 41.88 mg GAE/100g when the concentration of mulberry leaf extract range at 0% (v/v) to 4% (v/v) (Figure 4 b). This is to the addition of mulberry extract which has the total phenolic content (Jouki et al., 2014b). It was also found that there is much reduction of DPPH scavenging activity and total phenolic content, which has decreased 80% and 92%, respectively when the mulberry leave extract (100%) was added into the film in 4% (v/v) concentration.

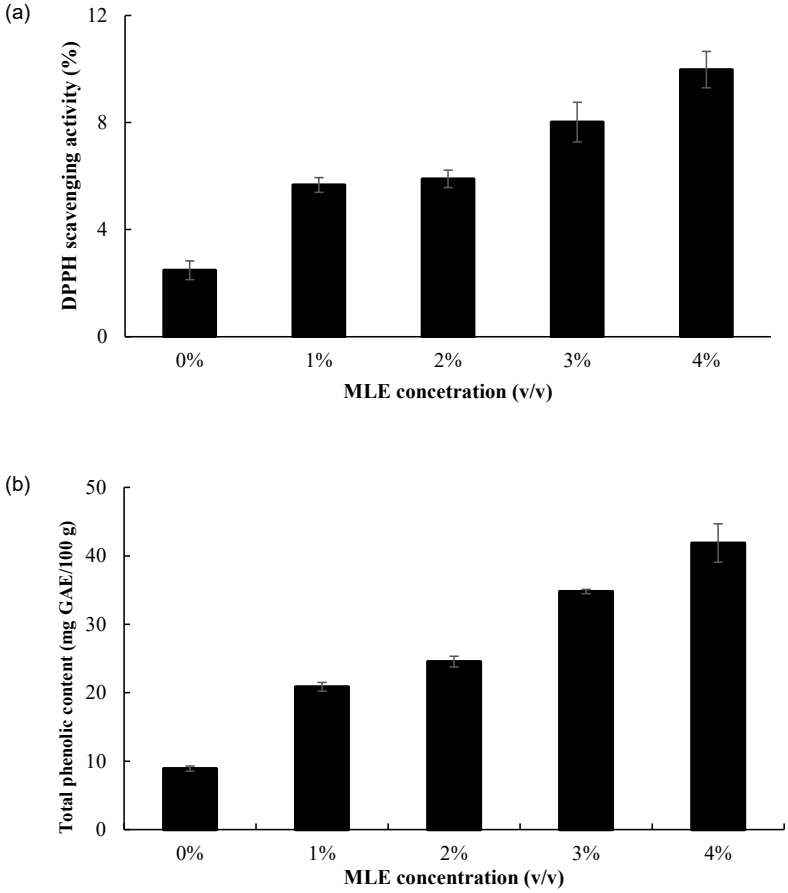

Figure 4. (a) DPPH free radical scavenging activity and (b) total phenolic content of sodium alginate edible films with mulberry leaf extract

Antimicrobial Activity of Sodium Alginate Edible Films with Mulberry Leaf Extract

There was no antibacterial activity found for the sodium alginate edible films with mulberry leaf extract. However, antimicrobial activity was found in the mulberry leaf extract, this may be due to the lesser amount of mulberry leaf extract incorporated into the films as compared to the extract on its own (with 1.46 mm inhibition zone).

This was in agreement with the study of Benavides et al. (2012), where a low concentration of oregano essential oil incorporated into edible film showed no antimicrobial activity. In addition, Chan et al. (2020) also found no antimicrobial acidity in *Melastoma malabathricum* incorporated film, despite the inhibition zone found in the extract.

CONCLUSIONS

With a higher concentration of mulberry leaf extract incorporated, the sodium alginate film shows an increase in thickness which is important for the mechanical aspect of the film. Increased mulberry leaf extract concentration produces films that are darker, greenish, and yellowish. This may decrease consumer acceptance. Besides, the increase of mulberry leaf extract produced a film that is low in tensile strength, a decrease in elongation at the break while not affecting its puncture force. Mechanical properties of the film when added with extract is still weak, hence further study on improving mechanical strength is suggested, by optimizing the concentration of glycerol. The films with mulberry leaf extract contain higher scavenging activity and total phenolic content compared with the film without extract. As the sodium alginate edible film with 3% (v/v) of mulberry leaf extract had higher tensile strength, it was considered as the best formulation among the 4 concentration, although overall the tensile strength is still quite low. The 3% mulberry leaf extract film can be further improved to be a potential film wrapping the foods for enhancing the antioxidant properties and total phenolic content.

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Scanning Electron Microscopy Analysis of Early Floral Development in *Renanthera bella* J. J. Wood, an Endemic Orchid from Sabah

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ABSTRACT

Renanthera bella J. J. Wood is an endemic orchid to Sabah with beautiful bright red petals. Most of the floral development of orchids is significantly affected by seasons and geographical factors. To date, the flower development stages of *R. bella* have never been studied. This study was conducted to observe the morphological changes of flower initiation and early development by apical dissection and scanning electron microscopy (SEM). The floral organs were dissected and fixed in 1.5% glutaraldehyde and dehydrated with alcohol. Characteristic stages of the initial flowering pattern were recorded until the flower was fully open and become senescent. *Renanthera bella* showed a typical acropetal pattern starting with early flowering from the base to the apex, forming a raceme-type inflorescence. Its flower development was divided into ten stages, which started with the flower bud appearance and the initiation of primordia. Next, the flower sepal started to develop within seven days of bud appearance. The final stage occurred after 25 days of observation when the bud opened, with an average bud length and diameter of 1.94 ± 0.56 cm and 0.50 ± 0.29 cm, respectively. The *R. bella* flower maturity stage was achieved between 38 to 40 days after anthesis, with the average length and diameter of petals and sepals increasing to 2.49 ± 0.23 cm and 0.38 ± 0.06 cm, respectively. A capsule successfully formed after one week of pollination. It reached the maturity stage at approximately 15 weeks after pollination. The orchid capsule started to break and expose the seeds inside after 21 weeks. This analysis emphasized the early floral development pattern, which could help estimate the length of flower maturity and pollination.

Keywords: Anther, flower development, pollen, *Renanthera bella*

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INTRODUCTION

Renanthera bella J. J. Wood is an epiphytic orchid endemic to Sabah. This species is distributed from the Mount Kinabalu region to the Lahad Datu district (Chan et al., 1994). According to Pridgeon et al. (2014), *R. bella* can be found at an elevation between 400 to 1200 m and is confined to low-stature hill forests. *Renanthera bella* produces beautiful, attractive blotched-pink to crimson red flowers with a yellowish-cream to apricot yellow base. This species has been widely over-collected and is classified as endangered in Appendix I of the Convention on the International Trade of Endangered Species of Wild Flora and Fauna (Chan et al., 1994). Given the challenges it faces, no studies on the pattern of *R. bella* floral development have been conducted.

Most orchid species are at risk of habitat loss due to various human activities, such as natural habitat destruction and overcollection. Most attempts to culture endangered and rare orchid species *in vitro* have been supported by anatomical and morphological observations of flower development (Arditti & Pridgeon, 1997; Burgeff, 1932). Various reports on molecular genetic analysis of flower development of orchids have been made over the years, such as the floral organ-specific genes (Pan et al., 2014), the role of MADS-box genes in the flower development and evolution (Becker & Theissen, 2003; Tsai et al., 2004; Zahn et al., 2005), and the regulatory mechanisms underlying orchid flower development (Xu et al., 2006). However, the morphological structure of buds and flowers in *R. bella* has not been well-identified.

Scanning electron microscopy (SEM) is used to increase the accuracy of plant and flower surface observations, as well as the development of floral organs. The images provide a more detailed view of the inner floral organs and the leaf surface features of the stomatal guard cells, epidermis, and hairs (if present). SEM was previously used to observe the stoma and micro-level character of the abaxial and adaxial surfaces of *in vitro* and *in vivo Passiflora edulis* leaves (Veeramohan et al., 2013).

Studies on the developmental stages of the inflorescence are essential as they relate to the control of plant reproduction, the pattern of plant growth, the timing of plant production, and how flowers interact with air movement for flowering (Friedman & Harder, 2005). An understanding of plant reproductive biology is essential in plant conservation efforts. In the present study, SEM was used to observe the morphological changes of flower initiation and early development until full bloom by apical dissection. During the observation, it was observed that different flower parts exhibit various patterns of development in their morphological characters. This study can provide basic background information that can be used to predict the maturity of the capsule that is important for seed germination.

MATERIALS AND METHODS

Plant Sources

Three-year-old *R. bella* plants were obtained from the greenhouse of the Institute for Tropical Biology and Conservation (ITBC), Universiti Malaysia Sabah (UMS). The plants were maintained in the greenhouse under natural light, 80% relative humidity, with a temperature of 28/24 ± 2°C day/night. The plants were grown in charcoal and coconut husk medium and fertilized using commercial fertilizer. Measurements for the *R. bella* flowers were recorded for length, diameter, and morphological changes. The *R. bella* flowers were hand-pollinated in September 2017 to form capsules. The plant species were identified by Mr. Johnny Gisil from ITBC and by a botanist from the Poring Orchid Conservation Centre (POCC; 0602′ 20.7″N, 116 06′ 38.2″E). This research was performed at the Tissue Culture Laboratory, ITBC, UMS (06003′ 33″ N, 116 012′ 29″E) under 24 ± 2°C temperature, 80% humidity, and 8/16 hours of light/dark condition.

Observation of Flower Bud Initiation and Development

The flower bud initiation and development of *R. bella* were recorded over two and a half months, starting with bud initiation, to the opening stages, and the formation of the full bloom or mature flower. The method of Smyth et al. (1990) was carried out with minor modifications. The samples were dried in the desiccator (for 1 h) instead of a critical point dryer. Additionally, the concentration of glutaraldehyde used was only 1.5% to prevent damage to the cells and tissues of the flower, as well as the thin surface. Data on bud length, bud width, lip length, lip width, pollen and anther size, and mature petal and sepal length and diameter were recorded weekly. The experiment was conducted using three replicates of plants with a total of eight floral organs per plant. The data was determined by calculating their mean and standard deviation. In addition to these data, the changes in the development of the flowers were recorded from time to time.

Dissection of Floral Organs

The floral organs were dissected into different flower parts or tissue such as bud, anther, pollen, petal, sepal, lip, and column for further observation of their morphology and physiology under the SEM. Both immature and mature floral organs were dissected for the comparison of their development.

Scanning Electron Microscopy (SEM)

The inflorescence and the young buds of *R. bella* were harvested from the greenhouse and dissected immediately into various floral tissues such as bud, anther, pollen, petal, sepal, lip, and column. The bud was held tightly with a pair of forceps and dissected using a blade by cutting the base part of the petal and sepal vertically to reveal the inner tissue (lip, column, anther, and pollen) under the stereomicroscope. The tissues were then fixed in 1.5% glutaraldehyde and incubated at 4°C for 24 h. The samples were then rinsed three times in 0.1 M phosphate buffer with an interval of 10 min. Then, the tissue

materials were dehydrated in a series of ethanol (35%, 50%, 70%, 90%, and 100%) for 30 min at each ethanol concentration. The samples were desiccated for 1 h before mounted on a metal SEM stub and coated with gold in a sputter coater for further observation on the SEM (Zeiss, Germany), and the image was recorded (Smyth et al., 1990).

RESULTS AND DISCUSSIONS

The Morphology of Flower Initiation and Inflorescence Formation

The studies of morphological development of flower bud formation in *R. bella* were based on the methods of physical observation and measurement. To date, the studies and in-depth analysis of *R. bella* flower development are scarce, making it difficult for comparison study.

Initially, the inflorescence started from the interstitial leaf (arrow; Figure 1A) and continue to develop and elongate rapidly after only five days (Figures 1B and 1C). It can grow to 30 cm and longer. Buds and flower organs arise at the peak of the inflorescence in the acropetal chain, where the flower buds begin to mature from the base to the top (Figure 1D). Usually, the inflorescence emerged from the fourth node below the apical leaf (Sakanishi et al., 1980). On the seventh day, cream and brown flower buds with a diameter of 0.02 cm appeared on the apical meristem and formed a raceme type of inflorescence (Figure 1E; stage 1). A raceme is an unbranched,

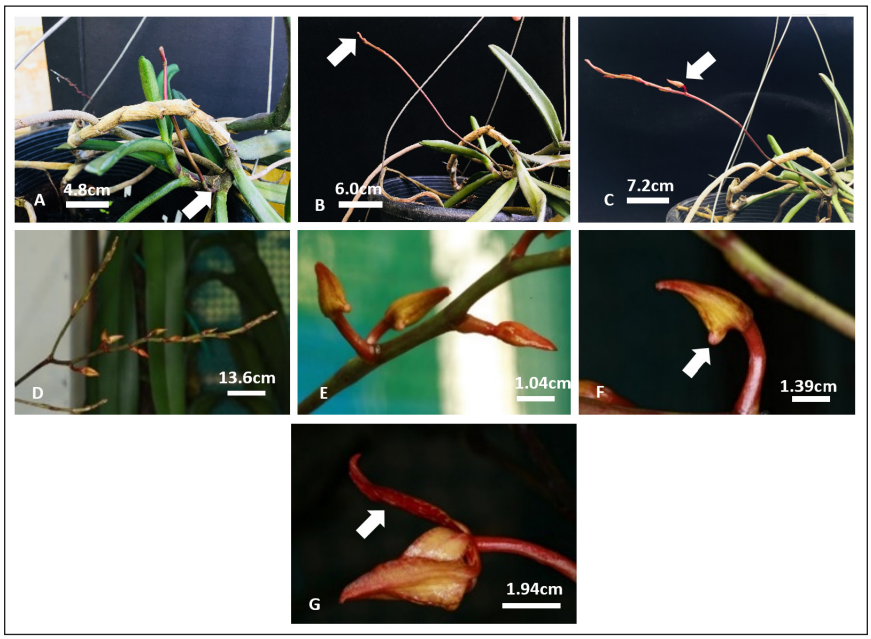

Figure 1. Inflorescence development of *Renanthera bella*. (A) Inflorescence started to arise from the interstitial leaf (arrow); (B) Inflorescence elongated and primordia bud started to appear (arrow); (C) Flower bud started to develop (arrow); (D) *Renanthera bella* flower development with acropetal growth pattern; (E) Formation of raceme inflorescence; (F) Lip started to arise and appear at the bottom of the bud (arrow); (G) Single dorsal sepal started to open (arrow)

elongated inflorescence with pedicellate flowers maturing from the bottom upwards (Harris & Harris, 2004). The lip starts to arise and appear at the bottom of the bud (Figure 1F), whereas the bud begins to open from its dorsal sepal (Figure 1F). There are approximately 7 to 15 flower buds in a single inflorescence. After 25 days, a single dorsal starts to open (Figure 1G).

The description of flower development has been reported in several studies. Previously, the flower development of *Malus* x *domestica* Borkh. (comprising 8 morphological stages) and *Arabidopsis* (comprising 12 stages) were recorded (Foster et al., 2003; Smyth et al., 1990). The overall results indicated that the early development of the inflorescence of *R. bella* followed a typical acropetal development pattern (Harris et al., 1991; Naghiloo & Claen-Bockhoff, 2017), whereby a single dorsal started to open after 25 days (Figure 1G). Xu et al. (2006) reported that most of the angiosperm flowers had four whorls, namely sepals, petals, anthers, and pistils. Based on the observations and measurements from the inflorescences, the process development of *R. bella* flower from its initiation until the bud opening can be separated into ten stages, as shown in Table 1.

Bud Development

The studies of bud development in *R. bella* were based on the methods of physical measurement and SEM analysis, as described by Wang et al. (2014). Continuous observations of early primordial growth and flowering were carried out until the buds reached maturity (bud opening). The flower bud size increased weekly, and the buds started to open after 25 days of observation.

Table 1
Morphological features in Renanthera bella *flower development pattern*

Stages	Development event	Flower development period (days)	Remarks
1	Flower bud started to appear and form a raceme type of inflorescence	7	The flower bud is creamy white, while the inflorescence stalk is light green
2	Flower bud primordia started to develop	7	The flower buds are creamy-white and brown
3	Sepals developed	12	The sepals are creamy red
4	Immature column developed	16	The column is red-brown
5	Lip developed and can be observed between lateral bud sepals.	16	The lip is creamy
6	Stigma papillae appeared	16	-
7	Pollen not fully developed	16	The pollen is light yellow and kidney-shaped
8	Anther cap enclosed the pollen	16	The anther cap is dark brown and has a straight line with a deep curve
9	Sepal enclosed the flower bud	21	The adaxial sepal is pale red
10	Bud started to open	25	Bud is red maroon

Renanthera bella inflorescences will continue to develop under 27 ± 2°C, 10/14 h light/dark with 70% – 80% humidity. Overall measurement on the length and diameter of flower buds was recorded for 5, 12, 18, and 25 days of observation and summarized in Figure 2.

Figure 2A shows the early flower bud that is covered with leaf primordial (arrow). Within seven days of observation, the primordial leaf sheath elongated, and the bud size increased (Figure 2B). Then, the lower part of the lip (Figure 2C) and sepal line (Figure 2D) started to appear and emerged from the bottom of the flower bud on the 16th day of observation. The flower bud developed and elongate then tapered at the apex (Figures 2E–2G). Some differences were noted on the surface character of the epidermal cell for abaxial and adaxial buds, where the abaxial immature bud character showed proximity to the epidermal cell and the presence of several stomata guard cells (Figure 2H), while the adaxial surface of the bud displayed a linear epidermal shape (Figure 2I). The flower induction process involved the triggering of the flowering bud by environmental factors, which result in the change of development patterns, leading to the formation of flowers.

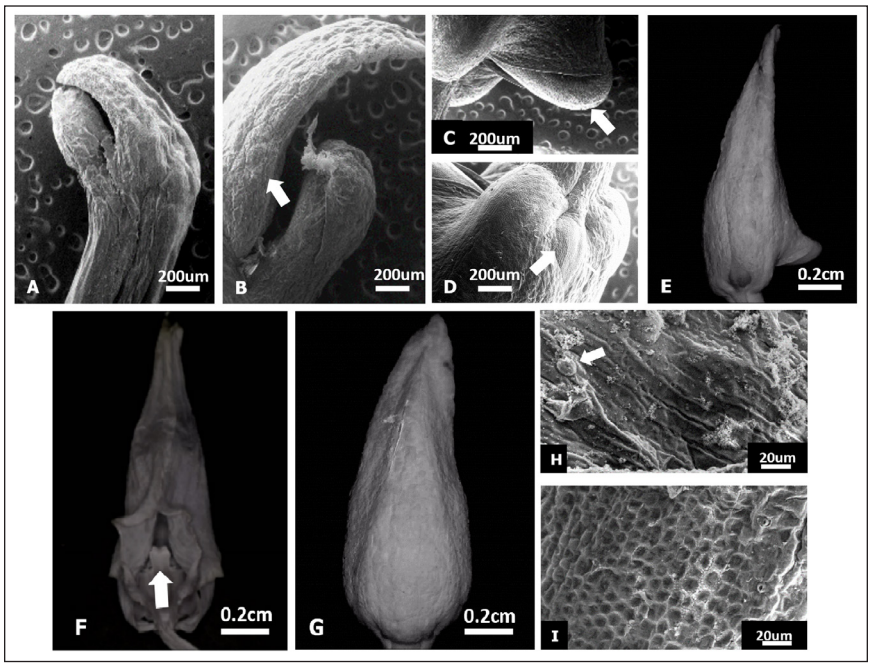

Figure 2. Scanning electron microscope analysis of flower bud development of *Renanthera bella*. (A) Early-stage of epidermal shape; (B) Floral bud with enclosed by young leaf primordium (arrow); (C) Lip appears at the bottom of the bud (arrow); (D) Line of lateral sepal on the immature bud (arrow); (E) Front view of mature flower bud; (F) Lower bud with tapered apex showed the appearance of lip (lateral view) (arrow); (G) Bud reaches a diameter of 0.5 cm and starts to open; (H) Abaxial surface of immature bud showing the proximity of epidermal cell and stomata guard cell (arrow); (I) Adaxial surface of bud with epidermal cell in an irregular shape

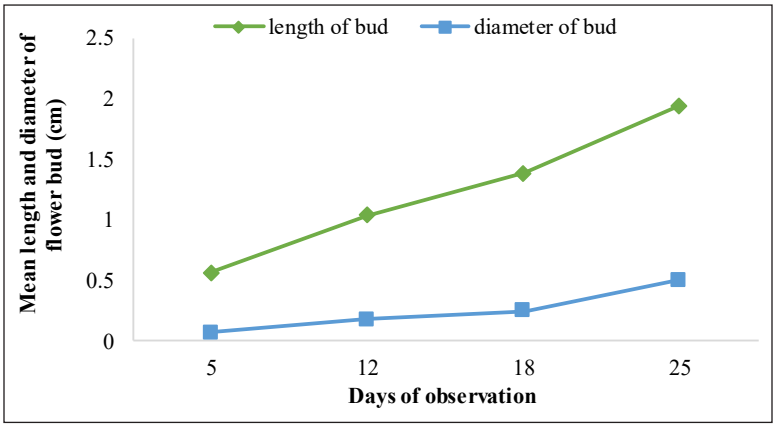

Figure 3. Length and diameter of the flower bud of *Renanthera bella* within 25 days of observation

The flower structure has been studied in various ways. Studies of natural history and evolutionary biology of flowers have emphasized understanding the ultimate (evolutionary) causes of a wide range of variants, such as color, symmetry, meristic arrangements (e.g., flower organ number), size, pollination syndrome and others (Alvarez-Buylla et al., 2010).

Lip and Column Development

The differences between the columns and the lip are shown in Figure 4A to Figure 4D through the front and lateral view. The column consists of the androecium, the male reproductive part, and the modified filaments and styles that fuse in orchid flowers. The immature column appears straight from the sides and has an average length of 0.50 mm (Figure 4A; stage 4), while the mature column reaches 3.5 mm in size and appears curved on the side (Figure 4B). The column contains anther, pollen, and stigmatic papillae that are part of the pollination process (stage 6). The stigmatic papillae have a rostellum character, and their position is below the column (Figure 4C). At its mature level, the column changes to a curve formation (arrow) and has a smooth surface without hair (Figure 4E), while the immature column is straight and has a hairy surface (arrow; Figure 4F).

Lips appeared below the bud between the lateral sepals after 14 days (stage 5). The lip is a modified petal of the orchid flower. The immature *R. bella* lip has an average size of 0.09 mm and is tapered at the apex. The *R. bella* lip is broad and slightly spread at the base (Chan et al., 1994). *Renanthera bella* has gynandrous stamens that fuse with the pistil to form the reproductive part called the column.

Figures 4G–4H show the front and lateral view of the appearance of the mature lip as it tapered to the apex, while Figure 4I shows a vertical view of the lip that forms the hole inward. The nectary primordia initiate under the column and become mature and elongated at the base (Figure 4J). Moreover, the surface of the epidermal cells of the mature column is circular and thick, with wrinkles lining their cell walls (Figure

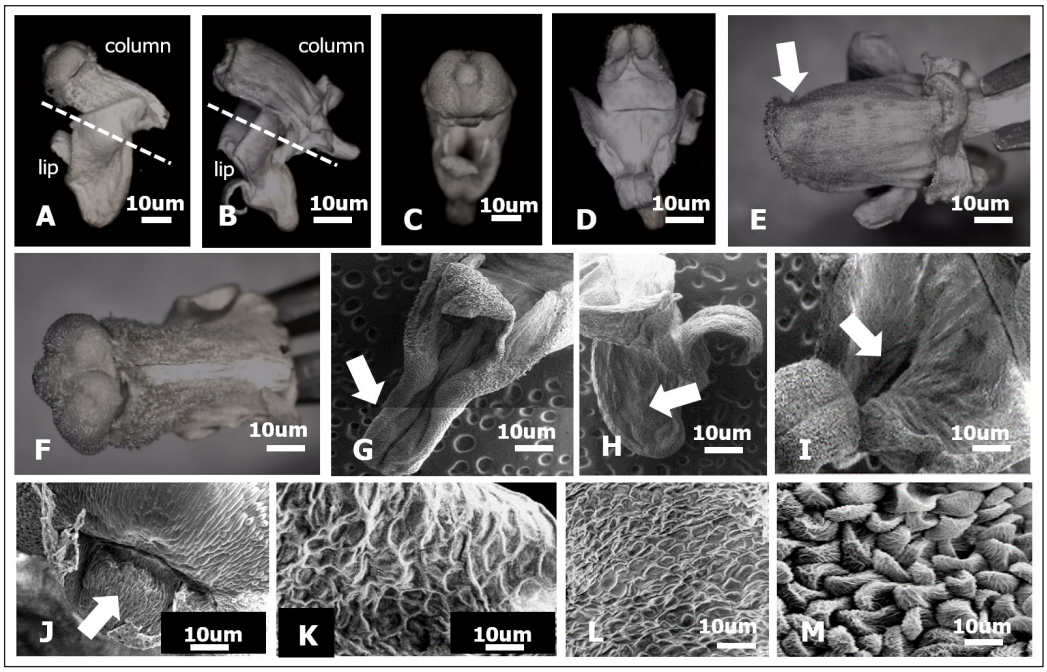

Figure 4. Lip and column development of *Renanthera bella*. (A) Lateral view of immature column and lip; (B) Lateral view of matured column and lip; (C) Front view of immature column and lip; (D) Front view of matured column and lip; (E) Vertical view of mature column change to curve formation (arrow); (F) Vertical view of an immature column; (G) The matured lip is tapered to the apex (front view) (arrow); (H) Lateral view of matured lip (arrow); (I) Vertical view of the mature column forms the hole inward (arrow); (J) Nectary primordia under the column (arrow); (K) The surface of the mature column; (L) The surface of the immature column with thickening circle shape; (M) Epidermal cell from the adaxial surface of mature lip showing dams hemisphere shape (*Note*. Sepal and petal dissected away to reveal inner organ)

4K). An epidermal cell in the middle of the column develops a thick, irregular circular shape that curves inward (Figure 4L). The cell surface of the lip tip has a hemispheric shape (Figure 4M).

Anther and Pollen Development

In stage 7, pollen is formed, while in stage 8, the anther cap develops and encloses the pollen for protection. *R. bella* has an anther cap structure that fuses in front of the column structure from the edge view, while the pollen is located inside the anther cap for protection before pollination at maturity (Figure 5A). The immature anther cap is round and curved with a small circle that rises and forms a line pattern in the middle (Figure 5B). The whole structure of the anther cap has puberulent hair, while the mature anther cap is also hairy on its surface, with a faint line in the middle and a small circle arising in the middle that begins to disappear (Figures 5A–5B). Furthermore, *R. bella* mature pollen has a thick, wrinkled shape (Figure 5C). The anther sac is curved inward to protect the diploid pollen before pollination (Figure 5D), while the pollen stalk in the inner surface of the cell has hexagonal epidermal cells (Figure 5E).

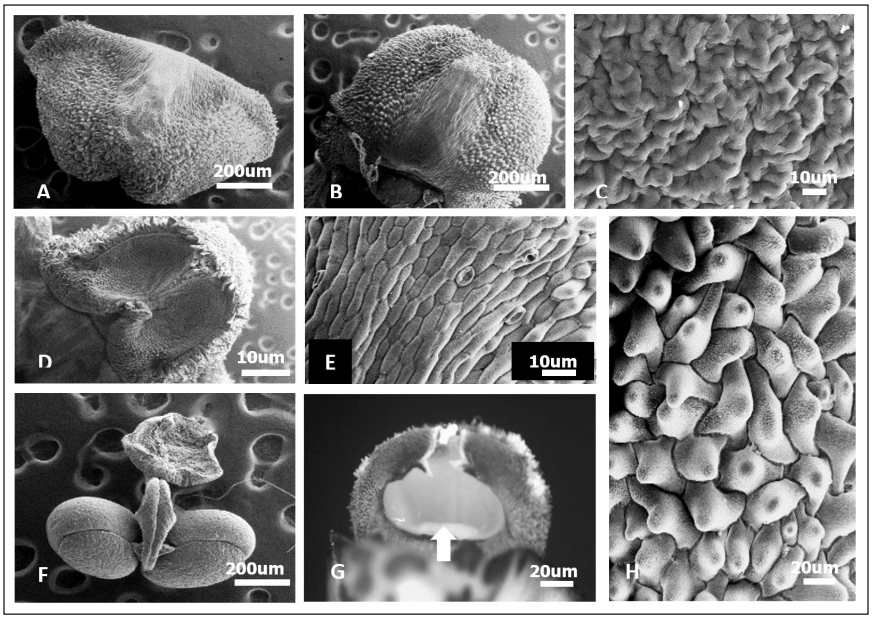

Figure 5. Anther and Pollen Development of *Renanthera bella*. (A) Vertical view of immature anther cap; (B) Matured sepal with pubescent hair; (C) The surface of mature pollen; (D) Vertical view of matured anther sac after pollen removed; (E) The surface of pollen stalk; (F) Mature pollen of *R. bella*; (G) Matured stigmatic papillae (arrow); (H) Vertical view of pollen surface (*Note*. Sepals and petals dissected away to reveal inner organ)

Renanthera bella pollen appears as two masses with kidney-like shapes (Figure 5F). Additionally, the stigma papillae have a sticky hole surface for pollination purposes (Figure 5G). Pollen begins to form together with column formation and develops along with the anther and cap. The pollen size in mature *R. bella* is approximately 0.05 mm. Mature anther of the epidermal cell surface of the epidermis shows a conical shape (Figure 5H).

Petal and Sepal Development

Data on the length and diameter of the outer flower organ (sepal and petal) is recorded in Table 2. The sepal line begins to appear, and curves inward to cover the apex (Figure 6A; stage 9). The primordial petals develop at the same time as the sepals, and they proliferate to enclose the bud (Figure 6B). The sepals on the young flower buds overlap with each other and cover the flower's internal organs (Figure 6C). The basal portion of the petal is broad and extends and tapers into the apex portion. The adaxial cell surface at the tip of the sepals begins to mature and forms a hexagonal cell when mature (Figure 6D). Stomata are observed on the adaxial surface of the sepals. The mature stomata are arranged far away from each other on the surface of the epidermal cell. Each stoma has two thickened guard cells (Figure 6D). Additionally, the mature septic surface of the sepals does not have a definite shape (Figure 6E). At maturity, the average length of the sepals was 0.38 ± 0.06 cm during

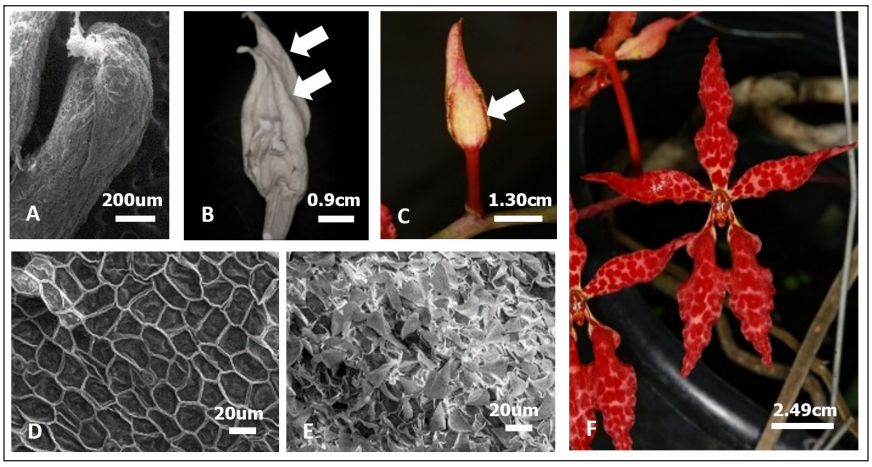

Figure 6. Petal and sepal development of *Renanthera bella*. (A) Sepal line begins to appear and curves inward to cover the apex; (B) Petals also develop an elongated first compare to sepals (arrow); (C) Line of immature sepal appears (arrow); (D) Enlarge the view of the adaxial surface of immature bud; (E) Enlarge the view of abaxial surface of immature bud; (F) Mature flower of *R. bella* (full bloom)

Table 2
Length and diameter of outer flower organ on the primary inflorescence of Renanthera bella *(n=3)*

Days (Age)	27	30	33	35
Length of lateral sepal	2.01 ± 0.16 cm	2.15 ± 0.14 cm	2.28 ± 0.10 cm	2.49 ± 0.23 cm
Diameter of lateral sepal	0.31 ± 0.06 cm	0.34 ± 0.05 cm	0.38 ± 0.07 cm	0.38 ± 0.06 cm
Length of petal	1.70 ± 0.05 cm	2.10 ± 0.08 cm	2.36 ± 0.14 cm	2.39 ± 0.14 cm
Length of stalk	0.81 ± 0.50 cm	0.86 ± 0.49 cm	0.89 ± 0.54 cm	1.01 ± 0.55 cm

growth in this experiment. The epidermal surface of the abaxial and adaxial sections is similar to the sepals. The development of the petal just precedes the anthesis process, and the petals can reach a maximum average length of 2.39 ± 0.14 cm. Like most of the orchid species, *R. bella* flowers consist of four whorls, namely sepals, petals, anthers, and pistils, with a unique feature (Xu et al., 2006). The first whorl of orchid flowers is made up of one dorsal and two lateral petaloid sepals (Figure 6F). Stage 10 occurs when the sepals start to open and elongate rapidly to form the mature flower. The bud begins to open from its dorsal sepal.

Capsule Development in *Renanthera bella*

The formation of the *R. bella* capsule was investigated between September and October. Figure 7 shows the length and diameter of the *R. bella* capsule after 21 weeks of observation. The formation of the capsule was recorded in terms of length and diameter until it matured, starting from the day of hand pollination. Based on the observations, there was a drastic increase in the length of the capsule in the fifth week after pollination, with the diameter of the capsule increasing steadily. For the first six weeks, the diameter of the capsule increased

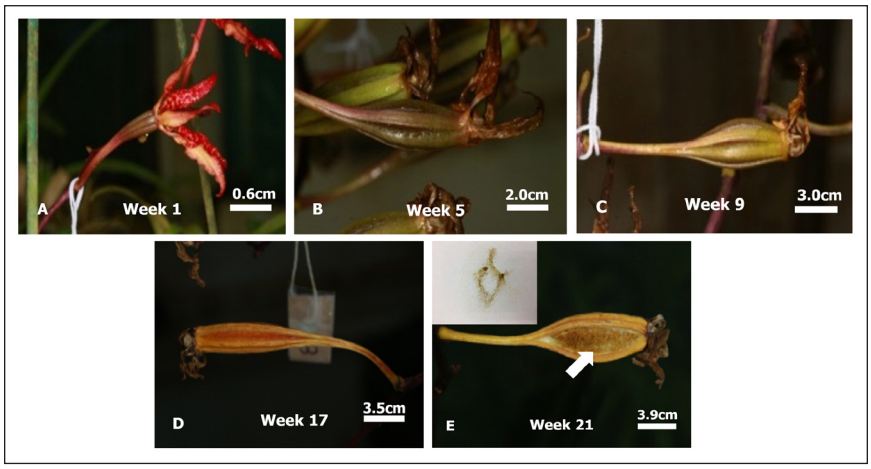

Figure 7. Formation of the *Renanthera bella* capsule after hand pollination. (A) Flower petal starts to shrink and the stalk starts to swollen; (B) Flower petal started to wilt, and the capsule formed; (C) Capsule increase in size; (D) Capsule change its color from green or brown to yellowish in a mature capsule; (E) Over matured capsule with an exposed seed (small figure)

steadily. However, the rate of length of the capsule growth started to decrease after week 5, with only a total of 1 cm growth recorded.

After six weeks, the capsule reached a diameter of 0.6 cm and a length of 3.0 cm. However, the length and diameter of the capsule are not the primary factors in determining the maturity level of seed. According to Malarkodi and Srimathi (2007), the maturity level of a seed can be attributed to the physiological and functional changes that occur from the flower bud formation until full bloom event or the anthesis stage. From the observation, after week 21, the *R. bella* capsule started to break out, showing that it has overreached the mature phase (Figure 7E). The formation and development pattern of the capsules after pollination for week 1 until week 21 are shown in Figures 7A–7E.

CONCLUSIONS

In conclusion, the study demonstrated ten stages of the early flower development pattern of *Renanthera bella*. During the process, flower bud initiation, morphological characteristics of flower events, and capsule formation were revealed. The time required for *R. bella* to produce mature and full bloom flowers from bud inflorescence formation ranged from 7 to 25 days. In addition, approximately after weeks 21, *R. bella* seeds reached maturity. More studies of orchid flower organ identities are required to understand the evolution of the unique structures better. Further work is also needed to identify the essential genes that may be involved and significant for the floral development process.

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Preliminary Study on the Effect of Nitrogen and Potassium Fertilization, and Evapotranspiration Replacement Interaction on Primary and Secondary Metabolites of *Gynura procumbens* Leaves

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ABSTRACT

Despite progressive reports on pharmacological properties in *Gynura procumbens*, many are overlooking the importance of agronomic requirements for high yields and phytochemical content that vary due to environmental variations. The study was carried out to examine the effects of nitrogen (N), potassium (K), and evapotranspiration replacement **(**ER) on growth and phytochemical content. Treatments affected parameters significantly ($p \leq 0.05$) with a stronger effect on physiological and biochemical attributes ($p \leq 0.0001$). Highest and lowest yield of biomass and phytochemical content were observed under N0K30(70) and N90K0(25), respectively. Treatments interaction was highly significant ($p \leq 0.0001$) in Cond, TPrC, and TFC, ($p \leq 0.05$) in CF and PWP, and not significant ($p > 0.05$) in Photo, TCC, and TPC. The 75% ER had significant ($p \leq 0.05$) output of biomass and phytochemical content. As ER decreased from 100 to 25%, the Photo and CF were reduced. Phytochemical content displayed a significant negative relationship with PWP. Caffeic acid, kaempferol, and ferulic acid demonstrated as lead compounds. Following this, there were correlations between phenolic biosynthesis-regulated gene and gene expressions such as PAL, CHS, and F3H primers.

Keywords: Biosynthesis, flavanone3-hydroxylase, *Gynura*, metabolite, phenylalanine ammonia-lyase, phytochemical

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INTRODUCTION

Enzymes in photosynthesis and metabolite processes require nitrogen (N), potassium (K), and water as cofactors. Several studies have reported associations between single or interaction of N-, K-, and water-dependent enzymes such as pyruvate kinase, starch synthase, nitrate reductase, and Rubisco for specific metabolic regulation (Pant et al., 2015). Due to the increasing establishment of gene expression and protein modification role in metabolic regulation, studies of N, K, and water availability-dependent reactions have also come into the mechanisms. To understand how N, K, and water influence the metabolite, the studies must assess metabolic regulation at different biological levels including photosynthesis, phytochemical, and gene expression to identify target rates of N, K, and water. Concurrently, the functional genes are determined and their roles verified (Mongkhonsin et al., 2016).

Among the parameters, water content is most affecting metabolites composition because water is characterized as a source of energy to synthesize organic compounds in photosynthesis. Water supply has an important role in growth and essential to determine the yield and quality of plants. Deficit or surplus water content is an environmental stress and is a significant factor influencing growth and productivity (Selmar & Kleinwächter, 2013). Identifying growth stages and compounds profile will allow water supply regimes to optimize crop yield and efficiently use of water resources. Rates of water intervals affecting plant performance. Water treatment, water use efficiency, and developmental stages have influenced overall plant performance including growth and metabolic activities. Generally, there are positive and negative effects exhibited by water treatment, depending on target treatment. There will be a target on growth and biomass, or biosynthesis of metabolites is a concern. The amount of water supply and selected rates of N and K will affect plant behavior regarding the biosynthesis of the metabolite (Kleinwächter & Selmar, 2014).

Besides, cellular and molecular regulation influences the biosynthesis and, therefore, affects the metabolites composition. Moreover, metabolites content and composition, and consistency in growth and development are especially susceptible to environmental factors such as water availability and fertilization due to plant heterogeneity. Therefore, it is significant to determine optimum evapotranspiration rate to optimize the productivity of the water unit, and at the same time to determine whether fertilizer supply may enhance or diminish the tolerance of plants to drought and possibly *vice versa*. Therefore, the general objective of the study was to examine the effects of different rates of N, K, and evapotranspiration replacement (ER) interaction on the growth and phytochemical content of *Gynura procumbens* (*G. procumbens*). And, specific objectives were to examine the correlation between total phenolic content biosynthesis and gene expressions such as phenylalanine ammonia-lyase and chalcone synthase to different rates effect of N, K and ER interaction,

and to identify optimum harvesting time for biomass yield and total content of metabolite.

MATERIALS AND METHODS

Treatments

The study was a three-factorial experiment. The first factor was two selected rates of N and K, *viz*. 0 (0.00 g total per plant) and 90 (1.08 g) kg N/ha (N0 and N90), applied in the form of urea and 0 (0.00 g) and 30 (0.36 g) kg K/ha (K0 and K30) applied in the form of muriate of potash. The second factor was four rates of ER, *viz.* 100 (percent, % of water replacement of total water lost volume per plant) as a control, 75 (75%), 50 (50%), and 25 (25%) per plant (100% ER, 75% ER, 50% ER, and 25% ER), applied manually at alternate days of irrigation frequency. The N and K were split into three fertilization phases (three months), and each phase (each month) was about 33.3% of total N and K fertilizer applied in the first week of the month. Meanwhile, the third factor was three harvestings (H) times, *viz*. 4, 8, and 12 weeks after treatment (WAT). These gave combined treatments of 216. Each combined treatment gave 72 plants per block, giving a total of 216 plants.

Total Plant Dry Weight

Three plants per treatment were harvested randomly and separately at 4, 8, and 12 WAT. The plants were separated into leaves, stems, and roots manually. Fresh plant materials were weighed and put in labeled paper bags. Then, dried at 40°C in a forced draft oven until constant weight attained to obtain dry weight (TPDW) (Oyedeji et al., 2014). The biomass of fresh and dry weight was measured using an electronic weighing machine. The unit of weight used was g. Dried samples were ground to a fine powder (0.25 mm) using a grinder and kept until analysis.

Leaf Gas Exchange Rate

The measurement was obtained from LI-COR® Environmental with a closed infrared gas analyzer. The measurements were carried out using fully expanded young leaves numbered three and four from plant apex to record photosynthetic carbon assimilation rate (Photo) and stomatal conductance to water (Cond). The unit of photosynthetic rate and stomatal conductance used was mol H_2O $m^{-2}s^{-1}$ and µmol CO_2 $m^{-2}s^{-1}$, respectively. The data were stored in the console and analyzed by the Photosyn Assistant software (Ibrahim et al., 2014).

Plant Water Potential Rate

The measurement was obtained from the pressure chamber instrument. The measurements were carried out using fully expanded young leaves numbered three and four from plant apex to record water potential rate (PWP). The leaf was cut from the stem and placed in a chamber with the cut petiole surface (0.5 cm) protruding through the rubber chamber lid. The pressure was applied to the leaf in the chamber and reading was taken when the first water appeared at the cut surface of the petiole. The unit of water potential used was Mpa (Jamaludin et al., 2015).

Chlorophyll Fluorescence Rate

The measurements were carried out using fully expanded young leaves numbered three and four from plant apex to record chlorophyll fluorescence rate (CF). Leaves were darkened for 15 min by attaching light-exclusion clips to the central region of the leaf surface. The rate was measured using a portable chlorophyll fluorescence meter. Measurements were recorded up for 5 s. The unit of quantum efficiency rate used was Fv/Fm (Ibrahim et al., 2017).

Total Chlorophyll Contents

Total chlorophyll content (TChlC) was measured spectrophotometrically using a fresh weight basis (Loh et al., 2002). Only fully expanded young leaves numbered three and four from plant apex were used in the analysis. Leaf disk of 3 mm in diameter was obtained using puncher to acquire 15 consistent sizes. The leaf disks were immersed and incubated in 20 mL of 80% acetone for homogenization in an aluminum foil-covered glass bottle for approximately 24 h at 5°C until all green color had bleached out. Finally, 15 µL solution was transferred into 96 well plates to determine the absorbance of chlorophyll *a* (Chl *a*), *b* (Chl *b*), and carotenoids (Car) using UV–Vis spectrophotometer at wavelengths of 645, 662, and 470 nm optical density (OD), respectively. The Chl *a*, Chl *b*, and Car content was calculated as µg g^{-1} fresh weight as per standard equations recommended:

Chl *a* = [(12.47 × OD at 662) - (3.62 × OD at 645) × 10] / (1000 × wt)

Chl *b* = [(25.06 × OD at 645) - (6.50 × OD at 662) × 10] / (1000 × wt)

Car = [(1000 × OD at 4700 – (1.29 × Chl *a*) – (53.78 × Chl *b*)] / 220

Total Carbohydrates Content

Total carbohydrates content (TCC) was measured spectrophotometrically using the Anthrone and Hofreiter method (Hansen & Moller, 1975). Samples (1g each) were weighed 1 g into a 50 mL conical tube. Then, hydrolyze by keeping it in a boiling water bath for three hours with 5 mL of 2.5 M hydrochloric acid and cool to room temperature. Next, neutralize with solid sodium carbonate until the effervescence ceases. Next, the volume was made up to 50 mL and centrifuged at 5,000 × *g* for 5 min. The supernatant separated and filtered with filter paper. The 1 mL aliquot was taken for analysis. Beforehand, the standards (liquid chromatography-grade glucose) were prepared by taking 0.0 (serves as blank), 0.2, 0.4, 0.6, 0.8, and 1 mL of the working standard. The volume was made up to 1 mL in all tubes including the sample tubes by adding distilled water. Cool the contents of all tubes on ice before adding ice-cold Anthrone reagent. Then, 4 mL of Anthrone reagent was added and heated for 8 min in a boiling water bath. The blanks used were absolute methanol. Finally, cool rapidly, then 15 µL solution was transferred into 96 well plates and read the sample absorbance at

630 nm using a UV–Vis spectrophotometer. From the graph (concentration of standard stock solution versus sample absorbance readings), calculate the quantification of carbohydrate present according to the formulation. The concentration of TCC was calculated according to the equation obtained from the standard glucose graph:

$$y = 0.01x + 0.1813$$

$$R^2 = 0.995$$

The TCC in the sample was expressed as mg glucose equivalent g^{-1} dry sample.

Total Protein Content

Total protein content (TPrC) was measured spectrophotometrically using the Lowry method (John, 1995). A 1g sample was weighed into 50 mL conical tube and extracted with 10 mL of 100% methanol (1:10 w/v) at room temperature for 24 h and centrifuged at 7,000 × *g* for 10 min. The supernatant separated and filtered with filter paper. Then, 0.2 mL of extract was pipette out and the volume was made up to 1 mL with distilled water. The 5 mL of alkaline copper reagent was added to all tubes and allowed it to stand for 10 min. Then, 0.5 mL of Folin's Ciocalteau reagent was added and incubated in the dark for 30 min. The blanks used were absolute methanol. Finally, 15 µL solutions were transferred into 96 well plates to determine the absorbance. The intensity of color developed was read at 660 nm using UV–Vis spectrophotometer. Beforehand, the standards [liquid chromatography-grade bovine serum albumin (BSA)] were prepared by dissolving 20 mg BSA in 10 mL of the same diluents for the samples. Then, dilute to 200, 400, 600, 800, 1,000, and 1,200 µg/mL. From the graph (actual protein content versus absorbance readings), calculate the quantification of protein present in the sample tube. The concentration of TPrC was calculated according to the equation obtained from the standard BSA graph:

$$y = 0.0621x + 0.1554$$

$$R^2 = 0.9904$$

The TPrC in the sample was expressed as mg BSA equivalent g^{-1} dry sample.

Total Lipid Content

Total lipid content (TLiC) was measured using the Folch method (Shams et al., 2015). A 1g well-ground sample was weighed into 50 mL conical tube and extracted with chloroform and methanol (2:1, v/v) (20 mL) (1:20 w/v) for homogenization at room temperature for 24 h and centrifuged at 7,000 × *g* for 10 min. A lipid extract was purified to eliminate contaminants by pouring the extracts into a beaker through filter paper containing activated charcoal to remove coloring matters. A clear supernatant obtained was then further purified with 0.2 mL of aqueous 0.9% (w/v) sodium chloride. Purified lipids were transferred into evaporated and concentrated dryness at 40ºC, and the residue weighed. Quantification of crude lipids was performed based on dry weight determination. The weight of extract gives TLiC which was expressed as mg g^{-1} dry sample.

Total Phenolic Content

Total phenolic content (TPC) was measured spectrophotometrically using the Folin-Ciocalteu colorimetric assay method. A 1g sample was weighed into 50 mL conical tube and extracted with 10 mL of 100% methanol (1:10 w/v) at room temperature for 24 h and centrifuged at 7,000 × *g* for 10 min. The supernatant separated and filtered with filter paper. Then, 1 mL of extract was pipetted out into 15 mL conical tube and 2 mL (10% v/v) of Folin-Ciocalteu reagent was added to the extracted sample and incubated for five min. Later, 1.6 mL (7.5%) of the sodium carbonate solution was added into the sample. The sample mixture was then vortexed and incubated in the dark for one hour at room temperature. The blanks used were absolute methanol. Finally, 15 µL solutions were transferred into 96 well plates to determine the absorbance. The absorbance of the samples was measured at 760 nm using UV–Vis spectrophotometer. Beforehand, a series of standard solutions (liquid chromatography-grade caffeic acid) (0.1 to 2 mg/mL) were prepared. From the graph (actual phenolic content versus absorbance readings), calculate the quantification of the phenolic present in the sample tube (Teoh et al., 2016). The concentration of phenolic compounds was calculated according to the equation obtained from the standard caffeic acid graph:

$$y = 0.0098x + 0.0427$$

$$R^2 = 0.9942$$

The TPC in the sample was expressed as mg caffeic acid equivalents, CAE, in mg/g dry sample.

Total Flavonoid Content

Total flavonoid content (TFC) was measured spectrophotometrically using aluminum chloride complex colorimetric assay method (Mongkhonsin et al., 2016). A 1g sample was weighed 1 g into 50 mL conical tube and extracted with 10 mL of 100% methanol (1:10 w/v) at room temperature for 24 h and centrifuged at 7,000 × *g* for 10 min. The supernatant separated and filtered with filter paper. Then, 1 mL of extract was pipetted out into a 15 mL conical tube, mixed with 5 mL of distilled water and 0.3 mL of 5% sodium nitrite solution. The mixture was mixed well and allowed to stand for 6 min. Then, 0.6 mL of 10% aluminum chloride solution was added. After 5 min, 2 mL of 1M sodium hydroxide was added to the mixture and made up to 10 mL with distilled water. The blanks used were absolute methanol. Finally, 15 µL solutions were transferred into 96 well plates to determine the absorbances. The absorbance of the samples was measured at 510 nm using UV–Vis spectrophotometer. Beforehand, a series of standard solutions (liquid chromatography-grade kaempferol) (0.04 to 1.80 mg/mL) were prepared. From the graph (actual flavonoid content versus absorbance readings), the quantification of flavonoid present in the sample tube was calculated. The concentration of flavonoid compounds was calculated according to the equation obtained from the standard kaempferol graph:

$y = 0.0108x + 0.0435$

$R^2 = 0.9933$

The TFC in the sample was expressed as mg kaempferol equivalents, KE, in mg/g dry sample.

Preparation of Plant Extract

A 1g well-ground sample was weighed into 50 mL conical tube and extracted with 10 mL of 100% methanol (1:10 w/v) at room temperature for 24 h, sonicated at normal mode for 5 min and centrifuged at 7,000 × *g* for 10 min. The supernatant separated and filtered with filter paper. The methanolic-extract was transferred into evaporated and concentrated dryness at 40 ºC using a rotary evaporator. It was weighed, re-dissolved in 1.5 mL liquid chromatography-grade methanol and filtered through a sterile membrane filter, 0.45 µm; 25 mm in 2 mL amber glass HPLC vials and ready for further chromatographic analysis (Li et al., 2016).

Thin Layer Chromatography

The stationary phase used was 20 × 20 cm, 0.25 mm TLC plate pre-coated with silica gel 60 F_{254} on aluminum sheets and the mobile phase used was a mixture of toluene, ethyl acetate and formic acid (5:4:1). Marker or reference compounds used were caffeic acid, cinnamic acid, chlorogenic acid, gallic acid, ferulic acid, and vanillic acid for phenolic compounds. Meanwhile, kaempferol, quercetin, myricetin, and rutin for flavonoid compounds (Ismail et al., 2017). The 10 µL of each tested sample and reference compound was applied as a 6 mm band, 2 mm apart, 10 mm from the lower, upper, left, and right edges of the plate using a microsyringe. In the glass tank, 50 mL developing solvent was poured and allowed to saturate for 5 to 10 min at room temperature. Migration (as in linear ascending development) distance of the developing solvent on the plate is 80 mm from the lower edge of the plate or equivalent to the time allowed for the development and maximal separation of the active compounds present in the samples was 15 to 25 min. The plates were then dried at 100ºC using a forced draft oven for 3 to 5 min. Dried plates were visualized under UV light at 254- and 366- nm. The color and distance of unknown spots were compared with the marker or reference compound. The R_f values were calculated using the formula of migration distance of the sport/migration distance of the solvent.

Total RNA Extraction

Samples were ground into fine powder in liquid nitrogen. About 100 mg of ground samples were transferred into 1.5 mL Eppendorf tube®. Total RNA was extracted from each sample using the Universal Plant Total RNA Extraction Kit (Spin-column) (BioTeke®, China) according to the manufacturer's protocol. The 1 mL Lysis Buffer PL™ was added into the tube and incubated for 15 min at 65ºC. The mixture was centrifuged at 12,000 × *g* for 10 min at 4ºC and the supernatant was pipette into RNase-free Filtration Column. The column was centrifuged at 12,000 × *g* for 10 min

and the flow-through was transferred into 1.5 mL Eppendorf tube®. One volume of 70 % ethanol was added, and mixtures were thoroughly mixed. Mixtures were then pipetted into Spin-column AC and centrifuged at 10,000 × *g* at 4ºC for 45 s. Flow-through was discarded and both Spin-column AC and collection tubes were reused. The 500 µL of Buffer RE was pipette to the center of Spin-column AC and the column was centrifuged at 10,000 × *g* for 45 s. Flow-through was discarded. Seven hundred (700) µL of Buffer RW was added and the column was centrifuged at 12,000 × *g* for 60 s followed by flow through the disposal. Five hundred (500) µL of Buffer RW was again added and the column was centrifuged at 12,000 × *g* for 60 s. Flow-through was discarded. Spin-column AC was replaced into the collection tube and spin for 2 min to remove excess ethanol. Spin-column AC was placed on 1.5 mL Eppendorf tube®. The 50 µL of pre-heated (at 75ºC) RNase-free water was applied into the center of Spin-column and left at room temperature for 2 min. The tube was centrifuged at 12,000 × *g* for 1 min. The RNA extracts were stored at -80ºC for further analysis.

RNA Quantification

The RNA extracts were quantified and characterized for purity using Nanophotometer Pearl UV-Vis spectrophotometer (IMPLEN™, Germany).

Synthesis of Complementary DNA (cDNA)

Synthesis of cDNA was carried out using TransScript® II One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech™, China). The method was conducted according to the manufacturer protocol, consisting of 200 ng of each RNA samples, 1 µL anchored oligo $(dT)_{20}$ primer (0.5 µg/µL), 10 µL 2X TS II reaction mix, 1 µL TransScript® II RT/RI enzyme mix, 1 µL gDNA remover, and nuclease-free water. The total volume of the reaction is 20 µL. The mixtures were incubated for 15 min at 50ºC and the reaction was terminated by heating the samples at 85 ºC for 5 s. The 5 µL of stock cDNA was aliquot into 45 µL of dH_2O to be used for RT-PCR runs.

Real-time PCR Analysis

The analysis was carried out using the KAPA SYBR® FAST qPCR Master Mix (2X) Kit (Kapa Biosystems™, USA). The primers sequence of *Gynura bicolor* (*Gb*) phenylalanine ammonia-lyase (PAL) (*Gb*PAL), chalcone synthase (CHS) (*Gb*CHS), flavanone3-hydroxylase (F3H) (*Gb*F3H) and one globular protein gene as a control gene (Tubulin) were used according to Fukuoka et al. (2014) (Table 1). The primer sequence was checked and verified using Primer3 software before synthesis. The qPCR samples were prepared to reach a final volume of 10 µL. None template control (NTC) was also prepared but without a cDNA template. The qPCR analysis was

Table 1
The primer used for qPCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Size of product (bp)
PAL	CTTACTTGACCGGCGAAAAGG	TTTGCACATAGCCGTGAACAC	2181
CHS	CCTTGACACAAGCCTTTACTCCTT	AGGGTGCGCGATCCAA	1197
F3H	ACCTTGTTGCTTCAGGACCAA	ATCCAACTCTTGCCACCATCA	1068
Tubulin	TGGAGGAGACCTGGCTAAGGT	CGGGAGAAGACTTCAGCAACA	275

Note. PAL = phenylalanine ammonia-lyase; CHS = chalcone synthase; F3H = flavanone-3-hydroxylase; Tubulin = globular protein; bp = base pair

conducted using the QuantStudio 12K Flex Real-Time PCR System. The qPCR method was set up for 40 cycles.

Statistical Analysis

The collected data were subjected to analysis of variance (ANOVA) and correlation using SAS® 9.4 software. The analyses were done in triplicate and expressed as mean (n=3) ± standard error (SE) from the dependent treatments (Jaafar et al., 2012). All the variables from measurements were analyzed using the General Linear Model with N, K, and ER supply management. Any differences between treatment means were analyzed by two-way analysis and compared using Duncan's multiple range test (DMRT) at *p*-value ≤0.05 levels. The regression model that best fitted the data, evaluated by an F-test, was chosen.

RESULTS

Total Plant Dry Weight, Leaf Gas Exchange Rate, Plant Water Potential Rate, Chlorophyll Fluorescence Rate, and Total Chlorophyll Contents

The effect of N, K, and ER rates as well as harvest time on TPDW, Photo, Cond, PWP, and CF were recorded in Tables 2, 3, and 5. Meanwhile, the effect on TChlC was listed in Tables 2, 4, 5, and 6.

Total Carbohydrates Content

The TCC was statistically significant in all N and K interaction treatments, increasing with increasing harvesting time and significantly different with decreasing rate of ER including the control plants ($p \leq 0.05$). The content was highest at Week 12 compared to Week 4 (Figure 1, Tables 2 and 4). The TCC are significantly correlated with Photo, TPrC, TPC, and TFC at r=-0.360; $p \leq 0.01$, r=0.745; $p \leq 0.0001$, r=0.515; $p \leq 0.0001$ and r=0.262; $p \leq 0.05$, respectively. However, the TCC is not significantly correlated with TLiC at r=0.218; $p > 0.05$ by a linear function (Tables 5 and 6). The contents of soluble sugar vary in plants and are subjected to different moisture conditions and nutritional status (Cheng et al., 2014). And, drought stress can also increase organic compounds required for cell osmotic adjustment, such as soluble sugars. The report is following the result when TCC was high at Week 12 compared to Week 4 with no significant difference ($p > 0.05$) in decreasing the rate of

Table 2
Probability of greater F (P>F) for the ANOVA on the effect of nitrogen, potassium and evapotranspiration rates, and harvest times on primary and secondary metabolite variables

Source	NK	ER	H	NK x ER	NK x H	ER x H	NK x ER x H	CoV
df	1	3	2	3	2	6	6	
TPDW	ns	ns	****	*	ns	ns	ns	28.399
Photo	ns	ns	****	*	**	ns	ns	41.464
Cond	**	****	****	***	****	****	****	15.191
PWP	****	****	****	ns	ns	ns	*	17.713
CF	****	****	***	ns	**	**	*	8.765
TChlC	****	****	****	ns	**	****	****	2.737
TCC	*	**	****	ns	ns	*	ns	9.729
TPrC	****	****	****	****	****	****	****	3.814
TLiC	ns	**	*	**	ns	ns	ns	48.295
TPC	ns	ns	****	*	****	****	ns	14.454
TFC	***	****	****	****	****	****	****	7.082

Note. All analyses are mean ± standard error of mean (SEM), N = 72 using DMRT. * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$; *** significant at $p \leq 0.001$; **** significant at $p \leq 0.0001$; ^{ns} not significant at $p > 0.05$; N = nitrogen rates; K = potassium rates; ER = evapotranspiration replacement rate; H = harvest time; CoV = coefficient of variation; df = degree of freedom; TPDW = total plant dry weight; Photo = photosynthetic rate; Cond = stomatal conductance rate; PWP = plant water potential rate; CF = chlorophyll fluorescence rate; TChlC = total chlorophyll content; TCC = total carbohydrate content; TPrC = total protein content; TLiC = total lipid content; TPC = total phenolic content; TFC = total flavonoid content

Table 3
Effect of treatments and harvest times on growth and physiology variables

	TPDW	Photo	Cond	CF	PWP
NK					
N0K30	1.087^{a}	3.131^{a}	0.090^{a}	0.525^{b}	0.767^{b}
N90K0	1.047^{a}	3.627^{a}	0.081^{b}	0.599^{a}	0.982^{a}
ER					
100	1.004^{a}	3.362^{a}	0.088^{a}	0.772^{a}	0.725^{c}
75	1.064^{a}	3.342^{a}	0.093^{a}	0.660^{b}	0.810^{bc}
50	1.206^{a}	3.144^{a}	0.071^{b}	0.506^{c}	0.911^{b}
25	0.992^{a}	3.668^{a}	0.088^{a}	0.311^{d}	1.053^{a}
H					
Week 4	0.517^{c}	4.817^{a}	0.049^{c}	0.561^{b}	0.890^{a}
Week 8	0.748^{b}	2.461^{b}	0.057^{b}	0.596^{a}	0.760^{b}
Week 12	1.935^{a}	2.859^{b}	0.149^{a}	0.529^{c}	0.975^{a}
Interaction					
NK	0.578^{ns}	0.140^{ns}	0.005^{**}	$<.0001^{****}$	$<.0001^{****}$
ER	0.144^{ns}	0.733^{ns}	$<.0001^{****}$	$<.0001^{****}$	$<.0001^{****}$
H	$<.0001^{****}$	$<.0001^{****}$	$<.0001^{****}$	0.0001^{***}	$<.0001^{****}$
NKxER	0.013^{*}	0.043^{*}	0.0003^{***}	0.167^{ns}	0.336^{ns}
NKxH	0.410^{ns}	0.004^{**}	$<.0001^{****}$	0.009^{**}	0.287^{ns}
ERxH	0.289^{ns}	0.845^{ns}	$<.0001^{****}$	0.005^{**}	0.158^{ns}
NKxERxH	0.072^{ns}	0.666^{ns}	$<.0001^{****}$	0.013^{*}	0.015^{*}

Note. All analyses are mean ± SEM, N = 72 using DMRT. ^{a,b,c,d} Means with the same letter vertically within each factor are not significantly different ($p > 0.05$)

Table 4
Effect of treatments and harvest times on biochemical assay variables

	TChlC	TCC	TPrC	TLiC	TPC	TFC
NK						
N0K30	5.10036^{b}	182.912^{a}	26.3667^{a}	20.750^{a}	242.330^{a}	61.2039^{a}
N90K0	5.40067^{a}	172.856^{b}	23.9453^{b}	25.972^{a}	228.309^{a}	57.0561^{b}
ER						
100	5.42556^{a}	184.548^{a}	25.2528^{b}	20.222^{b}	238.37^{a}	61.116^{a}
75	5.22928^{b}	167.331^{b}	24.4800^{c}	22.722^{b}	239.06^{a}	61.157^{a}
50	5.07211^{c}	186.898^{a}	26.2978^{a}	18.444^{b}	241.03^{a}	61.033^{a}
25	5.27511^{b}	172.759^{b}	24.5933^{c}	32.056^{a}	222.81^{a}	53.214^{b}
H						
Week 4	7.96933^{a}	157.724^{c}	20.2771^{c}	19.208^{b}	186.220^{c}	51.616^{c}
Week 8	3.79367^{c}	172.062^{b}	24.2454^{b}	22.875^{ab}	215.884^{b}	60.473^{b}
Week 12	3.98854^{b}	203.866^{a}	30.9454^{a}	28.000^{a}	303.853^{a}	65.301^{a}
Interaction						
NK	<.0001****	0.018*	<.0001****	0.056^{ns}	0.087^{ns}	0.0001***
ER	<.0001****	0.003**	<.0001****	0.004**	0.358^{ns}	<.0001****
H	<.0001****	<.0001****	<.0001****	0.033*	<.0001****	<.0001****
NKxER	0.354^{ns}	0.064^{ns}	<.0001****	0.001**	0.044*	<.0001****
NKxH	0.007**	0.067^{ns}	<.0001****	0.105^{ns}	<.0001****	<.0001****
ERxH	<.0001****	0.022*	<.0001****	0.281^{ns}	<.0001****	<.0001****
NKxERxH	<.0001****	0.363^{ns}	<.0001****	0.122^{ns}	0.268^{ns}	<.0001****

Note. All analyses are mean ± SEM, N = 72 using DMRT. ^{a,b,c,d} Means with the same letter vertically within each factor are not significantly different (p>0.05)

Table 5
Correlation of growth and physiology variables

	H	TPDW	Photo	Cond	CF	PWP	TChlC	TCC	TPrC	TLiC	TPC	TFC
H	1.000											
TPDW	0.811 ****	1.000										
Photo	-0.430 ***	-0.282 *	1.000				0.556 ****	-0.360 **	-0.357 **	-0.046 ns	-0.121 ns	-0.365 **
Cond	0.729 ****	0.590 ****	-0.037 ns	1.000								
CF	-0.070 ns	-0.087 ns	-0.0002 ns	-0.048 ns	1.000		0.034 ns	-0.062 ns	-0.095 ns	-0.230 ns	0.074 ns	0.105 ns
PWP	0.139 ns	0.232 *	0.190 ns	0.169 ns	-0.419 ***	1.000						

Note. All analyses are mean ± SEM; N = 72 using DMRT

Table 6
Correlation of biochemical assay variables

	H	TChlC	TCC	TPrC	TLiC	TPC	TFC
H	1.000						
TChlC	-0.831 ****	1.000					
TCC	0.634 ****	-0.465 ****	1.000				
TPrC	0.857 ****	-0.663 ****	0.745 ****	1.000			
TLiC	0.252 *	-0.158 ns	0.218 ns	0.131 ns	1.000		
TPC	0.685 ****	-0.455 ****	0.515 ****	0.761 ****	0.120 ns	1.000	
TFC	0.401 ***	-0.327 **	0.262 *	0.300 *	0.117 ns	0.188 ns	1.000

Note. All analyses are mean ± SEM; N = 72 using DMRT

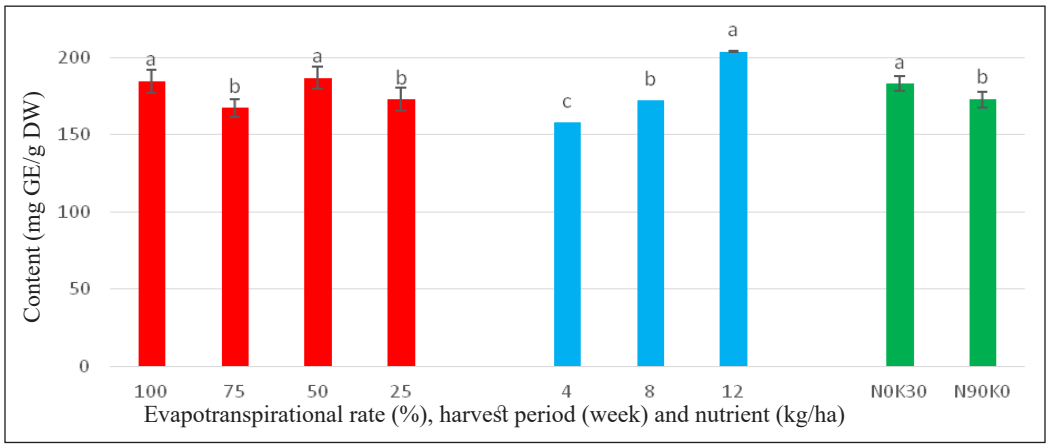

Figure 1. Effect of N, K and ER rates, and H times on total carbohydrate content

ER (Figure 1). However, N deficiencies had increased the content of soluble sugars when TCC was high in N0 than in N90 where ER was not significant (p>0.05) (Figure 1). Meanwhile, K deficiency had inhibited the growth of more than carbon assimilation, which resulted in the accumulation of carbohydrates in the leaves. This response may increase the substrate available for secondary metabolism. However, the results were less significant (p≤0.05) in K30 (182.912 mg GE/g DW) and K0 (172.856 mg GE/g DW) (Figure 1). Carbohydrates are the main organic solutes involved in osmotic adjustment which may lead to a decrease in leaf osmotic potential to maintain turgor.

This is an important adaptive mechanism in plants subjected to deficit irrigation (Wang et al., 2015). The accumulation of soluble TCC is also due to a reduction in soluble sugar transportation under water stress, however, the result reported less significant ($p \leq 0.05$) to TCC and ER.

Total Protein Content

The TPrC was statistically significant in all treatments with increasing harvesting time ($p \leq 0.0001$) (Figure 2, Tables 2 and 4). The 50% ER in N0K30 treatment and 25% ER in N90K0 had shown the highest protein content, meanwhile, 25% ER in N0K30 and N90K0 have shown the lowest (Figure 2). The content was highest under 50% ER in N0K30 at Week 12 compared to 25% ER in N90K0 at Week 4 (Figure 2 and Table 4). The TPrC was significantly correlated with Photo, TCC, TPC, and TFC at r=-0.357; $p \leq 0.01$, r=0.745; $p \leq 0.0001$, r=0.761; $p \leq 0.0001$ and r=0.300; $p \leq 0.05$, respectively. However, it was not significantly correlated with TLiC at r=0.131; p>0.05 by a linear function (Tables 5 and 6). The contents of proline (a proteinogenic amino acid in the biosynthesis of proteins) vary in plants and are subject to different moisture conditions and nutritional status (Mohd Zain & Ismail, 2016). And, drought stress can also increase organic compounds required for osmotic

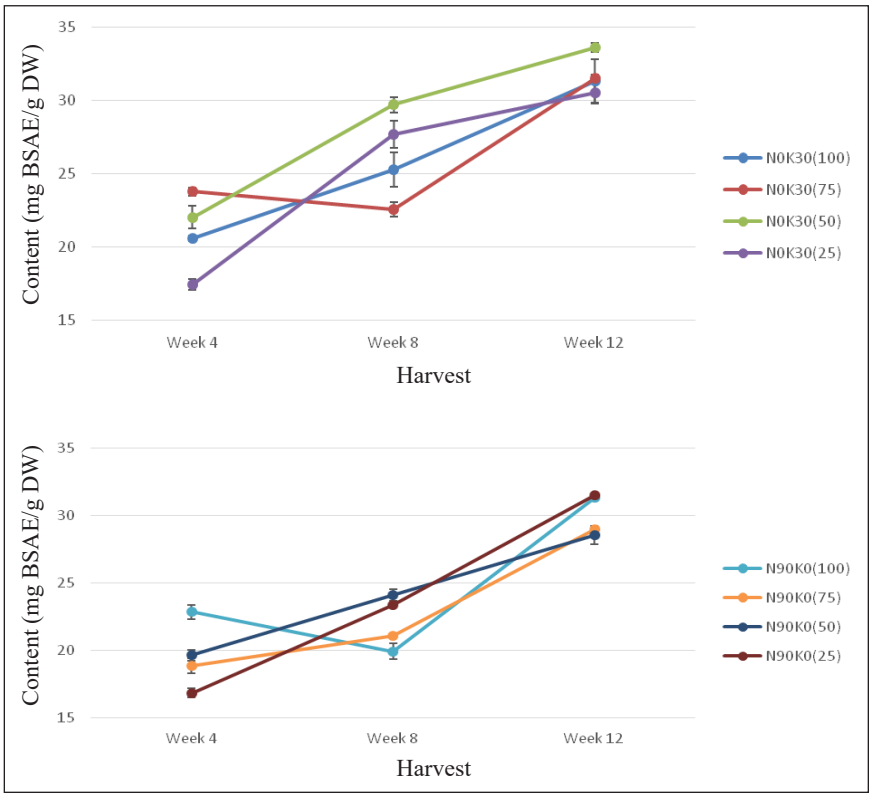

Figure 2. Changes in total protein content as affected by the interactions between rates of N, K and ER, and H times

adjustments, such as proline. The report is following the result when TPrC was high at Week 12 compared to Week 4, especially in N90K0 when 25% ER was reported high (Figure 2). Additionally, K is actively regulated in solute transport, protein synthesis, and enzyme activation point to a close relationship between K and metabolism (Armengaud et al., 2009). Potassium increases the plant's drought resistance through its functions in protein synthesis when TPrC was generally high in N0K30 (17–34 mg BSAE/g DW) compared to N90K0 (16–32 mg BSAE/g DW) (Figure 2).

Total Lipid Content

The TLiC was statistically not significant in all N and K interaction treatments, increasing with increasing harvesting time and significant difference with decreasing rate of ER including the control plants ($p \leq 0.05$). Total lipid content was highest in 25% ER at Week 12 compared to the lowest in 50% ER at Week 4 (Figure 3, and Tables 2 and 4). The TLiC was not significantly correlated with Photo, TCC, TPrC, TPC, and TFC at r=-0.046; p>0.05, r=0.218; p>0.05, r=0.131; p>0.05, r=0.120; p>0.05 and r=0.117; p>0.05, respectively by a linear function (Tables 5 and 6). As water stress rates increased, the oxidative stress in cells and tissues was enhanced, thus implying the occurrence of lipid peroxidation under high water stress (Jaafar et al., 2012) in the respective treatments, 20.222 mg/g DW (100% ER), 22.722 mg/g DW (75% ER), 18.444 mg/g DW (50% ER), and 32.056 mg/g DW (25% ER) (Figure 3). The formation of Malondialdehyde (MDA) was considered as a measurement of lipid peroxidation induced by a high-water stress rate.

Total Phenolic Content

The TPC was statistically consistent in all N and K interaction treatments, increased with increasing harvesting time, and no significant difference with decreasing rate of ER including the control plants ($p \leq 0.05$).

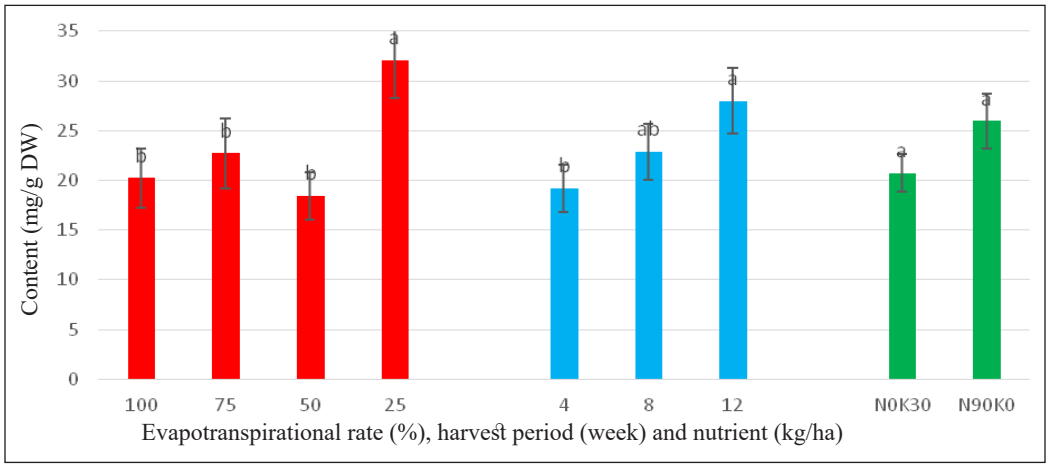

Figure 3. Effect of N, K and ER rates, and H times on total lipid content

Total phenolic content was highest at Week 12 compared to Week 4 (Figure 4, Tables 2 and 4). The TPC are significantly correlated with TCC and TPrC at r=0.515; p≤0.0001 and r=0.761; p≤0.0001, respectively. However, not significantly correlated with Photo, TLiC, and TFC at r=-0.121; p>0.05, r=0.120; p>0.05 and r=0.188; p>0.05, respectively by a linear function (Tables 5 and 6). There were studies which reported the effects of long-term and constant N limitation on carbon allocation for growth and synthesis of phenolics as well as N deficiency increases phenolic content in plants (Caretto et al., 2015). It was following the results when TPC was high in N0 at Week 12 compared to N90 when ER treatment was not significant (p≤0.05) (Figure 4). The increased content of phenolics in plant tissues was either as existing pools or by inducing their *de novo* synthesis (Romagni, 2009). Additionally, the accumulation of TCC had provided a signal of an increase in the production of secondary metabolites. However, less significant content of TCC in this study was translated into non-significant production of TPC corresponded (Figures 1 and 4, Table 6).

Total Flavonoid Content

The TFC was statistically significant in all treatments with increasing harvesting time (p≤0.0001) (Figure 5, Tables 2 and 4). The 75% ER in N0K30 treatment and 50% ER in N90K0 had shown the highest flavonoid content, meanwhile, 25% ER in N0K30 and 75% ER in N90K0 have shown the lowest. The content was highest under 50% ER in N90K0 at Week 12 compared to 75% ER in N90K0 at Week 4 (Figure 5). The TFC was significantly correlated with Photo, TCC and TPrC at r=-0.365; p≤0.01, r=0.262; p≤0.05 and r=0.300; p≤0.05, respectively. However, not significantly correlated with TLiC and TPC at r=0.117; p>0.05 and r=0.188; p>0.05, respectively by a linear function (Tables 5 and 6). The event was probably because, in resource-limited environments, carbon partitioning to constitutive secondary metabolism often increases, which enhances

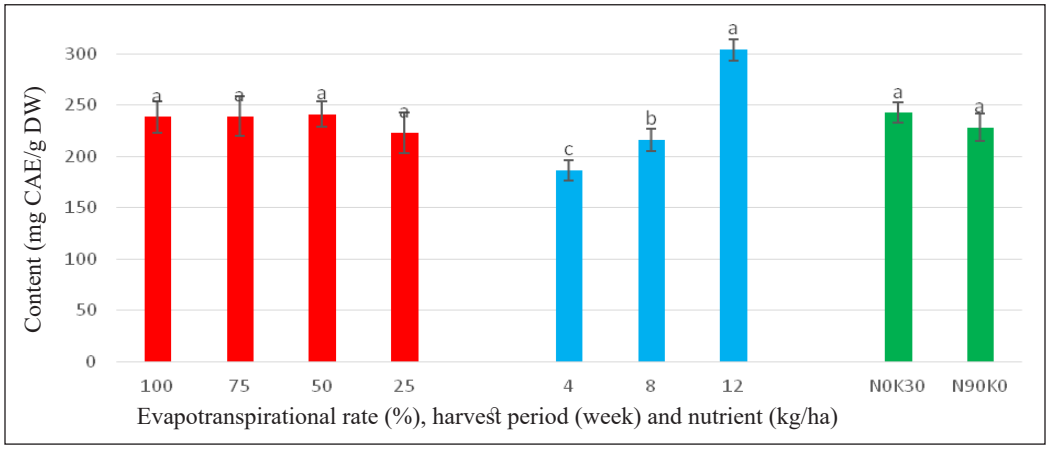

Figure 4. Effect of N, K and ER rates, and H times on total phenolic content

resistance to attack by pathogens and stress tolerance (Caretto et al., 2015). The results indicated the limitations in plant growth were generally accompanied by higher secondary metabolite content. And, N deficiency induces the synthesis of ascorbic acid, flavonoids, and flavonols. The result was following the report when TFC was generally high in N0 (40–80 mg KE/g DW) compared to N90 (20–80 mg KE/g DW). Coinciding with this prediction, the TFC increased with K starvation (N90K0), and an appropriate K rate (N0K30) (Figure 5) could maintain the concentration of flavonoids. In addition, there was an increase in TFC (70 mg KE/g DW and 83 mg KE/g DW) under high water stress (50% ER and 25% ER) (Figure 5), respectively to an accumulation of soluble TCC in plants is also as a result of reduced transportation of soluble sugar under water limitation (Figure 1) (Kleczewski et al., 2010). Possibly, there was a reduction in maximum quantum yield (0.01–0.49 Fv/Fm) (Tables 2 and 3) with increasing content of secondary metabolites as water field capacity being reduced (25% and 50% ER) again demonstrated the possible production of secondary metabolites under increasing water stress (Figure 5).

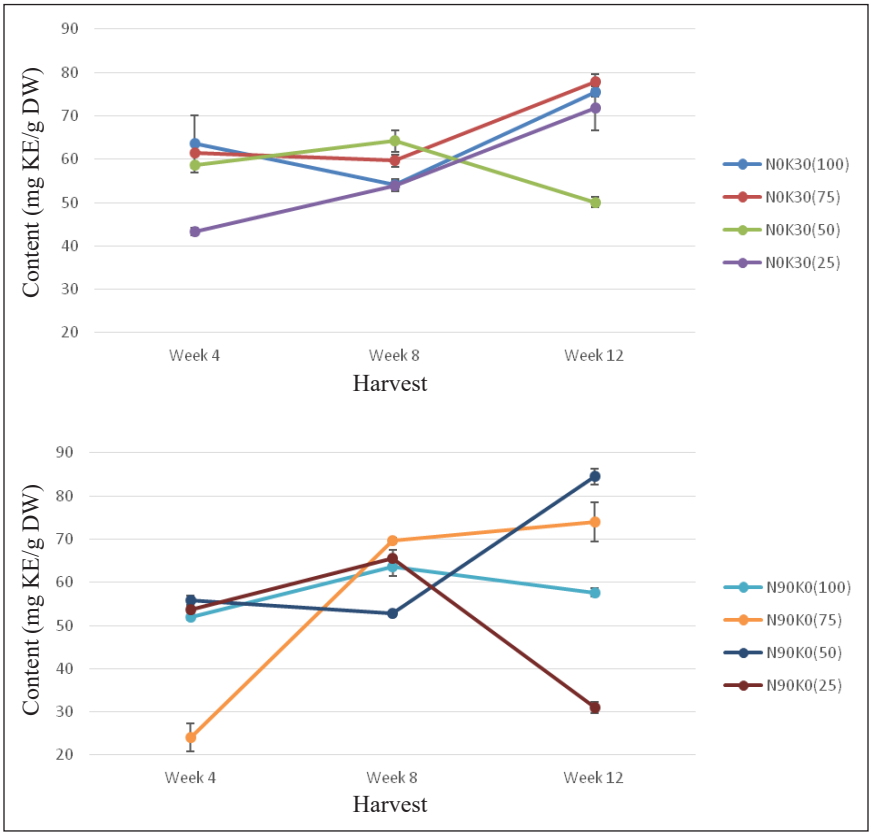

Figure 5. Changes in total flavonoid content as affected by the interactions between rates of N, K and ER, and H time

Thin Layer Chromatography

Qualitative analysis of the leaves methanol extract was performed using a mixture of toluene: ethyl acetate: formic acid (5:4:1 v/v) was used as the mobile phase (Sasidharan et al., 2011) yielded a good resolution of caffeic acid and ferulic acid with bands at R_f=0.64 and 0.69, respectively (Figure 6) for phenolic content. Meanwhile, the analysis also yielded a good resolution

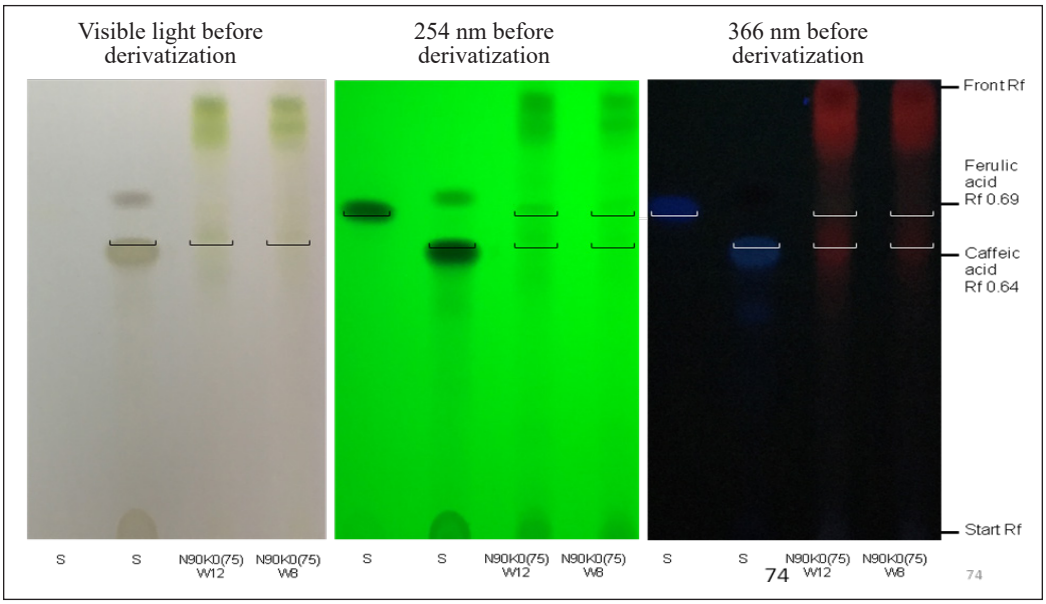

Figure 6. Thin-layer chromatography developed-profiles of caffeic and ferulic acid standard solution

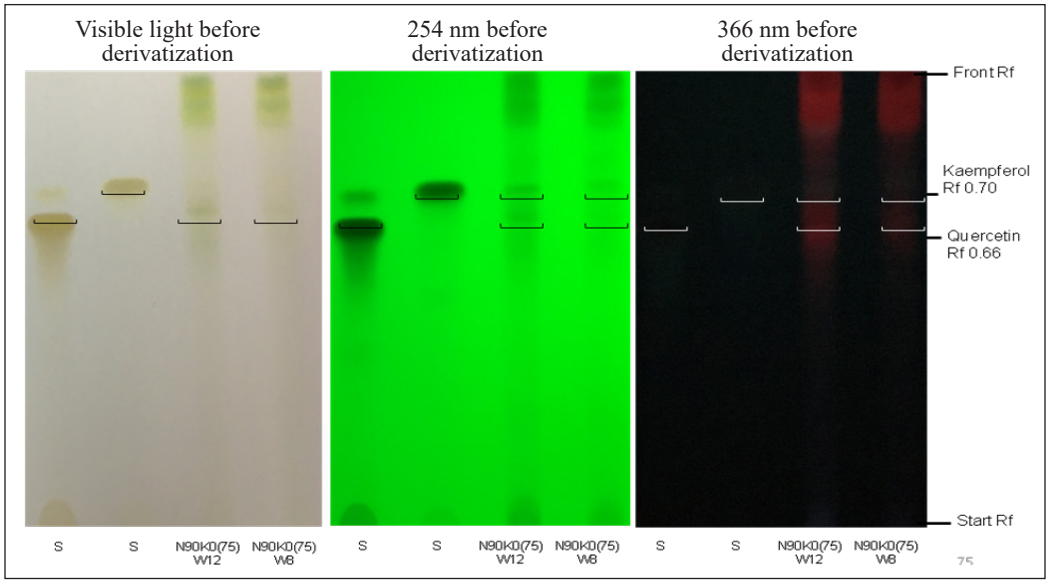

Figure 7. Thin-layer chromatography developed-profiles of kaempferol standard solution

of kaempferol with bands at R_f=0.70 (Figure 7) for flavonoid content. Caffeic acid, ferulic acid, and kaempferol were identified and quantified at visible light, 254 and 366 nm OD. The results revealed the methanol extract at 1 g/10 mL of 100% methanol contained caffeic acid and ferulic acid in accordance with TPC analysis, highest in N90K0(75) treatments at Week 12 compared to N90K0(75) at Week 8 (Figure 4). Meanwhile, kaempferol in accordance with TFC analysis, was highest in N90K0(75) at Week 12 compared to N90K0(75) at Week 8 (Figure 5). This TLC procedure can be used as a fast phytochemical markers screening method for *G. procumbens* leaf samples and herbal formulations.

Phenolic Gene Expression Profile

The analysis was to determine and identify *G. procumbens* phenolic gene expression and relation with selected phenolic biosynthetic genes. The analysis involved mother plant (control sample), and high and low phenolic content samples at Week 8 and 12 after treatment. The samples were selected based on the TPC analysis (Figure 4). The samples were subjected to real-time PCR analysis to substantiate the metabolites content is matched in quantification measurement with a spectrophotometer as well as to identify the responsible gene in modulating the metabolites synthesis. The control plants have shown the lowest content (Relative quantification, RQ 1) of phenolic content compared to other treatment samples. Phenolic content at Week 8 (RQ 1.482 and 0.974) were under the TPC analysis, however, in Week 12 (RQ 3.926 and 8.37) had contradicted the TPC analysis, where low and high TPC had shown a reversal in phenolic content RQ (Figure 8).

The phenolic metabolism was assessed by monitoring the leaf phenolic content together with the expression of three phenolic biosynthetic genes regulation [phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and flavanone3-hydroxylase (F3H)] and one globular protein gene as a control gene (Tubulin). The mechanisms underlying plant adaptation may involve several processes, such as accumulation of signaling proteins and transcription factors or epigenetic modification (de Rosas et al., 2017). Although the transcript levels of phenolic biosynthesis genes were repressed during fertilizer and water re-supply, the decrease in phenolic content in tissues was slower and appeared to be metabolite specific. From a mechanistic viewpoint, the response to fertilizer and water deficiency implies an early reprogramming of gene expression that starts within the first month of limitation, whereas the effects on growth and metabolic content have been proven to be significant between two and three months later.

The increase in the content of secondary metabolites in the present work could be related to an increase in PAL activities under low water field capacity replacement. Suggesting up-regulation of secondary metabolite production with increased PAL activity. This is basically due to PAL is an enzyme, which synthesizes a precursor for total phenolics and flavonoids

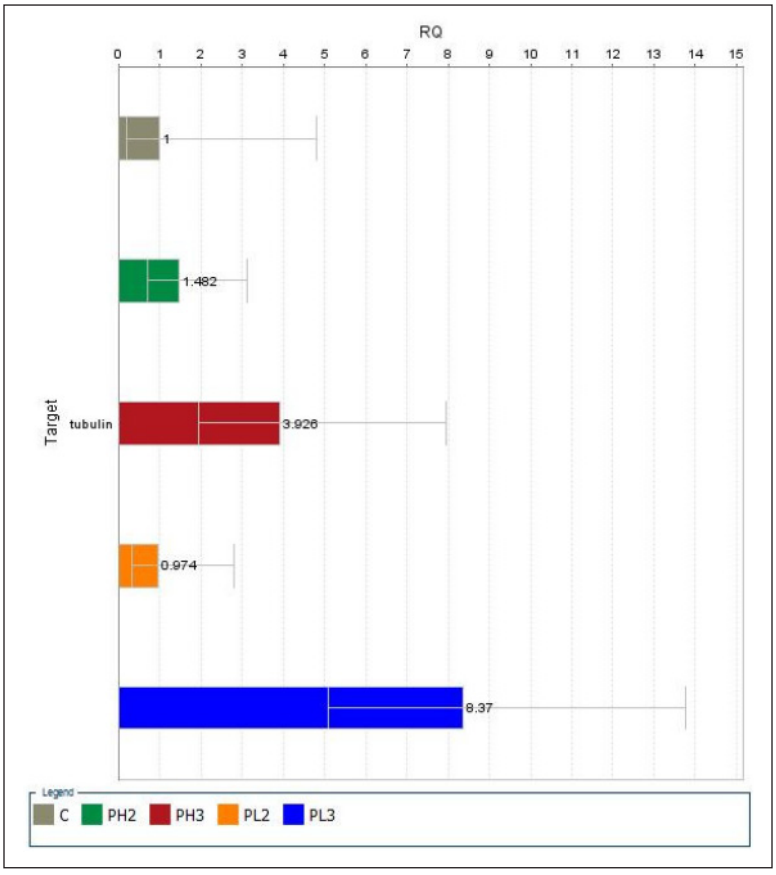

Figure 8. RT-qPCR relative quantification of phenolic compounds gene expression on four samples (PH2 to PL3) and one control sample (C) extracted with the spot method. Serial dilutions of crude extraction were used to calculate the relative quantification using the relative standard curve method. Results from each sample were normalized to the highest value (from sample PL3) and expressed as relative quantification (RQ). Bars represent the standard error of differences between the means at $p \leq 0.05$. C = control plants (mother plants); PH2 = high phenolic (8 WAT); PH3 = high phenolic (12 WAT); PL2 = low phenolic (8 WAT), and PL3 = low phenolic (12 WAT)

biosynthesis (Koyama & Goto-Yamamoto, 2008). High water stress in the present study may have increased the availability of phenylalanine pool as less protein is used for plant maintenance under high water stress, hence, more phenylalanine is available to produce secondary metabolites. These results suggest the up-regulation of secondary metabolites production in *G. procumbens* under high water stress may be due to an increase in PAL activity due in turn to the increased availability of phenylalanine under stress conditions. However, the synthesis of phenolics in plant tissues depends on genetics, the organ, and the developmental stage and is also greatly affected by environmental factors including N, K, and water availability (Turnbull et al., 2018).

DISCUSSION

Water is crucial for productivity and quality. However, the requirements are differed according to plant types and growing media. Deficit or surplus water will induce water stress-related metabolic responses, and due to reduced Cond, the uptake of CO_2 decreases significantly (Tables 2 and 3). Thus, the consumption of reduction equivalents ($NADPH^+H^+$) for CO_2 fixation via the Calvin cycle declines, accordingly, generating large oxidative stress and over-supply of reduction equivalents. Consequently, metabolic processes shifted towards biosynthetic activities which consume reduction equivalents. Accordingly, the synthesis of reduced compounds, such as phenols, is enhanced (Figure 4 and Table 4). As stress-related metabolic changes affect all plant processes extensively, the synthesis of secondary metabolites also is affected (Ren et al., 2014). The present study has shown a discrete effect on growth, physiology, and metabolite content in the following manner, N0K30>N90K0 and ER 75>50>100>25%. The highest and lowest yield of biomass and metabolite content was recorded under N0K30(70) and N90K0(25), respectively. The results also showed the influence of treatments are highly significant ($p \leq 0.0001$) in Cond, TChlC, TPrC, and TFC, ($p \leq 0.05$) in CF and PWP, and not significance ($p > 0.05$) in Photo, TCC, TLiC, and TPC (Tables 2, 3 and 4). Meanwhile, 75% of ER was significantly affected biomass, primary, and secondary metabolites content in all treatments (Tables 2, 3, and 4).

Total biomass and phenolic content negatively interacted with physiology, primary metabolic, and polyphenolic content (flavonoid) (Tables 5 and 6). The study has shown no-significant difference ($p > 0.05$) in Photo has caused TPDW to be significantly affected (Tables 5). Meanwhile, maintaining the productivity of Cond, TChlC, and CF had affected the TCC, TPrC, TLiC, and TFC (Tables 5 and 6). The study suggesting the source (Photo) and sink (metabolites) invariably recorded during the plant growth and development was negatively interacted according to the GDB hypothesis in water stress and selected rates of N and K study (Le Bot et al., 2009). The justification proposes trade-off between primary (growth) and secondary (defense/ stress tolerance) metabolism emerging from developmental constraints in growing cells, and in direct competition for resources between primary and secondary metabolism in mature cells. Therefore, when moderate resource limitation constrains relative growth rate to a greater degree than the net assimilation rate, photosynthate accumulates in tissues and becomes available to support secondary metabolic processes, resulting in higher levels of constitutive secondary metabolites in tissues (Shitan, 2016). These variations may be due to the inherent physiological abilities of the treated plants to absorb and utilize the given fertilizers and water rates with the requirements for growth and development.

In this study, Photo was decreased significantly ($p \leq 0.05$) under water deficit

condition. The decrease is probably due to low CO_2 availability as a result of reduced stomatal and mesophyll conductance. The limited CO_2 assimilation of the leaf tissues may result in an increased allocation of photoassimilates to the secondary metabolites production (Ren et al., 2014). The increase in secondary metabolites production under low Photo was due to upregulated shikimic acid pathway activity under stressed conditions. Suggesting up-regulation of the shikimic acid pathway has involved in secondary metabolites production was under down-regulated Photo. Under high water stress, there is a limit on the translocation of carbon to sinks, with the remaining carbon accumulates as carbohydrates, leading to an increase in carbon pool allocated for secondary metabolism, with little or no competition with growth and development. However, in this study, the result has shown less significance of TCC with ER treatment and in harvest time (Tables 2 and 4). Therefore, the study relatively agrees that water stress usually enhances the production of secondary metabolites (Kleinwächter & Selmar, 2014). This is following plant resource allocation for growth influenced phenolic content in plant tissue, by affecting the plant CNB. When conditions are favorable, plants preferentially allocate carbon for growth. On the other hand, when water or fertilizers are low, carbon will often accumulate, and used for secondary metabolite synthesis (Soubeyrand et al., 2014).

CONCLUSION

The lowest rate of N, moderate of K and 75% ER had produced optimum biomass for required optimum primary and secondary metabolites content. Meanwhile, caffeic acid, kaempferol, and ferulic acid were demonstrated as lead compounds in this study. High phenolic content is probably due to the induction of phenolic-related biosynthesis genes regulation including PAL, CHS, and F3H, and attributed to defense response against ER rates. The N, K, and ER modulation are effective to promote phenolic content because the expressions of phenolic-related biosynthesis genes are high as a result of favorable abiotic influence.

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Relationship between Groundwater Level and Water Content in Oil Palm Plantation on Drained Peatland in Siak, Riau Province, Indonesia

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ABSTRACT

The decrease in groundwater level (GWL) on peatlands, especially in the dry season, can lead to extensive peat drying and making it more vulnerable to the occurrence of wildfire. This research aimed to study the relationship between GWL fluctuations and water content on the surface of peatlands. The study was carried out in a 14 to 17 years old palm oil plantation and a secondary forest located in Siak, Riau Province, Indonesia. Field observations were carried out by installing a water level data logger and soil moisture sensor at a depth of 10 cm and 30 cm from the peat surface, recorded at an hour interval for one year. The results showed that GWL fluctuation was highly correlated to the peat water content in the 10 cm layer both in oil palm plantation (R^2 = 0.65) and secondary forest (R^2 = 0.67). The peat water content in the 30 cm layer showed a low correlation with GWL fluctuation down to -90 cm in oil palm plantation (R^2 = 0.01), however, it was strongly correlated in secondary forests (R^2 = 0.89). Water capillarity in peat soils was able to increase to up to 10 – 30 cm layers from the surface, ranging from 284 to 476% w/w. The capillary water could rise to 68 to 76 cm. The result of the General Linear Model analysis showed that there was a significant influence of land cover, GWL, and peat bulk density on soil water content. Oil palm cultivation activities on peatlands increase the peat bulk density, which in turn increases water capillarity and soil water content.

Keywords: Capillarity, peat bulk density, soil moisture, tropical peatlands

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INTRODUCTION

The utilization of peatlands for agriculture in Indonesia, especially oil palm plantations, has reached a high number (i.e., 1.54 million ha from the total 13.4 million ha peatlands) in the past years (Ritung et al., 2019; Wahyunto & Dariah, 2013). Long-term use of peatlands and land drainage (Van Lanen et al., 2004) has resulted in a decrease in groundwater level, an increase in aeration, soil subsidence, and rapid compaction of peat (Rieley, 2007). In addition, peat excessive drainage may cause peat irreversible drying or hydrophobicity (Hooijer et al., 2010). This process may be accelerated if the peatlands' groundwater level (GWL) is too deep, especially during the dry season, causing the water capillarity inhibition. The critical water content where the irreversible drying occurs is 184 – 213% w/w for peat with hemic maturity and 118 – 126% w/w for peat with sapric maturity (Winarna et al., 2016). Putra (2003) reported that when the peat water content was lesser than 117% w/w, the flammability increased, thus might trigger a large-scale fire. Rein et al. (2008) also added that the peat soil water levels below 125% w/w could start a spontaneous peat ignition.

Over the past decades, Indonesia has experienced a series of drought-related major forest and land fires, such as those that occurred in 1982/83, 1997/98, 2009, and the most recent 2015 fire. Nearly 70 million people have been exposed to unhealthy air during the 2015 fire (Crippa et al., 2016). The fire had caused enormous economic and social consequences, for instance, the smoke/ haze pollution that caused billions of dollars economic loss (Varma, 2003; World Bank, 2016). When drought coincides with El Nino in the humid tropics area (e.g., Southeast Asia), the impact increases through forest fire which disrupts the global carbon cycle, including reduction of carbon stocks (Hooijer et al., 2010; Huijnen et al., 2016; Page et al., 2002; Page & Hooijer, 2016) and intensifies the danger of haze (Lee et al., 2016).

Peatlands can experience drought under an excessive drainage condition, thus increasing its susceptibility to fire (Szajdak & Szatylowicz, 2010). According to Wösten et al. (2008), peatlands' fire vulnerability will increase when GWL falls below 40 cm from the surface of peatlands (i.e., -40 cm). Agreeing to this, the Indonesian government has declared through its regulation (PP No. 71/2014 juncto PP No. 57/2016) that the peat ecosystems are considered degraded if the GWL is lower than -40 cm and an urgent mitigation act should be focused in these areas. Indeed, the construction of a massive drainage system has a real impact on peatland hydrology, causing a major decrease in GWL (Ishii et al., 2016). In turn, the decrease in GWL affects the distribution of moisture throughout the peat soil profile. GWL acts as a water reservoir in peatlands and the dynamics of peat water content are related to the bulk density and the water capillarity capacity of the peat soil (McLay et al., 1992). Agricultural development on peatlands has impacted the physical characteristics of peat that are relevant to the influence of humidity,

including total porosity and soil bulk density (Radjagukguk, 2000).

There have been several studies on the effect of groundwater level fluctuations on the upper-soil layer in peatlands, however, these studies did not cover peatland areas planted with oil palm, especially those planted for the long-term. The water capillarity characteristic of peat soils was shown able to maintain moisture on the soil surface (Nugraha et al., 2016) and is expected to support the sustainable use of peatlands for oil palm cultivation. Therefore, this study aimed to investigate the relationship between the groundwater level fluctuations and water content of peat soils in oil palm plantations and secondary forests by considering the peat soil bulk density of each type of land cover.

MATERIALS AND METHODS

Research Location

This study was conducted from January to December 2018 on tropical peatlands located in PT ABC, Koto Gasib Village, Siak Regency, Riau Province, Indonesia. Field observations were done at two different locations, i.e., oil palm plantation and an adjacent degraded secondary forest. The oil palm plantation was located at 0°43'34.10" N 101°45'33.40" E, while the secondary forest was located at 0° 44'44.70" N 101°46'24,20" E. The oil palm plants ranged from 14 to 17 years. The secondary forests were dominated by fast-growing species, e.g., *Macaranga* sp., *Acacia cracicarpa*, and *Acacia mangium*.

Characterization of Peat Soil

Field peat characterization was done at two study locations. Direct soil characterization was carried out in the field using peat drills; samples were taken and analyzed at the laboratory. Field peat soil characterization included was physical properties and maturity level of the soil. Peat samples were taken using cubicle metal boxes (10 cm x 10 cm x 5 cm) and samples were taken from different soil layers (i.e., 0 – 10, 10 – 20, 20 – 30, 30 – 40, and 40 – 50 cm) in three replications at each location. The laboratory analysis of peat soil characteristics included was water content, bulk density, and ash content.

The level of peat maturity was determined directly in the field by assessing the remnants of the fibers left in the hand upon squeezing (Wahyunto et al., 2005). Determination of water content and bulk density was carried out by gravimetry method with oven drying temperature at 65 ± 5°C for 2 x 24 hours until it reached a constant weight (Ng & Eischens, 1983). The determination of ash content was by the loss on ignition at 700°C.

Automatic Measurement of Fluctuations in Groundwater Level

Fluctuations in the groundwater level were measured using a 4-inch piezometer pipe completed with a water level data logger (HOBO U20L-40, USA). The water level data logger measured the pressure of the water column (kPa) in an hour interval. The data was then converted to the distance (meter) where the measurements were

conducted. Recorded data were downloaded using a USB Base Station. A unit of data logger was installed each in oil palm plantation and the secondary forest. The design of the water level measurement is presented in Figure 1. The water level height from the land surface was calculated using the following equation:

$$GWL = S - (H + X)$$

where:

GWL : Groundwater level (m)

S : Length of the sling to the sensor at the water level data logger (m)

H : Height of piezometer head pipe (m), which is the length of the piezometer body above ground

X : Height of the water column recorded by the sensor (m)

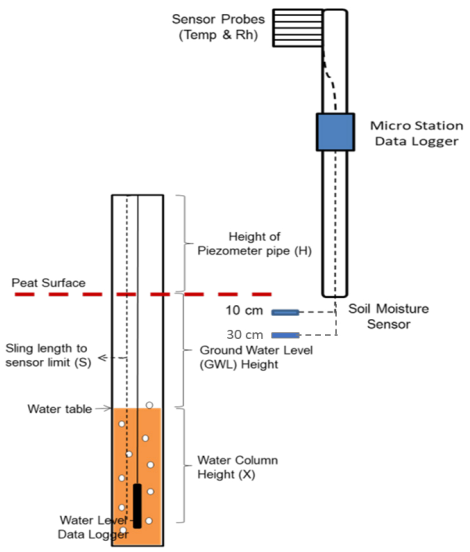

Figure 1. The design of groundwater level and peat water content measurement

Automatic Measurement of Peat Water Content

The automatic measurement of peat water contents was done using a soil moisture sensor (Decagon EC5 S-SMC-M005, USA) that can measure soil moisture content from 0 to 0.550 (v/v). Soil moisture sensors were installed at a depth of 10 cm and 30 cm from the soil surface at each studied location. Each sensor retrieved data every one hour and automatically stored it into the micro station data logger (Hobo H21-USB Micro Station, USA). The soil moisture sensor was installed close to the water level data logger (Figure 1). The peat water content in volume (v/v) recorded by the sensor was converted to percent weight (% w/w) by dividing it with the related bulk density of the soil at each depth.

Data Analysis

All collected data were tested for homogeneity of variance using Levene's test at $p < 0.05$ and the normality test using Kolmogorov-Smirnov at $p < 0.05$. The relationship between groundwater level and peat water content was analyzed using simple regression. General Linear Model analysis was used to study the effects of land cover, groundwater level, and peat bulk density on peat water content. Statistical analysis was conducted using SPSS version 16.0-2007 (SPSS Inc., USA). Groundwater level and soil moisture data processed using HOBOware Pro (Onset Computer Corp., Bourne, USA).

RESULTS AND DISCUSSIONS

Characteristics of Peat Soil

The peat soil characteristics are presented in Table 1. A sapric peat maturity level was found at the 0 – 20 cm layers of oil palm plantation and the 0 – 10 cm layer of the secondary forest (Table 1). Thus, in general, the oil palm plantation has a higher peat maturity compared to the secondary forest. The peat bulk density (BD) at 0 – 30 cm layer of oil palm plantation samples were relatively more compacted (0.09 – 0.15 g cm^{-3}) compared to the secondary forest (0.07 – 0.12 g cm^{-3}). In agricultural practice, compaction of peat due to draining of peatlands causes changes in the structure of peat, in particular the bulk density and cavity ratio (Camporese et al., 2006), and resulted in a higher BD in upper layers of the oil palm plantation compared to the secondary forest. The higher BD of the upper layers was also due to the higher degree of decomposition of peat at these layers.

Analysis of water content from each location showed that the water content surface was lower than the deeper layers of peat. The water content in 0-30 cm layers in the oil palm plantation ranged from 205 to 405% and in the 30-50 cm layer ranged from 461 to 474%. In the secondary forest, the water content in the 0-30 cm layers ranges from 208 to 316%, and in the 30-50 cm layers ranges from 401 to 490%. The higher water content in the deeper layers probably due to its closer distance to the groundwater level.

Dynamics of Groundwater Level

The observation of GWL dynamics in peatlands on the oil palm plantation and the secondary forest was carried out from January to December 2018. The

Table 1
Characteristics of peat soils at the study site

Land cover	Peat depth (cm)	Peat maturity	Peat water content (% w w^{-1})			
			Replicate 1	Replicate 2	Replicate 3	Avg.
Oil palm plantation	0 – 10	Sapric	138	323	153	205
	10 – 20	Sapric	198	315	318	277
	20 – 30	Hemic	415	405	396	405
	30 – 40	Hemic	501	469	414	461
	40 – 50	Fibric	540	440	441	474
Secondary forest	0 – 10	Sapric	300	163	160	208
	10 – 20	Hemic	225	247	202	225
	20 – 30	Fibric	335	314	298	316
	30 – 40	Fibric	504	304	395	401
	40 – 50	Fibric	466	430	575	490

Table 1 *(Continued)*

Land cover	Peat depth (cm)	Peat maturity	Bulk density (g cm^{-3})			
			Replicate 1	Replicate 2	Replicate 3	Avg.
Oil palm plantation	0 – 10	Sapric	0.18	0.11	0.16	0.15
	10 – 20	Sapric	0.11	0.12	0.12	0.12
	20 – 30	Hemic	0.10	0.09	0.08	0.09
	30 – 40	Hemic	0.09	0.09	0.09	0.09
	40 – 50	Fibric	0.09	0.08	0.08	0.08
Secondary forest	0 – 10	Sapric	0.09	0.13	0.14	0.12
	10 – 20	Hemic	0.07	0.10	0.09	0.09
	20 – 30	Fibric	0.07	0.07	0.08	0.07
	30 – 40	Fibric	0.08	0.09	0.08	0.08
	40 – 50	Fibric	0.08	0.07	0.06	0.07

Note. *WC = Water content (% w/w); BD = Bulk density (g cm^{-3})

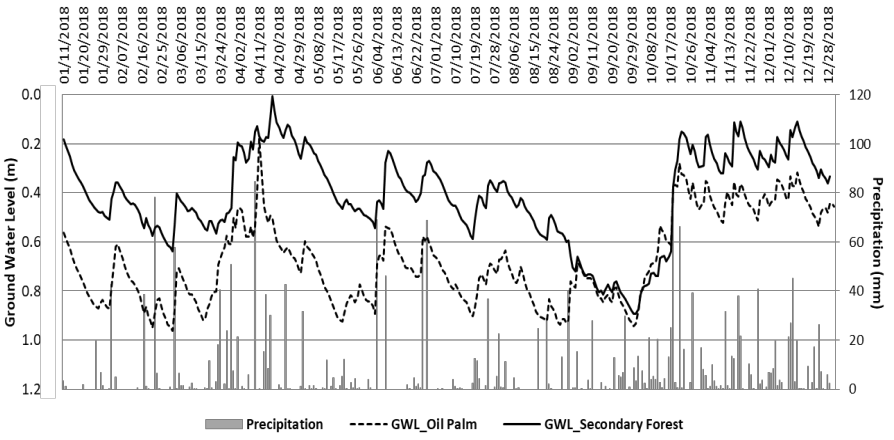

Figure 2. Groundwater level fluctuations in oil palm plantation and secondary forest

results showed that the fluctuations in the groundwater level were strongly influenced by the rainfall conditions at the studied sites (Figure 2). A similar GWL dynamics pattern was observed at the two studied locations. The annual rainfall at the research location in 2018 reached a maximum of 2,169 mm, with 2 wet periods in April-June and October-December, also 2 dry periods in January-March and July-September.

The wet period in October-December with rainfall of 8.62 mm day^{-1} reached the highest GWL average in both sites (-0.47 m in the oil palm plantation, -0.33 in the

secondary forest; see Table 2). On the contrary, the dry period in July-September with rainfall of 3.82 mm day^{-1} reached the lowest GWL average (-0.80 m in the oil palm plantation, -0.56 m in the secondary forest; see Table 2). In general, the GWL in the oil palm plantation is deeper compared to that in the secondary forest. A possible explanation was due to the development of the canal system in the oil palm plantation to facilitate the rooting conditions of oil palm. The exploitation of forest resources on peat surfaces further reduces the ability of ecosystems to withstand rainfall, thus, water flows faster into rivers and the water level decreases in the dry season (Rieley, 2007).

Table 2
Seasonal period and groundwater level (GWL) at the study site

Period (Month)	Rainfall (mm/day)	Average GWL (m)	Land cover
Wet (April - June)	6.45	0.67	Oil palm plantation
		0.41	Secondary forest
Wet (October – December)	8.62	0.47	Oil palm plantation
		0.33	Secondary forest
Dry (January - March)	5.3	0.75	Oil palm plantation
		0.53	Secondary forest
Dry (July - September)	3.82	0.80	Oil palm plantation
		0.56	Secondary forest

Relationship between Water Level Fluctuation and Peat Water Content

The observation of peat water content at the oil palm plantation was successfully done in January to December 2018, while the observation in the secondary forest was only done from January until July 2018 due to the damage in the soil moisture sensor. The observation results of the peat water content and water level fluctuations are shown in Figure 3.

In both locations, the fluctuations of water contents to a certain extent were affected by the fluctuations of the groundwater level. The effects of GWL were more pronounced in the surface up to the 10 cm depth in the secondary forest and up to 30 cm depth in the oil palm plantation. The decrease in the groundwater level decreases soil water content in the entire soil profile and resulting in the release of a number of groundwater volumes from the above layers (Kurnain et al., 2006). A high reduction of GWL in the oil palm plantation during the dry months (January-March and July-September) to around -0.90 m was related to

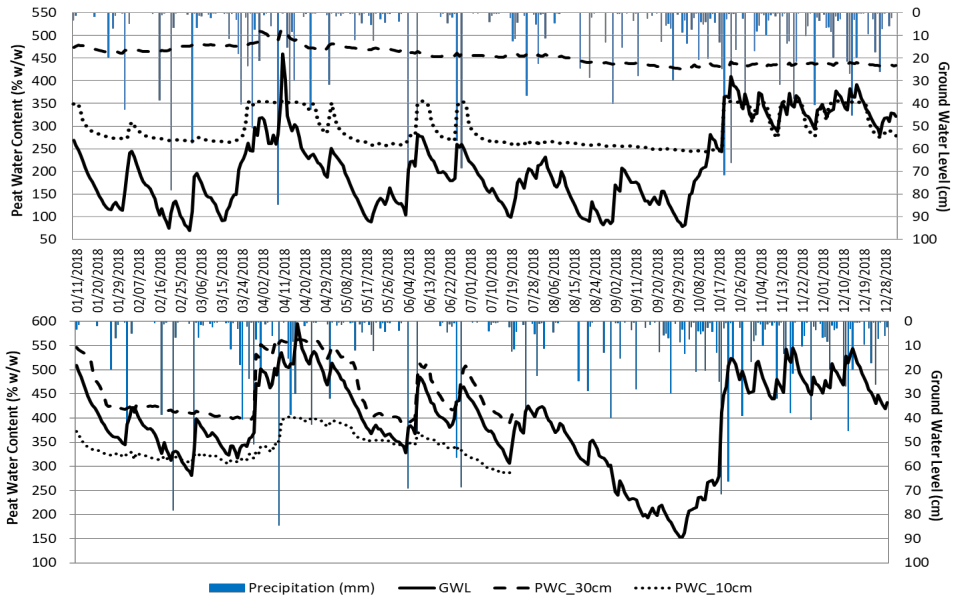

Figure 3. Groundwater level and peat water content in (a) oil palm plantation and (b) secondary forest (*Note*. GWL = groundwater level; PWC = peat water content]

the water content (10 cm layer: 244 - 267% in 10 cm layer 426 - 476% in 30 cm layer; see Figure 3a). During the dry months, GWL in the secondary forest only decreased to around -0.65 m and also related with its water content (284-312% in 10 cm layer and 389 – 415% in 30 cm layer; see Figure 3b). The observed water contents were much higher than the critical irreversible dry water content according to Winarna et al. (2016), which were 184-213% for hemic maturity and 118-126% for sapric maturity. The observed water contents were also much higher than the water content of a potentially burned peat material at 117% (Putra, 2003) or a peat material that can spontaneously ignite below 125% (Rein et al., 2008).

Fluctuations in peat water contents were strongly influenced by the groundwater level dynamics (Figures 3 and 4). A strong correlation was found between GWL depth and water content in the 10 cm layer in both sites (plantation $R^2 = 0.65$; $p < 0.05$ and secondary forest $R^2 = 0.67$; $p < 0.05$; see Figure 4a). A very strong correlation ($R^2 = 0.89$; $p < 0.05$) between GWL depth and water content in the 30 cm layer was found in the secondary forest (Figure 4b). Contrary to other results, there was only a very weak correlation ($R^2 = 0.01$; $p < 0.05$) between the GWL and the water content in the 30 cm layer in the oil palm plantation (Figure 4b). As presented in Figure 3a, there were only low fluctuations in the water content of the 30 cm layer (435 to 510%) although there were high fluctuations in the related GWL (about -0.18 to -0.94 m). The relatively stable water content in this

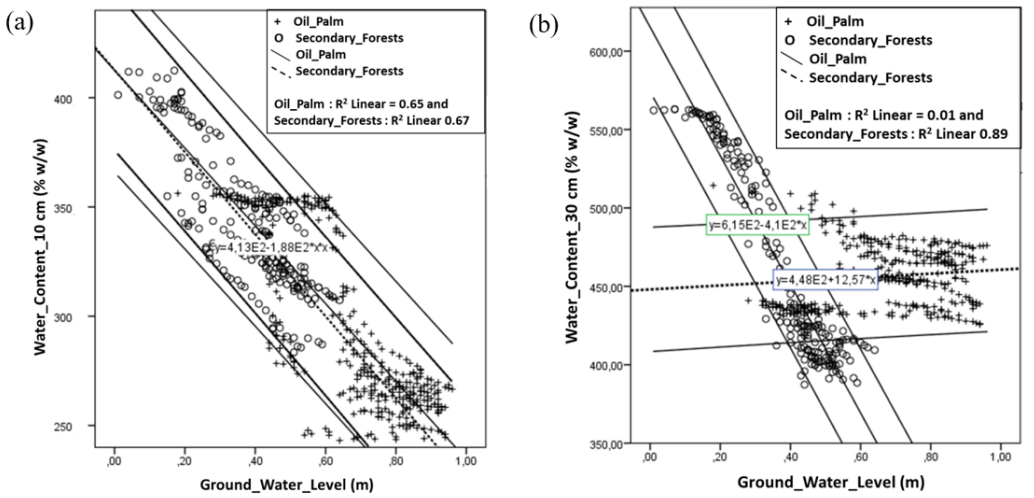

Figure 4. Correlation of groundwater level with peat water content in oil palm plantation and secondary forest on (a) 10 cm layer from the surface (a) and (b) 30 cm layer from the surface

layer was probably due to the higher bulk density and maturity of peat in the profile of the oil palm plantation (Table 1), allowing a higher raise of water capillarity compared to that in the profile of the secondary forest. Capillary water can replace water lost by evapotranspiration in the upper layers (Chesworth, 2008; McCarter, 2012; Yazaki et al., 2006). The rise of capillary water in this study can be observed from the rainfall data, water content, and GWL during the dry period in the oil palm plantation. During the dry period of February 5-16 and June 28 to July 8 with daily rainfall of 0.5 mm, peat water contents in the 30 cm and the 10 cm layers were around 455-466% and 284-294%, respectively. These numbers were much higher than the hydrophobicity water content according to Masganti et al. (2002) and Winarna et al. (2016). The peat water contents were related to the GWL fluctuation (-78 to -86 cm) in the oil palm plantation. Considering the recorded water content in the 10 cm layer, it can be suggested that the capillary water in the oil palm plantation could rise from about 68 to 76 cm. This result is higher compared to the laboratory study of Nugraha et al. (2016), where the water capillary increased for 50 cm.

Effects of Land Cover, Groundwater Level, and Bulk Density on Soil Water Content

There were two General Linear Model (GLM) models developed in this study. The model I studied the effect of land cover (F1), depth of soil moisture sensor (F2), and groundwater level (X1) on peat water content (Y) (Table 3). The results of the GLM model I showed the significant influence of each of the factors and interactions between land cover factors (F1), depth of soil moisture sensor (F2) and groundwater level (X1) on peat water content (Y) with $R^2 = 0.960$ at the significance level $\alpha < 5\%$. Model II studied

the effect of land cover (F1), depth of soil sample (F2), and bulk density (BD) on peat water content (Y) (Table 4). The results of the model II GLM analysis showed the significant effect of land cover factor (F1) and interactions between land cover (F1) and bulk density (BD) on peat water content (Y) with $R^2 = 0.955$ at the significant level $\alpha < 5\%$.

Table 3

Effect of land cover (F1), depth of soil moisture sensor (F2) and groundwater level (X1) on peat water content (Y)

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	5561113.51[a]	7	794444.78	2713.20	.000**
Intercept	11232491.28	1	11232491.28	38361.37	.000**
F1	11838.62	1	11838.62	40.43	.000**
F2	184386.09	1	184386.09	629.72	.000**
X1	742117.84	1	742117.84	2534.49	.000**
F1 * F2	63755.93	1	63755.93	217.74	.000**
F1 * X1	103420.29	1	103420.29	353.20	.000**
F2 * X1	2545.10	1	2545.10	8.69	.003**
F1 * F2 * X1	151419.36	1	151419.36	517.13	.000**
Error	231903.37	792	292.80		
Total	129349953.22	800			
Corrected total	5793016.89	799			

[a]) $R^2 = 0.960$ (Adjusted $R^2 = 0.960$); **) significant at $\alpha < 5\%$

Table 4

Effect of land cover (F1), depth of soil sample (F2) and bulk density (BD) on peat water content (Y)

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	416251.46[a]	19	21907.97	11.17	.000**
Intercept	22674.11	1	22674.11	11.56	.007**
F1	9898.57	1	9898.57	5.05	.048**
F2	16682.66	4	4170.66	2.13	.152
BD	1532.91	1	1532.91	0.78	.397
F1 * F2	16427.08	4	4106.77	2.09	.156
F1 * BD	12264.76	1	12264.76	6.25	.031**
F2 * BD	13895.27	4	3473.82	1.77	.211
F1 * F2 * BD	17278.75	4	4319.69	2.20	.142

Table 4 *(Continued)*

Source	Type III sum of squares	df	Mean square	F	Sig.
Error	19605.15	10	1960.51		
Total	4029419.829	30			
Corrected total	435856.606	29			

[a]) R^2 = 0.955 (Adjusted R^2 = 0.870); **) significant $\alpha < 5\%$

CONCLUSION

This study showed that the peat soil water content on the surface of peatlands was greatly affected by the fluctuations in the groundwater level (GWL), land cover, and peat density. Strong correlations were shown between GWL and peat soil water content in the 10 cm layer in oil palm plantations (R^2 = 0.65) and secondary forests (R^2 = 0.67). A very strong correlation was found between GWL and peat soil water content in the 30 cm layer at the secondary forest (R^2 = 0.89). The peat soil water content in the 30 cm layer of the oil palm plantation remains high despite the fluctuations in GWL, due to the higher bulk density which allows an increase in water capillarity. The capillary water in the oil palm plantation during the dry period could rise to 68 to 76 cm marked by the high peat water content in the 10-30 cm layers, ranging from 284 to 476%. The result of the General Linear Model analysis showed that there was a significant influence of land cover, GWL, and peat bulk density on soil water content. Oil palm cultivation on peatland increases the peat bulk density and water capillarity, also maintains a high peat soil moisture, thus reducing peat vulnerability to fire.

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***In vivo* Fecundity Evaluation of *Phaleria macrocarpa* Extract Supplementation in Male Adult Rats**

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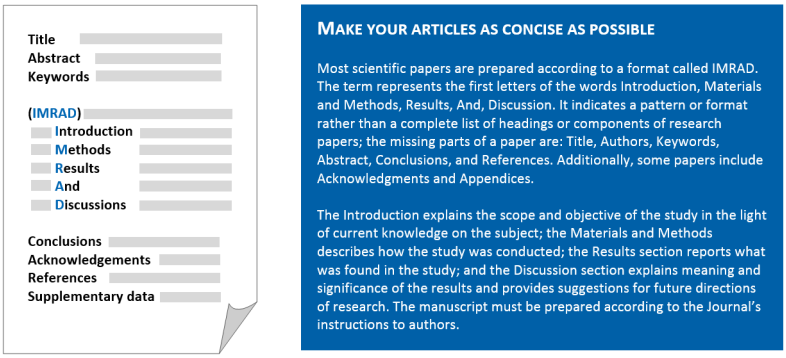

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Article in a newsletter	… ("Australians and the Western Front", 2009) ...	Australians and the Western Front. (2009, November). *Ozculture newsletter*. Retrieved June 1, 2019, from http://www.cultureandrecreation.gov.au/newsletter/

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Online	… (Tester, 2008) … Or Tester (2008) …	Tester, J. W. (2008). The future of geothermal energy as a major global energy supplier. In H. Gurgenci & A. R. Budd (Eds.), *Proceedings of the Sir Mark Oliphant International Frontiers of Science and Technology Australian Geothermal Energy Conference*. Canberra, Australia: Geoscience Australia. Retrieved June 1, 2019, from http://www.ga.gov. au/image_cache/ GA11825.pdf

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