Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

JTAS

VOL. 40 (1) **FEB. 2017**

PERTANIKA JOURNALS

A scientific journal published by Universiti Putra Malaysia Press

Journal of Tropical Agricultural Science
Journal of Tropical Agricultural Science

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About the Journal

Overview

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

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

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

The Journal is available world-wide.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

JTAS

Journal of Tropical Agricultural Science

AN INTERNATIONAL PEER-REVIEWED JOURNAL

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43400 UPM, Serdang, Selangor, Malaysia.
Tel: +603 8946 8855, 8946 8854
Fax: +603 8941 6172
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URL: http://penerbit.upm.edu.my

ABSTRACTING/INDEXING

Pertanika is now over 39 years old; this accumulated knowledge has resulted the journals being indexed in abstracted in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge [ESCI, BIOSIS & CAB Abstracts], EBSCO & EBSCOhost, ERA, DOAJ, AGRICOLA (National Agric. Library, USA), Cabell's Directories, Google Scholar, MyAIS, Islamic World Science Citation Center (ISC), ASEAN Citation Index (ACI) & Rubriq (Journal Guide).

Pertanika Journal of Tropical Agricultural Science
Vol. 40 (1) Feb. 2017

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Foreword

Welcome to the **First Issue 2017** of the Journal of Tropical Agricultural Science (JTAS)!

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

This issue contains **15 articles** which are regular research papers. The authors of these articles are from **Malaysia, China,** and **Indonesia**.

These papers cover a wide range of topics. In the first paper, a group of researchers report on the Magnesium in local edible Ulam *Centella asiatica* and its relation to their habitat soils in peninsular Malaysia (*Ong, G. H., Yap, C. K., Mahmood, M., Tan, S. G. and Hamzah, M. S.*). The next paper discusses the comparative histological evaluations of the submandibular salivary glands of EBN Swiftlets (*Aerodramus fuciphagus*) in man-made houses and natural caves (*Ibrahim, M. M., Zakaria, Z. A. B., Amin, F. M. and Omar, A. R.*). The other papers consist of the report of the physical, chemical and microbiological properties of different combination of soilless media and their effect on the vegetative component and nutrient content of Hempedu Bumi (*Andrographis paniculata*) (*Shara S. A., Zaharah S. S., Radziah O., and Puteri E. M. W.*); Identification and factors affecting *in-vitro* growth of an indigenous mushroom, *Boletus sp.* from Bachok, Kelantan, Malaysia (*Lau Meng Fei, Rosnida Tajuddin, Masratulhawa Mohd and Latiffah Zakaria*); Evaluation of nutritive value of seven Kenaf (*Hibiscus cannabinus* L.) varieties harvested depending on stubble height (*Bai Jie, Li Defang, Chen Anguo, Li Jianjun, Huang Siqi and Tang Huijuan*); Quantification of total phenolic compounds in Papaya fruit peel (*Jamal, P., Akbar, I., Jaswir, I. and Zuhanis, Y.*); Detection of *Leptospira* spp. in selected national service training centres and paddy fields of Sarawak, Malaysia using polymerase chain reaction technique (*Chai Fung Pui, Lesley Maurice Bilung, Lela Su'ut, Yee Ling Chong and Kasing Apun*); Xanthones from *Calophyllum inophyllum* (*Kar Wei Lee, Gwendoline Cheng Lian Ee, Shaari Daud and Thiruventhan Karunakaran*); Comparative study of antioxidant level and activity from leaf extracts of *Annona Muricata* Linn obtained from different locations (*Syed Najmuddin, S. U. F., Alitheen, N. B., Hamid, M. and Nik Abd Rahman, N. M. A.*); Supplementation of antioxidant BHT to different bull semen extenders enhances

semen quality after chilling (*Khumran, A. M., Yimer, N., Rosnina, Y., Ariff, M. O., Wahid, H., Kaka, A., Ebrahimi, M. and Aliyu, A. B.*); Physicochemical characteristics of Oil Palm Frond (OPF) composting with fungal inoculants (*Fadzilah, K., Saini, H. S. and Atong, M.*); Morphological and molecular identification of sea cucumber species *Holothuria scabra*, *Stichopus horrens* and *Stichopus ocellatus* from Kudat, Sabah, Malaysia (*Kamarul Rahim Kamarudin, Maryam Mohamed Rehan and Nur Aliah Bahaman*); Effect of combined application of poultry manure and inorganic fertiliser on yield and yield components of maize intercropped with soybean (*Almaz, M. G., R. A. Halim and M. Y. Martini*); Analysis of Gamma irradiated-third generation mutants of rodent tuber (*Typhonium flagelliforme* Lodd.) based on morphology, RAPD, and GC-MS markers (*Sianipar, N. F., Purnamaningsih, R., Gumanti, D. L., Rosaria, and Vidianty, M.*).

I conclude this issue with the article on forming process analysis in environmentally-friendly composite production from fibres of oil palm empty fruit bunches (*Arya, A. C.*).

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

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

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

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Pertanika J. Trop. Agric. Sci. 40 (1): 1 - 18 (2017)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Magnesium in Local Edible Ulam (*Centella asiatica)* and Its Relation to Their Habitat Soils in Peninsular Malaysia

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ABSTRACT

The aim of this study was to determine the Mg levels in *Centella asiatica* and their relationship to the habitat soils. Based on the levels of Mg in soils from the 12 sampling sites, its concentration was found to range from 13080 to 45350 µg/g dw. Although higher than the continental crust and European topsoils baselines, the soils of Peninsular Malaysia were considered 'unpolluted to moderately polluted' based on EF and 'deficiency to minimal enrichment' based on Igeo. As for plants, the highest Mg level was found in roots (3250 ± 815 µg/g dw), followed by leaves (2900 ± 565 µg/g dw) and stems (1660 ± 393 µg/g dw). This is in agreement with the transfer factor (TF). Based on correlation analysis and multiple linear regression analysis, Mg-Soil was found as a significant and the most important factor controlling the Mg uptake from the soils to the three plant parts. The direct relationships between Mg(plant)-Mg(Soil) also indicates that *C. asiatica* roots, leaves and stems are able to reflect the Mg levels of the sampling sites. Thus, the experimental transplantation studies under field and laboratory conditions confirmed the results from the field collected samples and indicated the roots, leaves and stems can be used as good biomonitors of Mg levels in the habitat soils.

Keywords: Biomonitor, *Centella asiatica*, Correlation studies, Magnesium, Transplantation

ARTICLE INFO

Article history:
Received: 04 June 2016
Accepted: 18 January 2017

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INTRODUCTION

Magnesium (Mg) is an alkaline earth metal and the eighth most abundant element in the Earth's crust. Although Mg is naturally present in soil, local sources such as industry, agriculture, sewage sludge, waste incineration and road traffic have increased the concentration of Mg in soil (Celik et al., 2005). Therefore, concern about the Mg concentration in the environment should arise to ensure the safety of human usage. During the mineral weathering process, Mg^{2+} cation, released from minerals in soil solutions, can be taken up by roots and stored in vegetation over shorter (months) or longer (hundreds of years) periods (Bolou-Bi et al., 2012). Mg can be accumulated in crops or plants and may lead to damage and alteration of animal or human physiological functions through the food chain. Biologically, high Mg levels can alter cellular ion balance and activity, especially Ca^{2+} activity, which directly affects neural and muscular functions (Barbare, 2005). In Malaysia, the levels of Mg are not found in the literature, especially in the local edible ulam, *Centella asiatica*. Furthermore, there are no baseline data for Mg in the plants and soils from Malaysia. Although there is no clear Mg pollution in Malaysia, the focus on Mg levels could shed some lights into the possibility of Mg pollution or to confirm that Mg is not an environmental concern in Malaysia at the moment.

The plant *Centella* has been used widely in folk medicine for hundreds of years to treat a wide range of illnesses (Brinkhaus et al., 2000). From the entire genus of *Centella*, only the *asiatica* species is found in commercial drugs today and acknowledged by WHO as one of the important medical plant species to be conserved (Zainol et al., 2003). In addition, locally, they are consumed as ulam. Therefore, public concerns over the potential ecotoxicological hazards posed by the presence of excessive Mg accumulation should be checked when using the plants to treat various illnesses and consuming it as a local vegetable.

Information on Mg levels in terrestrial soils in relation to *C. asiatica* is lacking in the literature. Hence, Mg contamination of natural soil resources due to urbanisation and industrialisation in Peninsular Malaysia can be understood better through this study. For this purpose, a low cost method to determine the extent of Mg contamination in local environments was assessed in this study. Therefore, the objective of this study was to determine the levels of Mg in *C. asiatica* and habitat surface soils, and later to relate the Mg levels in both plants and soils.

MATERIALS AND METHODS

Vegetation and soil sampling

Plant and soil samples were collected from 12 sampling sites from Peninsular Malaysia (Figure 1). Plants of 2-4 months maturity were collected and placed in plastic bags. At the same time, the surface soil of 3-5 cm depth (litters were removed) was collected into a plastic bag using a plastic scoop.

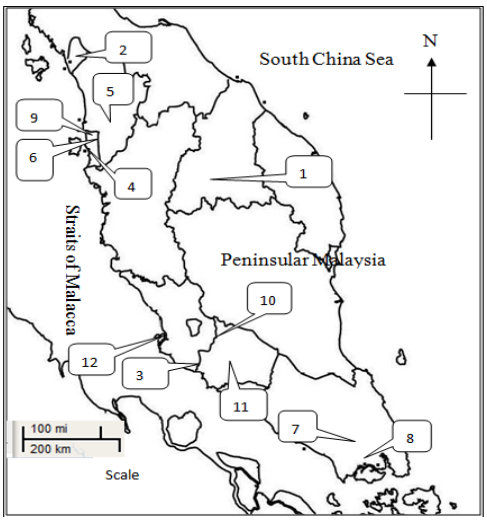

Figure 1. Map showing the sampling sites in Peninsular Malaysia

Note.

No	Sampling sites	Sites Description
1.	Wakaf Baru, Kelantan	Near a housing area.
2.	Arau, Perlis	Near an agriculture area.
3.	Universiti Putra Malaysia (UPM), Selangor	Near an agriculture area.
4.	Butterworth, Penang	Near an industrial and highway.
5.	Karangan, Kedah	Near an oil palm plantation.
6.	Permatang Pauh (PPauh), Penang	Near a housing area and highway.
7.	Pontian, Johore	Near a plant agriculture area.
8.	Kempas, Johore	Near a housing area.
9.	Kepala Batas (K.Batas), Penang	Near housing and agriculture areas.
10	Seremban, Sembilan	Near shop lots and road sides.
11	Senawang, Sembilan	Near an industrial area.
12	Port Klang (P.Klang), Selangor	Near port and industrial areas.

Transplantation Study

The transplantation studies were divided into experimental field and experimental laboratory conditions. All the plants used were obtained from the University Agricultural Park (UAP) at Universiti Putra Malaysia. The plants were planted together in the same condition for two months to reach the maturation stage before they were transferred or transplanted to specified locations. Four sites were selected for this transplantation study. Based on observations and reported studies, UPM was regarded as an unpolluted site, while Seri Kembangan (SK) and Balakong were taken as potentially receiving industrial wastes and Sg. Juru as a highly industrial area in Penang (Yap et al., 2009). Prior to the transplantation study, surface soils were collected from the four sites and their Mg levels were analysed. The results showed that the Mg levels (µg/g dw) in the surface soils were 15130 ± 2920 for UPM, 21650 ± 1110 for SK, 26050 ± 3160 for Balakong, and 32420 ± 1840 for Sg. Juru. Based on the comparison of the continental crust guidelines of Wedepohl (1995) (22000 µg/g dw) and Taylor (1964) (23300 µg/g dw), the four sites could be categorised as 'low Mg' at UPM, 'semi-high Mg' at both SK and Balakong, and 'relatively high Mg' at Sg. Juru.

In the experimental field condition study, the randomly selected plants were transplanted from UPM to SK, Balakong and Sg. Juru for three weeks (week 0 till week 3). After 3 weeks, the plants were

back-transplanted to the control site and were left to grow for 3 more weeks (week 3 till week 6). As for the experimental laboratory condition, the method was the same as that of the field condition; the difference was that the plants were planted in trays containing soils taken from the four selected sites (UPM, SK, Balakong and Sg. Juru). The duration of 3 weeks for transplantation was chosen because according to USEPA (1996), obvious effects could be observed on plants after 2 weeks of transplantation. In the transplantation study, three replicates were conducted for each site. The field study consisted of traps with the measurements of 75 cm × 75 cm × 10 cm and the laboratory study consisted of trays with the measurements of 60 cm × 35 cm × 10 cm.

Sample Treatments

In the laboratory, the plants were separated into leaves, stems and roots. The roots of the plants were washed with clean water under running tap so that no soil particles would be adsorbed on the root surface. Later, the leaves, stems and roots were rinsed with distilled water. The separated plant parts and soil samples were dried in an oven (65°C) for 5 days, which resulted in constant dry weight. The dry samples obtained were ground in an electronic agate homogenizer to yield a homogenous powder (± 2mm mesh size) and ensure that the elements within each sample were uniformly distributed. The homogenous samples were shaken manually and stored in polyethylene vials with weights ranging from 0.15-0.20g. The vials were all heat-sealed until further analysis.

Neutron Activation Analysis (U.S.EPA, 2001; IAEA-TECDOC-1360, 2003)

The TRIGA MARK II reactor at Agensi Nuklear Malaysia (NUKLEAR MALAYSIA) in Bangi, Selangor (Malaysia), was used to perform irradiations on the samples. Briefly, a pneumatic transfer system (PTS) was used and each sample was irradiated for a period of 30-60 seconds on the same position for a short irradiation to enable immediate counting of short-lived isotopes including Mg, Ti, Mn, K and Ca. In particular, the ^{27}Mg determined in this study was a short-lived radioisotope with a half-life of 9.46 minutes (Holden, 2004).

After irradiation in the reactor, the radioactivity measurement of the samples were carried out after a proper cooling time by using various close-end coaxial high purity germanium detectors (Model GC3018 CANBERRA Inc and Model GMX 20180, EG4G ORTEC Nuclear Instrument) and their associated electronics. The cooling time varied from 5-20 minutes for 1st gamma-ray counting for ^{27}Mg determination.

Data Verification

Certified reference material (CRM) IAEA-SOIL-7 was prepared using the same conditions and it was also used as quality control for each batch. The recovery of Mg based on IAEA-SOIL-7 was 96.56% (CRM certified value: 11300.00 ± 565.00µ/g dw; measured value: 10911.59 ± 1050.14µ/g dw).

Transfer Factor

The ratio of the Mg concentration in the plant to that in the soil was defined as the transfer factor (TF). The TF was based on total Mg in the soil, which was formulated by Alexander et al. (2006), as follows:

$$TF = CplantCsoil$$

where C_{plant} is the Mg concentration in the plant and C_{soil} is Mg concentration in the soil. Taking the source of Mg into account, the second formula is as follows:

$$TFadded = Cplant\text{-}Ccontrol\ plantCsoil\text{-}Ccontrolsoil$$

where C_{plant} is the Mg level in the test plant, $C_{control\ plant}$ is the Mg level in the control plant, C_{soil} is the Mg level in the test soil, and $C_{control\ soil}$ is the Mg level in the control soil.

Enrichment Factor

The calculation for differentiating metal origins by human activities or from natural sources is known as enrichment factor (EF). It can also be used to analyse the degree of anthropogenic influence in soil. It was calculated by using the following formula (Buat-Menard & Chesselet, 1979):

$$EF = \left(\frac{Cn\ (sample)/Cref\ (sample)}{Bn\ (baseline)/Bref\ (baseline)}\right)$$

where C_n (sample) is the concentration of the examined metal, C_{ref} (sample) is the concentration of the reference metal, B_n (baseline) is the content of the examined metal, and B_{ref} (baseline) is the content of the reference metal.

In order to normalise Mg, Titanium (Ti) was selected because it is a conservative element that is known to be derived mainly from crustal weathering (Schütz & Rahn, 1982). The baseline values were selected from the element's concentrations in the continental crust (Mg - 22000 µg/g dw and Ti - 4010 µg/g dw by Wedepohl, 1995) (Mg - 23300 µg/g dw and Ti - 3800 µg/g dw by Taylor, 1964). Since Malaysia does not have these baseline values, the values were based on the global average values. The levels of EF are categorised as reported by Han et al. (2006): <2 (deficiency to minimal enrichment), 2-5 (moderate enrichment), 5-20= (significant enrichment), 20-40 (very high enrichment) and >40 (extremely high enrichment).

Geoaccumulation Index

The geoaccumulation index (Igeo) was calculated based on the following equation (Müller, 1969):

$$Igeo = Log2\left(\frac{Cn}{1.5xBn}\right)$$

where C_n is the concentration of the examined metal, and B_n is the content of the reference metal. Factor 1.5 is the background matrix correction factor due to lithogenic effects. Since there are no background Malaysia values of the metals of interest, we adopted the earth crust

values as in the EF calculation (Wedepohl, 1995; Taylor, 1964). The following Igeo classifications are given according to Müller (1969): < 0 (practically unpolluted), 0-1 (unpolluted to moderately polluted), 1-2 (moderately polluted), 2-3 (moderately to strongly polluted), 3-4 (strongly polluted), 4-5 (strongly to very strongly polluted) and >5 (very strongly polluted).

Concentration Factor and Accumulation Rate

Concentration factor (CF) is a way to identify the uptake level of Mg in plants for transplantation studies. It was calculated based on the following formula (Yap et al., 2004):

$$CF = \frac{Mg_{\text{end of accumulation}}}{Mg_{\text{initial}}}$$

Where, the end of accumulation was based on the length of time for the accumulation of Mg in the plants after three weeks (21 days). The Mg accumulation rate (AR) was calculated based on the following formula (Yap et al., 2004):

$$AR = \frac{Mg_{\text{exposed}} - Mg_{\text{initial}}}{\text{Day(s) of Mg exposure}}$$

Elimination Factor and Elimination Rate

Elimination factor (EF) was used to determine the elimination rate of Mg in plants for the transplantation study, and it was calculated according to Yap et al. (2004):

$$EF = \frac{Mg_{\text{end of elimination}}}{Mg_{\text{initial}}}$$

Where, the end of elimination was based on the length of time for the elimination of Mg from the plants after three weeks (21 days). The Mg elimination rate (ER) was calculated according to Yap et al. (2004):

$$ER = \frac{Mg_{\text{exposed}} - Mg_{\text{initial}}}{\text{Day(s) of Mg elimination}}$$

Statistical Analysis

Correlation analysis (CA) and multiple linear regression analysis (MLRA) were performed based on all mean values. They were log10(mean + 1) before CA and MLRA in order to reduce the variance (Zar, 1996). Both analyses were done by using STATISTICA version 8 software.

RESULTS

Based on Figure 2, the Mg levels in soils from the 12 sampling sites ranged from 13080 to 45350, with the mean value of 26730 µg/g dw. Meanwhile, the highest level was recorded in Seremban. Based on the data presented in Table 1, the ranges of EF varied from 0.53 to 4.53, with Kalangan being the highest, and the least was from Arau. The Igeo values were ranged from -1.42 to 0.46. For all the sampling sites, the roots (3250 ± 810 µg/g dw) showed

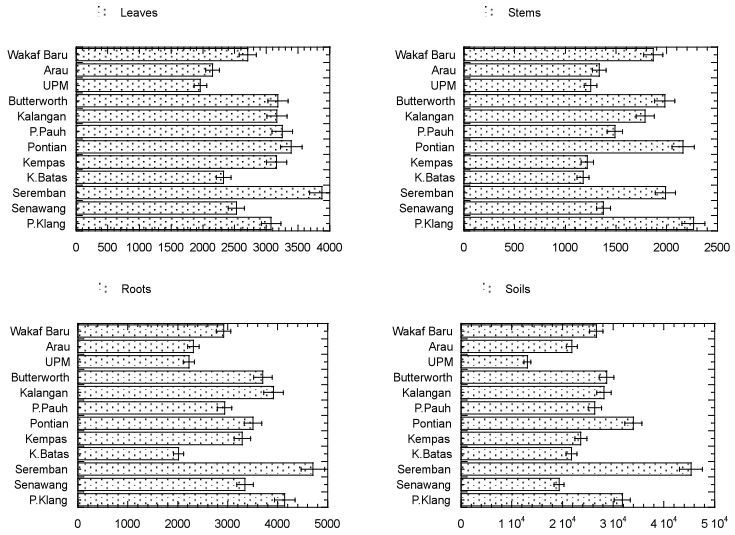

Figure 2. Mg concentrations (mean ± SD, µg/g dry weight) in leaves, stems and roots of *Centella asiatica* and habitat surface soils collected from 12 sampling sites in Peninsular Malaysia

Table 1
Levels of enrichment factor (EF) and geoaccumulation index (Igeo) of Mg from 12 sampling sites in Peninsular Malaysia

	Sites	EF[a]	EF[b]	Igeo[a]	Igeo[b]
1.	P.Klang	1.58	1.41	-0.05	-0.14
2.	Senawang	0.97	0.87	-0.77	-0.85
3.	Seremban	2.46	2.20	0.46	0.38
4.	K.Batas	1.21	1.09	-0.60	-0.68
5.	Kempas	1.03	0.93	-0.48	-0.56
6.	Pontian	0.97	0.87	0.04	-0.04
7.	P.Pauh	2.80	2.51	-0.32	-0.41
8.	Kalangan	1.84	1.64	-0.23	-0.31
9.	Butterworth	1.84	1.65	-0.20	-0.28
10.	UPM	0.60	0.54	-1.33	-1.42
11.	Arau	0.75	0.67	-0.60	-0.68
12.	Wakaf Baru	0.93	0.84	-0.31	-0.39

Note: a: Background values by Wedepohl (1995); b: Background values by Taylor (1964)

the highest Mg accumulation, followed by leaves (2900 ± 565 µg/g dw) and stems (1660 ± 393 µg/g dw). In leaves and roots, the samples from Seremban were highest in Mg concentration, while P. Klang showed the highest Mg concentration in stems.

In Table 2, the accumulation of Mg increased for all parts when transplanted from control to semi-polluted and polluted sites under field condition (week 0 to week 3). The increases were highest for Juru, followed by SK and Balakong for roots, leaves and stems. However, the accumulation decreased (week 3 to week 6) after the transplantation from the semi-polluted and polluted sites back to the control sites. The accumulation was still highest in Juru, followed by SK and Balakong. For the transplantation study under laboratory conditions, the trend was exactly similar to the transplantation study under field conditions, with lower

Table 2
Concentrations (mean ± SD, µg/g dry weight) of Mg in leaves, stems and roots of Centella asiatica for transplantation studies under field and laboratory conditions

Field	week	Leaves			Stems			Roots		
Juru	0	1120	±	94	930	±	54	1230	±	69
	3	1580	±	20	956	±	47	2412	±	23
	6	1514	±	37	950	±	32	2326	±	45
Balakong	0	1120	±	94	930	±	54	1230	±	69
	3	1390	±	55	950	±	33	2204	±	77
	6	1216	±	45	936	±	21	1873	±	68
SK	0	1120	±	94	930	±	54	1230	±	69
	3	1411	±	66	939	±	47	1687	±	52
	6	1402	±	73	934	±	68	1398	±	43
UPM	0	1120	±	94	930	±	54	1230	±	69
	3	1124	±	45	936	±	46	1230	±	53
	6	1119	±	55	932	±	71	1228	±	46
Laboratory	week	Leaves			Stems			Roots		
Juru	0	1120	±	94	930	±	54	1230	±	69
	3	1547	±	13	950	±	62	2382	±	79
	6	1414	±	47	944	±	48	2226	±	61
Balakong	0	1120	±	94	930	±	54	1230	±	69
	3	1215	±	64	950	±	53	2177	±	68
	6	1192	±	45	941	±	27	1570	±	35
SK	0	1120	±	94	930	±	54	1230	±	69
	3	1410	±	58	944	±	47	1643	±	71
	6	1314	±	45	937	±	13	1314	±	46
UPM	0	1120	±	94	930	±	54	1230	±	69
	3	1124	±	46	936	±	46	1230	±	53
	6	1119	±	54	932	±	71	1228	±	46

concentrations of Mg accumulated (Table 2). In Table 3, the overall values for CF and AR were highest for Juru under field and laboratory conditions. Hence, the EF varied for different sites, and the ER was fastest for Balakong (Table 3).

Table 3
Concentrations (mean ± SD, µg/g dry weight) of Mg in leaves, stems and roots of Centella asiatica for transplantation studies under field and laboratory conditions

Sites	Field conditions			Laboratory conditions		
	Leaves	Stems	Roots	Leaves	Stems	Roots
CF						
Juru	1.41	1.03	1.96	1.38	1.02	1.94
Balakong	1.24	1.02	1.79	1.09	1.02	1.77
SK	1.26	1.01	1.37	1.26	1.01	1.34
AR						
Juru	21.79	1.14	56.38	20.36	0.86	54.95
Balakong	12.92	0.85	46.48	4.57	0.85	45.20
SK	13.92	0.31	21.86	13.86	0.56	19.79
EF						
Juru	0.96	0.99	0.96	0.91	0.99	0.93
Balakong	0.87	0.99	0.85	0.98	0.99	0.72
SK	0.99	1.00	0.83	0.93	0.99	0.80
ER						
Juru	-2.96	-0.28	-4.09	-6.29	-0.28	-7.43
Balakong	-8.32	-0.65	-15.76	-1.08	-0.42	-28.93
SK	-0.44	-0.22	-13.77	-4.58	-0.31	-15.66

The TF values for all the 12 sampling sites are shown in Figure 3. The values ranged from 0.085-0.150 for leave, 0.044-0.096 for stem and 0.093-0.173 for root. Based on the mean values, the TF is highest in the root (0.126), followed by leave (0.113) and stem (0.064).

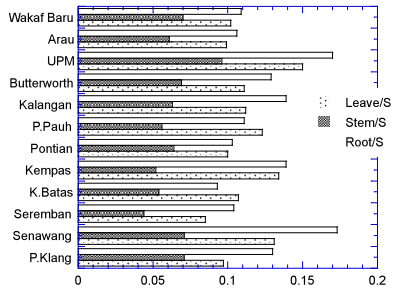

Figure 3. Transfer factors in the different leaves, stems and roots of all the sampling sites. Note: S= soil

The TF values for the three transplantation sites, both under field and laboratory conditions, are shown in Figure 4. The field condition values are higher in the leave (0.009-0.043) and root (0.026-0.064) compared to those (leave: 0.007-0.030; root: 0.013-0.058) under laboratory conditions. Based on the roots under both conditions, Sg. Juru showed the highest TF values, followed by Balakong and SK.

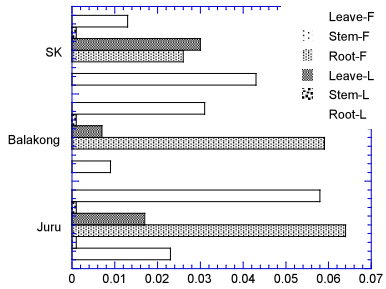

Figure 4. Transfer factors in the different leaves, stems and roots of the transplanted sites ate Seri Kembangan (SK), Balakong and Sg. Juru. Note: S= soil

Based on the leaves under both conditions, SK showed the highest TF values, followed by Sg. Juru and Balakong, while the stems did not show any significant variations of TF values in the sites.

DISCUSSION

Mg in the soil samples

When compared to the continental crust guidelines of Wedepohl (1995) (22000 µg/g dw) and Taylor (1964) (23300 µg/g dw), the eight samplings sites were higher than both the guidelines, particularly for the Seremban soils which was significantly ($P<0.05$) highest (Figure 2). Also, all the soil samples from 12 sampling sites were higher than the European topsoils baseline 11,800 ± 11,730 (<100–24,600) µg/g dry weight (Salminen et al., 2005). Therefore, these sites could be categorised as 'higher than background Mg level'; however, the interpretation of EF and Igeo values is needed to determine whether or not these sites were polluted by Mg.

The present average Mg level (26730 ± 8150 µg/g dw) in the soils from Peninsular Malaysia was higher than some reported data in the literature. For example, Jodral-Segado et al. (2006) reported the average Mg levels (µg/g dw) in agricultural soils of Southeastern Spain as 14230 ± 2250, sewage sludge as 15340 ± 1880, industrialised zone as 14360 ± 1890 and non-industrialised zone as 14160 ± 2250. They explained that the lower Mg levels could be attributed to natural sources. De Temmerman et al. (2003) also reported low Mg level in the soils (1380 µg/g dw) from Belgium. However, the soils from Turkey were reported to contain a high Mg level of 75200 µg/g dw (Hooda et al., 2004), and this elevation might be due to several factors, including geographic location, type of rock, pH, nature of drainage water, clay content, cation exchange capacity, weathering and climatic conditions, and type of plants grown (Jo & Koh 2004; Scheuner et al., 2004; Yanai et al., 2004).

The present EF values ranged from 0.53 and 4.53 (Table 1). Eight sites showed EF below 2, indicating 'deficiency in minimal enrichment', while only four sites showed EF more than 2, which indicated that the EF was in 'moderate enrichment'. This is in agreement with most reported studies, where data were usually below 5. Hernández-Mena et al. (2011) reported that the EF of Mg level in the city of Guadalajara, Mexico, was less than 5 (> 5; very strongly polluted), which was attributable to geological origins. This result is similar with the present study due to the different activities from different sampling sites. This indicated that the EF of Mg in Peninsular Malaysia was

not significant. Most of the Igeo values were less than 0 (unpolluted) and only two sites showed less than 1 (unpolluted to moderately polluted). The present Igeo values (-1.42 to 0.46) indicate 'deficiency to minimal enrichment'. Therefore, the Igeo values indicate a similar conclusion about the Mg status in Peninsular Malaysia as the EF. Hence, we can conclude the Mg level in the soils of Peninsular Malaysia did not indicate pollution by this metal.

Mg in the Plant Samples

The higher Mg levels found in roots than leaves from this study could be due to the large surface area of roots because root hairs elevate the adsorption and absorption of metals and facilitate nutrient uptake (Yap et al., 2010). This is supported by the TF values found in the present study, in which the Mg uptake in the different plant parts showed highest in the roots, followed by leave and stem. Roots adhere to the soil all the time to facilitate the absorption of water and nutrients. The uptake of metals must pass through the roots before reaching the other parts of plants (Ong et al., 2011). Therefore, the exposure of roots towards Mg in soil is higher, increasing the chances of Mg accumulation in roots. Hence, less amount of Mg was able to be transported to the upper parts of plants. This was supported by Shtangeeva et al. (2011), who showed that the roots of wheat had highest Mg levels (1650 ± 1000 μg/g dw), followed by the leave (1560 ± 544 μg/g); and higher in the root of rye (1870 ± 420 μg/g dw) than in the leave (1720 ± 295 μg/g dw).

The TF values found in the present study also indicated variations of Mg uptake in the different sampling sites. The highest TF values in the root from Sg. Juru could explain it being an industrial polluted site (Yap et al., 2009) and the Mg level in the soils was also the highest. Therefore, more Mg was being transferred to the plant root.

The present Mg levels of the leaves were comparable to some reported studies in the literature. For example, Oladipo et al. (2012) reported Mg concentrations (μg/g dw) in the leaves of some medicinal plants from Northern Nigeria (*Boerhavia diffusa*; 6640; *Euphoria hirta*, 1960; *Senna occidentalis*, 4730; *Senna obtusofolia*, 2510; *Cyprus dilatatus*, 3550; *Mitracarpus villosus*, 2670). Łozak et al. (2002) reported the Mg concentration (5778 μg/g) in mints leaves. This indicated that Mg concentration in *C. asiatica* from Peninsular Malaysia was within the average range as reported in the literature.

Mg in Transplantation Studies

In Table 2, samples from the four sites showed a similar trend as the samples from the 12 sampling sites (Figure 2). Mg concentration was highest in the roots, followed by the leaves and stems. This result is also supported by the TF values in which root showed the highest value. The accumulation of Mg increased in all the plant parts when they were transplanted from the 'low Mg' at UPM to 'semi-high Mg' area in SK and Balakong and 'relatively high Mg' at Sg. Juru under the field conditions from week 0 to week 3 (Table 2). In Table

3, all the CF values were higher than 1, indicating that the plants were able to uptake high concentration of Mg. Within 3 weeks, the plants were able to uptake at least 100% more Mg than the initial value. The range of AR was high starting from 0.31 to 56.38 µg/g dw per day, with the uptake of Mg being the highest in roots, followed by leaves and stems. This shows that C. asiatica can reflect the Mg levels in the environment through the uptake of Mg into the plants. Therefore, C. asiatica can be an ideal biomonitor due to its tolerance to a wide range of Mg concentration and its capability as a net accumulator over a short period of time (Rainbow & Phillips, 1993).

The accumulation of Mg in the plants decreased from week 3 to 6 due to its transplantation back to the control site. As shown in Table 3, the EF under field and laboratory conditions was at least 70%, indicating that Mg could be removed from all plant parts when the condition of the site was less contaminated than its previous site. The Mg was eliminated from plants at a rate of at least 0.22µg/g dw per day. However, the Mg concentration was still higher compared to the initial concentration at week 0, with roots having the highest Mg concentration, followed by leaves and stems. This showed that the elimination of Mg was not completed during the three weeks of transplantation due to the slower elimination rate compared to the accumulation rate. The accumulation and elimination of metals in plants were dependent upon the transplantation period (Hedouin et al., 2011). Hence, a longer time was required for Mg to be eliminated from the plants.

In order to understand the major controlling factor for the Mg uptake, the relationships of Mg levels with other major (Ca and K) and minor (Mn and Ti) metals between the plant and soils were investigated. Based on the data in Table 4, the correlation coefficients of Mg between plants and soils were found to be the highest between leaves-soils (R= 0.89, P< 005), followed by roots-soils (R= 0.77, P< 005) and stems-soils (R= 0.76, P< 005). It is interesting to note that positive significant correlations are also found for Leave-K(Soil), Stem-K(Soil), Root-K(Soil), Leave-Mg/Ti(Soil) and Root-Mg/Ti(Soil) (Table 4). In order to investigate other soils' metal, factors that could control the Mg uptake from the soils to the plant parts, regression analysis with inclusion of other soil factors are shown in Table 5. The inclusion of soil factors could

Table 4
The correlation coefficients of Mg concentrations between different parts of Centella asiatica and major cation ratios in the soils

Soil	Leave	Stem	Root
MgSoil	0.89	0.77	0.76
CaSoil	0.35	0.41	0.34
KSoil	0.68	0.59	0.63
MnSoil	0.32	0.06	0.13
TiSoil	-0.26	0.09	-0.14
Mg/Ca	0.25	0.11	0.18
Mg/K	0.52	0.46	0.41
Mg/Mn	0.04	0.26	0.19
Mg/Ti	0.76	0.43	0.59

Note: Values in bold are significantly correlated at P< 0.05

Table 5
Prediction equations for Mg levels (log10) transfer from soils to the plant parts based on multiple linear regression analysis

Prediction equations	R	R2	P
Mg(Leave)= 0.88+ 0.886 (Mg-Soil)	0.89	0.79	0.001
Mg(Leave)= 2.02 + 1.04(Mg-Soil)- 0.13(Ca-Soil)- 0.14(K-Soil)- 0.10(Mn-Soil)- 0.37(Ti-Soil)	0.92	0.85	0.019
Mg(Leave)= 1.83 + 0.43(Mg-Soil) + 0.11(Mg/Ca-Soil) + 0.12(Mg/K-Soil) + 0.09(Mg/Mn-Soil) + 0.49(Mg/Ti-Soil)	0.92	0.84	0.019
Mg(Stem)= 0.60 + 0.77(Mg-Soil)	0.77	0.59	0.003
Mg(Stem)= -0.90 + 0.52(Mg-Soil) + 0.27(Ca-Soil) + 0.22(K-Soil) + 0.01(Mn-Soil) + 0.32(Ti-Soil)	0.82	0.68	0.147
Mg(Stem)= -0.57 + 1.24(Mg-Soil) - 0.25(Mg/Ca-Soil) - 0.19(Mg/K-Soil) + 0.01(Mg/Mn-Soil) - 0.41(Mg/Ti-Soil)	0.82	0.67	0.153
Mg(Root)= 0.65 + 0.77(Mg-Soil)	0.77	0.59	0.003
Mg(Root)= 1.38 = 0.83(Mg-Soil) + 0.02(Ca-Soil) - 0.04(K-Soil) - 0.22(Mn-Soil) - 0.23(Ti-Soil)	0.79	0.62	0.213
Mg(Root)= 1.26 + 0.51(Mg-Soil) - 0.02(Mg/Ca-Soil) + 0.03(Mg/K-Soil) + 0.21(Mg/Mn-Soil) + 0.30(Mg/Ti-Soil)	0.79	0.62	0.216

usually improve the correlation performance between Mg level in the plant and in the soil compared to those based on only the Mg level in the soil (Liang et al., 2013). This is well indicated in the present study, as in Table 5. For example, R2 between log[Mg(Leave)] and log[Mg(Soil)] was 0.79. When Ca-Soil, K-Soil, Mn-Soil and Ti-Soil were combined, the controlled variance was improved to R2= 0.85, and when Mg/Ca-Soil, Mg/K-Soil, Mg/Mn-Soil and Mg/Ti-Soil were introduced in a separate equation, the regression coefficient rose to R2= 0.84. Similar patterns of R2 increments were also observed for Mg(Stem) and Mg (Root). However, these soil factor inclusions in the regression equations did not significantly increase the P values and the significant values. Therefore, this indicated that other factors could also control the Mg uptake; however, the direct correlations between the Mg(plant)- Mg(Soil) are still higher, indicating that the Mg level in the soils is the major controlling factor for the transfer of Mg to the three plant parts, as also evidently seen in Table 4. However, it is undeniable that the Mg contents in plants (oats, maize, yellow lupine and radish) were generally correlated with the accumulation of other macroelements and some microelements (Ciećko et al., 2005), which is affected by the presence of other cations such as ammonium, potassium, manganese and sulphur (Phillips & Chiy, 2002). Although it has been shown that the soil composition can be a factor in determining the growth of *C. asiatica* (Devkota & Jha, 2009), how the soil composition can influence major elemental uptake such as Mg is still unknown to our knowledge. In this study, it is assumed that the major controlling factors on the Mg bioaccumulation in *C.*

asiatica are the anthropogenic input and the environmental natural sources. However, other factors that can potentially affect the Mg uptake in the root, stem and leave of *C. asiatica* should not be ruled out.

According to a Freundlich-type function relationship, a strong relationship could still be expected between plant metal concentration and soil metal concentration (Efroymson et al., 2001). Furthermore, the Freundlich-type function is often used to describe metal transfer from the soil to plants (Krauss et al., 2002). Therefore, the significant and strong correlations of Mg levels between the three plant parts and soils indicated the Mg transfer from the soil to the plant. Mg is one of the secondary macronutrients, which are usually available in soils (Barker & Pilbeam, 2007). Mg is found in chlorophyll molecules and it is essential for photosynthesis. It also helps in the activation of many plant enzymes that are needed for growth and maintenance of cell ionic balance (Gums, 2004). Therefore, a significant correlation was found between soils and *C. asiatica*. The above results indicated that the three parts of *C. asiatica* are able to reflect the Mg levels in the soils. Therefore, the roots, leaves and stems of *C. asiatica* are good biomonitors of Mg enrichment.

CONCLUSION

Although higher than the continental crust and European topsoils baselines, the soils of Peninsular Malaysia are considered as 'unpolluted to moderately polluted' based on EF and 'deficiency to minimal enrichment' based on Igeo. The Mg concentrations in *C. asiatica* from Peninsular Malaysia were within the average range when compared to the reported data. The highest Mg level was found in the roots, followed by leaves and stems. Based on CA and MLRA, Mg-Soil was found as the significant and most important factor controlling the Mg uptake from the soils to the three plant parts. The direct relationships between Mg(plant)-Mg(Soil) also indicated that *C. asiatica* roots, leaves and stems are able to reflect the Mg levels of the sampling sites. The experimental transplantation studies, under field and laboratory conditions, confirmed the results from the field collected samples and indicated that the roots, leaves and stems could be used as good biomonitors of Mg levels in the habitat soils.

ACKNOWLEDGMENTS

The authors wish to acknowledge the financial support provided by Universiti Putra Malaysia through the Research University Grant Scheme (RUGS) [Vote no. 9322400].

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Pertanika J. Trop. Agric. Sci. 40 (1): 19 - 34 (2017)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Comparative Histological Evaluations of the Sublingual Salivary Glands of EBN Swiftlets (*Aerodramus fuciphagus*) in Man-Made Houses and Natural Caves

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ABSTRACT

One of the most precious edible bird's nests (EBN) is constructed by the white-nest swiftlet (*Aerodramus*). However, different swiftlet populations might have different food intakes as a result of their different habitat sources. This situation will likely influence the secretion of the salivary gland. EBN is built from the saliva of the swiftlets. The major function of the salivary gland is to secrete saliva. This study was conducted with the aim of defining and comparing the histological structures of the sublingual salivary gland and its mucin content found in two separate populations of house-farm and cave white-nest swiftlets. Samples were collected from Seri Iskandar, Perak, Malaysia (04º20.824´N, 100º52.826´E) and Gomantong caves, Sabah, Malaysia (5º31.46.5´N, 118º4.29.6´E). It was found that the largest visible salivary gland present in both populations was the sublingual gland. The glands were stained with hematoxylin and eosin (H & E) stain and a combination of Alcian blue (AB) with periodic acid-Schiff (PAS) stain. The H&E stain displayed a broad range of cytoplasmic, nuclear and extracellular matrix features. The parenchyma of the cave swiftlet population appeared foamy due to high mucous secretion whereas the cells of the house-farm population could clearly be seen to be separated because of less mucous secretion. There was a clear difference in density and abundance of mucous acini cells in which the samples from the cave population were compacted with these cells. AB-PAS stains revealed full complement of tissue proteoglycans and acidic-mucin, neutral-mucin and mixtures of acidic and neutral mucins. The cave population exhibited higher concentrations of acidic, neutral, and mucins mixture compared with those from the house-farm. This is probably

ARTICLE INFO

Article history:
Received: 17 October 2014
Accepted: 10 November 2016

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ISSN: 1511-3701

caused by several combinations of factors such as difference in dietary habit, habitat preference and age of the swiftlet.

Keywords: white-nest swiftlets, edible bird's nest (EBN), sublingual salivary gland, saliva, house-farm swiftlets, cave swiftlets

INTRODUCTION

A few species of swiftlets (genus *Aerodramus*) build edible nests that are consumed by humans, known as the 'caviar of the East' or as a medicinal food (Marcone, 2005). Edible bird's nest (EBN) refers to the nest produced by several different swiftlet species. Human consumption of these nests has been regarded as a symbol of wealth, power and prestige, while its use for its medicinal value by traditional Chinese medicine practitioners dates as far back as the Tang (618-907 AD) and Sung (960-1279 AD) dynasties (Lim & Cranbrook, 2002). The majority of EBNs traded worldwide comes from two heavily exploited species, the white-nest swiftlet (WNS) and the black-nest swiftlet (*A. maximus*). This species distribution ranged from the Nicobar Islands in the Indian Ocean to the sea caves in the coastal regions of Thailand, Vietnam, Indonesia, Borneo and the Palawan Islands of the Philippines (Lim, 2000; Lim & Cranbrook, 2014). Based on the recent systematic review by Cranbrook et al. (2013), WNS are divided into two large allopatric species, namely the grey-rumped Swiftlet *Aerodramus inexpectatus*, with subspecies *A. i. germani* and *A. i. perplexus*, and Thunberg's or the brown-rumped Swiftlet *Aerodramus fuciphagus*, with subspecies *A. f. fuciphagus* and *A. f. vestitus*. Species identification in the field has proven to be challenging because of the limited variation in size and plumage colouration of the swiftlets. Stresemann (1931) has characterised these birds based on their tarsal feathering, rump and shaft colouration as well as the length of the wing, tail and their furcation. The type of the nest was also considered to be one of the reliable taxonomic indicators among swiftlets as shown by Medway (1966a). In this study, the taxonomic classification for the species studied follows that of Cranbrook et al. (2013), in which the cave population is identified as *A. f. vestitus*, while the current domestic house-farm population is a potential hybrid species drawn from genetic mixing of two species of WNS (i.e. *A. inexpectatus* and Thunberg's swiftlet *A. f. fuciphagus*).

EBN is the nest of the swift that is made from its saliva, which contains sialylglycoconjugates (Matsukawa et al., 2011). The composition of the swiftlet's saliva resembles that of salivary mucin. Many studies have been carried out on the tonic effects of EBN, and it has been shown that EBN stimulates mitosis hormones and the growth factor for epidermal growth, resulting in repair of cells and stimulation of the immune system (Ng et al., 1986; Kong et al., 1987). The average crude protein content in the EBN has been reported by Marcone (2005) to be at 62%-63% and by Kathan and Weeks (1969) to be at 32.3%. Researchers have also found several carbohydrate

molecules in EBN including new sialic acid-containing compounds and glycoconjugates (Martin et al., 1977; Pozsgay et al., 1987; Reuter et al., 1989; Wieruszeski et al., 1987; Kakehi et al., 1994; Yu-Qin et al., 2000). However, the importance of sialic acid residues in EBN is still not clear.

The number and arrangement of salivary glands vary among the species. In mammals, there are three main pairs of salivary glands: (i) the submandibular (ii) the sublingual, which lie under the tongue, and (iii) the parotid, which lies at the back of the mouth between the upper and the lower jaw (Tomasi & Plaut, 1985). In birds, adaptation of the salivary glands is based on the type of food consumed. In general, a species that relies on a relatively soft diet has less developed salivary glands while insectivores and seed eaters have more developed and functional salivary glands (King & McLelland, 1984; Blanks, 1993; Taib & Jarrar, 2001). In contrast, swiftlets have numerous minor salivary glands in their lingual apparatus. This modification allows the swiftlets to produce massive amounts of salivary secretion, which may manifest significantly during the nest-building process (Shah & Aziz, 2014). Although the salivary glands of most birds are not conspicuous as those in mammals, the comparative morphology has been studied since the 1880s (e.g. Batelli & Giacomini, 1889) and most avian glands are histologically described as the mucous type (Jerret & Goodge, 1973). In addition, the presence of serous cells has also been recorded in the salivary glands of quail, *C. coturnix* (Taib & Jarrar, 1998).

The primary function of salivary glands is to secrete saliva, a fluid composed of water, electrolytes and various multifunctional proteins (Koller et al., 2000). The basic protective mechanism mediated by saliva is bacterial clearance. Saliva is also an essential fluid for the health of human teeth and oral mucosal surfaces and for maintaining microbial balance and supporting other oral functions. Other than that, saliva also contains antifungal and antiviral substances that make it part of the mucosal immune system (Tomasi & Plaut, 1985). On the other hand, swiftlets use saliva to construct their nest (Goh et al., 2001), and this is considered one of the swiftlet's prized assets because there are no other organisms with such ability. Swiftlet nests are constructed at the vertical concave of a cave wall in a half bowl-like shape into which the swiftlets' eggs are hatched (Marcone, 2005). The objectives of this study were to define the histological structures of the sublingual glands and to determine the mucin type of the glands from two different swiftlet populations. It was hypothesised that there are differences in the morphology and the amount of protein concentrations in the sublingual glands of swiftlets from house-farm and cave populations.

MATERIALS AND METHODS

Sample and Data Collection

During mist-netting, a number of captured swiftlets were released as there was no visible bulking salivary gland underneath the throat and lower mandible. Only 14 birds were selected based on this criterion.

It was believed that the swiftlets used were adult birds based on their overall body size as well as the presence of bulking salivary glands found under the lower mandible. Seven WNS were captured in Seri Iskandar (04º20.824´N, 100º52.826´E), where the distance of the sampling area from the bird-house is approximately 500 m. The other seven swiftlets were captured inside the Gomantong caves (5º31.46.5´N, 118º4.29.6´E). The swiftlets from Seri Iskandar, Perak were captured on 3 January, 2013, whereas the swiftlets from Gua Gomantong, Sabah were captured on 18 December, 2013. Only 12 birds with well-developed sublingual glands (three for each group) out of the 14 birds were selected to standardise the comparison. Only a small number of samples was collected for each group due to lack of cooperation from the house-farm/cave owners. The 20-m mist net (2.5 m height and 2 cm x 2 cm mesh size) with two shelves was deployed in the free land of Seri Iskandar, Perak, and the swiftlets were enticed by playback calls using a portable speaker (G-Shark S938). When the birds hit the net, they were quickly caught and transferred into wooden cages, which were then covered with cloth to reduce the stress of the birds. The birds were quickly transported to a laboratory in the Institute of Biosciences, Universiti Putra Malaysia (UPM). Sampling at Gua Gomantong, Sabah was conducted by deploying the nets directly to heights closest to the bird nests. Subsequently, the birds were euthanised and dissected at the Regional Veterinary Laboratory, Department of Veterinary Services and Livestock Industries, Kota Kinabalu, Sabah. The study protocol was approved by the UPM Animal Ethics Committee (AUP: 12R144/Apr12-March13).

Gross Examination

Before performing the dissection, the swiftlets were euthanised using approximately 1 mL pentobarbitone sodium (Nembutal®) at a dose of 80 mg/kg intravenously, which was injected through the brachial ulna vein (Close et al., 1996). The feather around the lower mandible was gently removed using alcohol; this was carried out carefully to prevent any distortion to the salivary glands. Subsequently, the dissection was carried out to expose the sublingual glands. The glands were subjected to gross examination under stereomicroscope (Nikon SMZ1500, Tokyo, Japan), and the weight of the glands was measured using a three-decimal place weighing balance (B303-S analytical balance, Mettler Toledo, Switzerland). Following that, the gender of the swiftlets was determined by observing the sexual reproductive organs.

Microscopic Examination

The tissues of the gland were then removed and fixed in Bouin's solution for 16 to 24 h and washed every 2 h using 50% alcohol for a total of three times and preserved with 70% alcohol (Adnyane et al., 2011). The samples were then transferred into a cassette, processed for 16 h and then embedded in paraffin wax (Bancroft & Gamble, 2008). The blocks were serially sectioned into 4-µm thickness using a microtome

(Leica RM-2155 rotary microtome; Leica Microsystem Inc., Bensheim, Germany). The sections were then deparaffinised, hydrated through graded alcohol with water and stained with hematoxylin and eosin (H&E) to demonstrate the general histological architecture of the tissue (Spector & Goldman, 2006). The tissue was also stained using a combination of Alcian blue-periodic acid-Schiff (AB-PAS) with a pH of 2.5 for the differentiation of neutral and acid mucins (Spicer & Meyer, 1960; Bancroft & Gamble, 2008). The slides were mounted with cover slips using the mounting medium (Entellan®, Merck, Germany) and left for 24 h in the open air. Finally, the stained slides were examined under a light microscope equipped with an image analyser (Olympus BX51; Olympus Optical Co. Tokyo, Japan).

Statistical Analysis

The data providing sublingual gland weight were calculated based on actual and relative weight, which was expressed as mean ±SD. Statistical comparisons were conducted between the two WNS populations from different habitats. Data were analysed using an independent-sample t-test for parametrics (IBM SPSS Statistic Ver. 21). The significant level was set at $p<0.05$.

H_0 = Mean relative weight of sublingual glands was the same for both the house-farm and cave population

H_1 = Mean relative weight of sublingual glands was significantly different for the house-farm and cave population.

RESULTS

Gross Examination

Gross examination of the salivary glands showed that there was a pair of major salivary glands (i.e. sublingual glands) present ventral to the lower mandible of the swiftlet (Figure 1). However, this structure

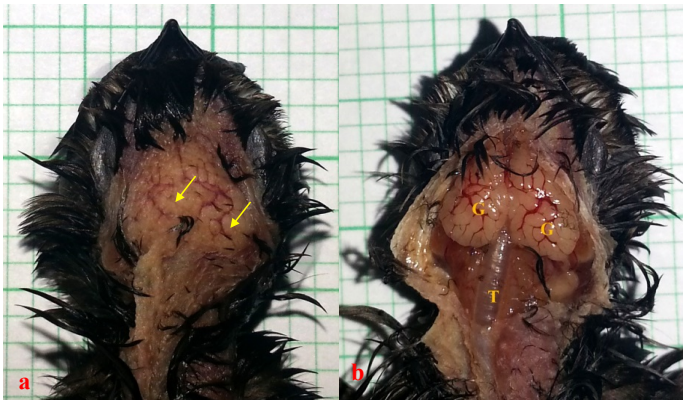

Figure 1. Photographs of the bird (a) before dissection showing the submandibular glands lying underneath the skin ventral to the lower mandible (arrows) (note that the feather around the lower mandible was removed) and (b) after dissection showing the exposed submandibular glands. The glands are enlarged and lobulated. G = submandibular glands; T = trachea; 1 grid = 1 mm

could only be seen clearly once the feather under the lower mandible of the bird was removed. The glands were present in a pair, and they were greatly enlarged compared with the other major salivary glands. An observation of the 3D images under the stereomicroscope showed a well-developed gland that appeared as a 'brain-like' coiled tubular structure with a soft white to pinkish appearance of the sublingual glands (Figure 2). The weight and the relative weight of the sublingual glands between the house-farm and cave WNS population are shown in Table 1.

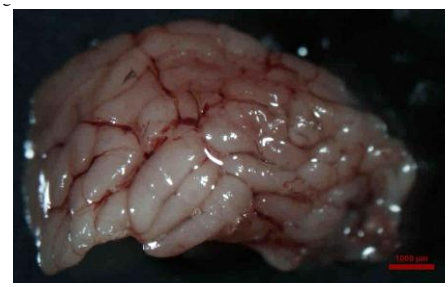

Figure 2. Photograph of the sublingual gland under high magnification (microvisualisation) showing the coiled tubular structure of the gland that appeared as a brain-like coiled tubular structure with soft white to pinkish colour. Magnification: x25; Scale: 1 mm

Table 1
Mean Values of Whole Body Weight and Sublingual Glands (Grams) of WNS from House-Farm and Cave Population

Location	House-Farm			Cave		
Sex	Male	Female	Mean (Total)	Male	Female	Mean (Total)
No. of animal	3	3	6	3	3	6
Body weight (g)	8.520 ± 0.494	9.033 ± 0.354	8.777 ± 0.476	10.283 ± 0.580	10.500 ± 1.212	10.39 ± 0.858
Sublingual gland weight (g)	0.050 ± 0.030[a]	0.079 ± 0.031[b]	0.065 ± 0.032[c]	0.072 ± 0.015[a]	0.050 ± 0.039[b]	0.061 ± 0.029[c]
Relative sublingual gland weight (%)*	0.604 ± 0.366[c]	0.879 ± 0.363[d]	0.721 ± 0.324**	0.702 ± 0.176[c]	0.500 ± 0.418[d]	0.574 ± 0.253**

*The relative sublingual gland weights was calculated based on the sublingual gland weight (g)/body weight (g) and presented in percent (%).
[a, b, c, d, e] The mean value of the sublingual glands with similar letter was not significantly different ($p>0.05$).
**Comparison between these two populations showed a significant p-value ($p<0.05$).

As shown in Table 1, the mean body weight of the cave population was 10.28±0.58 g for males and 10.50±1.21 g for females, whereas the house-farm population had a lower mean body weight (8.52±0.49 g for males and 9.03±0.35 g for females). The weight of the sublingual glands of the cave WNS was greater than that of the house-farm population in males, but the weight of the gland in females from the house-farm population was greater than that of the cave population although the body weight was slightly lower. However, based on the statistical test, the relative weight of the

sublingual glands between both populations was not significantly different ($p>0.05$). Comparison between these two populations was significantly different at $p<0.05$.

Microscopic Examination

Based on the H & E stain (Figure 3), cross sections of the cave WNS showed that the alveolus of the mucous acinus was wholly stained as compared with that of the house-farm birds. The parenchyma appeared foamy in cave swiftlets apparently due to the high mucous secretion and viscosity of the mucous cells, whereas the cells of house-farm birds could clearly be seen to be separated; it was expected there would be less mucous secretion and viscosity. The mucous acini were well positioned around the cell itself and were compartmentalised into several lobules by connective tissue. The nucleus could be seen at the base attached to the branch of the connective tissue. The irregular loose connective tissue was present between and encapsulating the glands, whereas the blood vessel was present in the middle and outside the cells (Figures 3a and 3d). There was no observable difference in mucous secretion between these two WNS populations (Figures 3b and 3e). The mucous acini cells could be seen to be separated from one another, but there was a clear difference in terms of density; the samples from the caves were compacted within these cells (Figures 3c and 3f). This was believed to be the cause of the foamy appearance of the gland tissues. The epithelial cells surrounding the gland tissues of the birds from cave populations (Figures 3d-3f) were observed, but the simple connecting ducts or the excretory ducts in both samples could not be observed. The presence of numerous acinus cells in both samples was weakly stained by the H & E stain; this could indicate that the sublingual glands of the WNS were only present within the mucous acini.

The sublingual gland tissue of both samples stained with AB-PAS (pH of the AB was 2.5) showed that the gland tissue was full of mucin secretion (Figure 4). Both samples contained mixtures of acidic and neutral glycoprotein-containing structures (mucin) because, histochemically, both showed positive reactivity to staining using a combination of AB and PAS stains. The staining area of the mixture of mucin and neutral mucin tissue was roughly the same in total area. However, it was apparent that samples from the cave also exhibited dominant blue staining, which indicated the occurrence of acidic mucins; this was not presented in the samples from the house-farm. Figure 4 shows the magnification of different levels of AB-PAS stain from both population samples. The samples from the house-farm population (Figures 4a-4c) clearly expressed different colours of staining, showing a mixture of mucin stained purple violet and the neutral mucin stained magenta. Meanwhile, as indicated by the intense colour stained, it was clear that mucous cells were abundant, the secretion was high in the cave samples and the cells were not well separated (Figures 4d-4f).

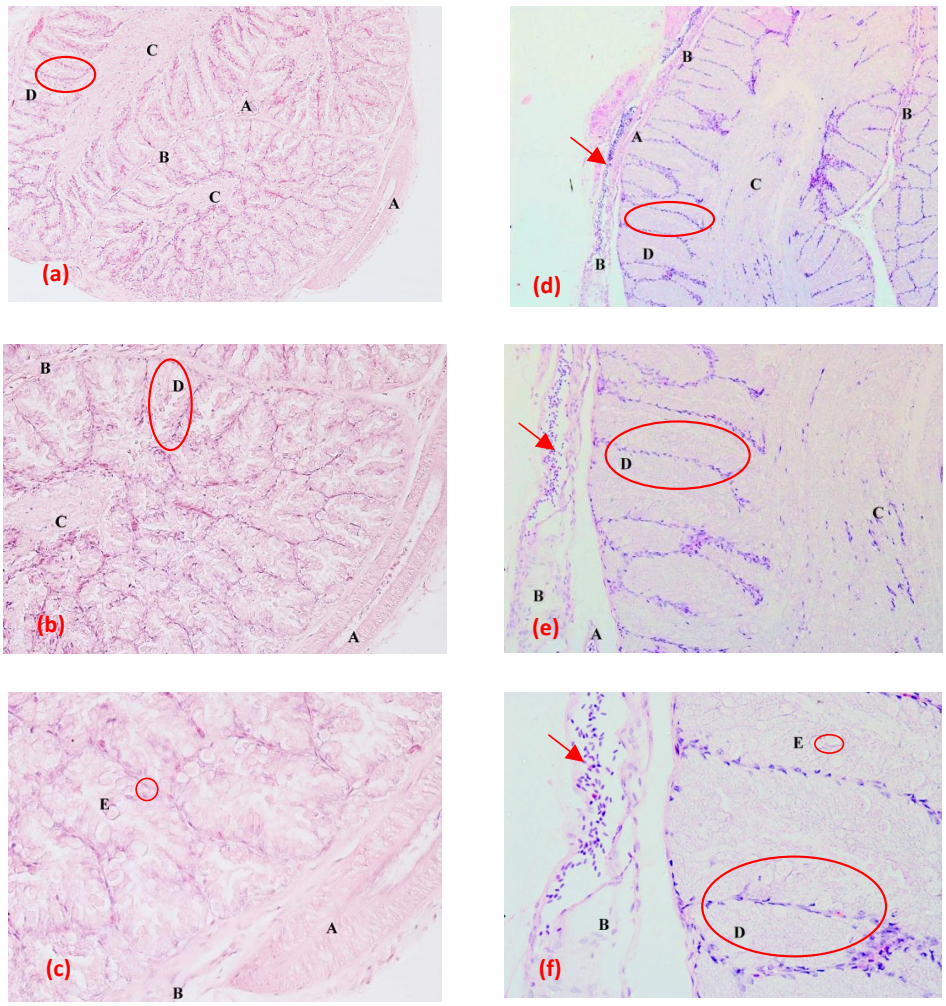

Figure 3. Photomicrographs of the cross-section of the sublingual glands of house-farm and cave WNS. Samples were stained with H & E at different levels of magnification. Cross-sections from (a-c) the house-farm and (d-f) the cave samples are shown. The magnification levels were set starting at (a and d) µ100, (b and e) µ200 and (c and d) µ400. The circles labelled D and E are the lobule of a gland cell and a mucous cell. The arrows show the epithelial cells present around the gland. A=blood vessel; B=loose connective tissue; C=mucous secretion

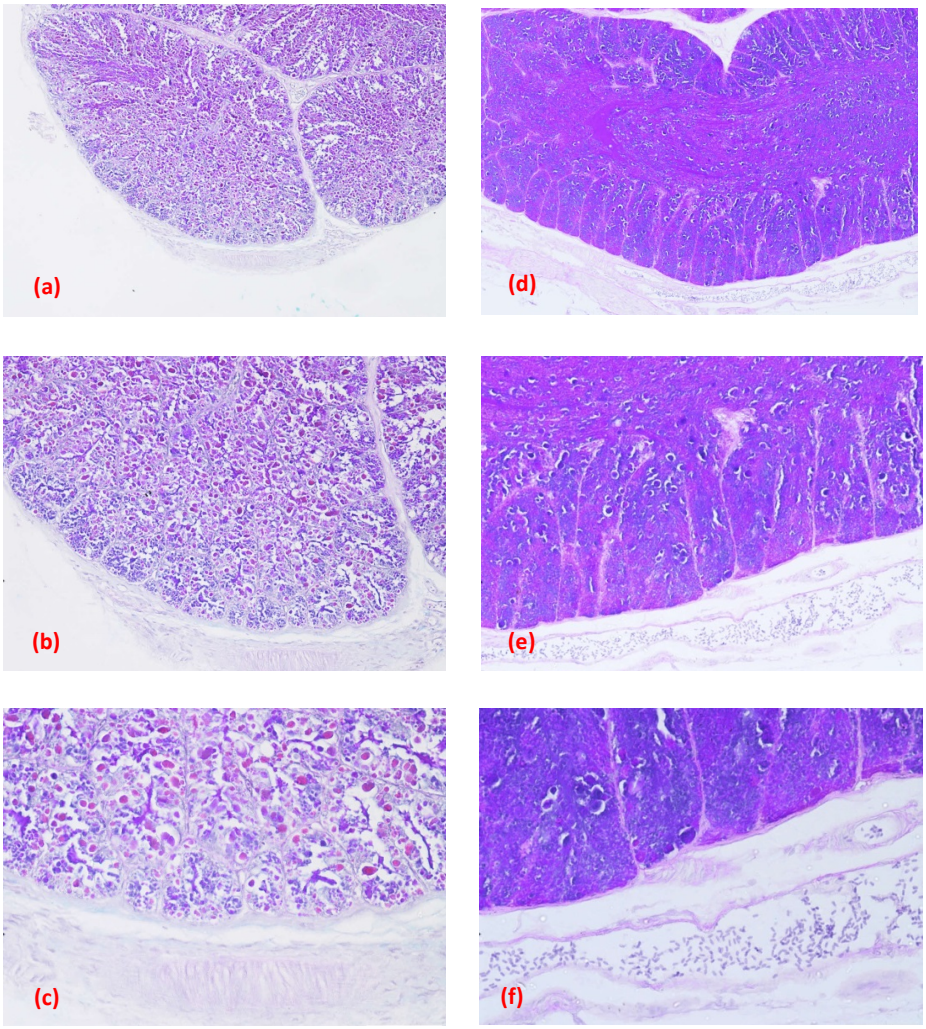

Figure 4. AB-PAS staining of the cross-sectional view of (a-c) house-farm and (d-f) cave WNS using the following magnification levels: (a and d) x100, (b and d) x200 and (c and f) x400

DISCUSSION

The EBN is wholly secreted by a pair of sublingual glands (Marshall & Folley, 1956) and this study found that the largest visible salivary gland present in WNS of both localities was the sublingual gland. The gland is a simple tissue mass with a soft to white pinkish appearance. According to Mese and Matsuo (2007), larger glands have more cells that will contribute to a higher production of saliva in either the stimulated stage or the unstimulated stage (resting saliva). The swiftlets were captured in January and December, which coincided with the active breeding season when the salivary glands expand (Lim & Cranbrook, 2014). Mating occurs throughout, but breeding is concentrated in the period from October to February (Langham, 1980). The sublingual glands appear to be the largest salivary gland; hence, the main source of saliva production in the WNS. This is in contrast with the salivary gland in humans, where the largest major salivary gland is the parotid gland (Ono et al., 2006).

Swiftlets are aerial insectivores that prefer foraging habitat across the tropical forest canopy (Waugh & Hails, 1983). It was hypothesised that the glands of cave WNS had more mass compared with the house-farm species due to its habitat preference and the high abundance and diversity of insect sources in the tropical forest. The hypothesis was derived based on food availability in the habitat as the size of the organ is most likely influenced by diet. Lewis et al. (1985) and Stoltzner (1977) stated that organ weight is often greatly reduced by dietary restriction. In addition, Lourie and Tompkins (2000) reported that the glossy swiftlets (*Collocalia esculenta*) live on forest feeds that have a higher percentage of Hymenoptera (i.e. bees and ants) (42% of total prey) and Coleoptera (i.e. beetles) (21% of total prey), whereas those that live in the urban areas preferred Diptera (i.e. flies) (71% of total prey). The percentage of crude proteins is 21.0% from Hymenoptera, 26%-30% from Coleoptera (Banjo et al., 2006) and 48% from Diptera (Odesanya et al., 2011). This shows that the glossy swiftlets from urban areas have more protein intake. In this study, none of the birds was subjected to a controlled diet, and the limited source of food intake could not be neglected. Table 1 shows that there was no significant difference in the mean weight and relative weight of sublingual glands between the two populations ($p>0.05$). This might be because of the small sample size of the study due to sampling limitation. Difference in the size of the glands between both specimens might as well influence the analysis. Lim and Cranbrook (2014) stated that the salivary glands of swiftlets expand in the breeding season. This difference is because of their different breeding seasons; harvesting might affect the breeding season of the swiftlets (Tompkins, 1999).

Based on the H & E staining, there was no visible serous cells in the swiftlets' sublingual glands, unlike in humans (Myers & Ferris, 2007) and rats (Miclaus et al., 2009). The only cells present in the swiftlets' sublingual glands were the mucous cells, as demonstrated by the pale-stained cytoplasm with flattened nuclei at the base of the

cell. Serous acini cells are darkly stained and generally spherical in shape (Ross & Pawlina, 2011). Mucous cells are associated with the secretion of viscous mucins stored in vacuoles (Ekstrom et al., 2012) and possessed a mixture of glycoconjugates with different nature (Arthitvong et al., 1999). EBN contains a high amount of glycoconjugates beneficial to humans (Nakagawa et al., 2007). Compared with the serous secretory granules, it contains less glycoconjugates and has a large amount of water and ions. The glycoconjugates of serous granules are acidic and termed 'seromucous' (Kademani & Tiwana, 2015). However, this mucous cell might not be the same as that found in other organisms. In rats, the serous acini of the submandibular gland are not identical with the serous acini that is present in the parotid gland (Miclaus et al., 2009). Other study showed that salivary glands are present with lumens that act as a passage for gland secretion to the oral cavity (Wells & Patel, 2010) and ducts (either in the form of intercalated, striated, excretory and main excretory ducts), which will modify the secretion of the acinar cells. However, it can only be observed under electron microscope observation (Amano et al., 2012) and is rarely observed in H & E stains. This sublingual glands need to be further studied with regards to its ultrastructure to observe the type of myoepithelium, secretory granules, plasma cells etc. Although the parotid glands mainly have serous acini, as in humans, the sublingual glands are mucous, whereas the submandibular glands are a mixture of the two, yet these acinar cells do not relate to all species. As diets vary from one species to another, in the same way, salivary glands vary from one another, as they are specialised mainly for diet (Tandler & Philips, 1998). The current results also showed that the sublingual glands of the WNS had a spherical outer layer and there was no demilunar structure as in cats and dogs (Shackleford, 1962). The sublingual glands might be suggested to be classified as mucous glands due to the absence of serous cells.

A combination of an AB-PAS stain can be used to differentiate neutral mucins from acidic mucins within a tissue section (Mowry, 1963), where the differentiation is based on the net charge of the molecule (Filipe, 1979). Mucins are the determinants of the functional and physical properties of mucous, which is highly glycosylated and has high molecular weight proteins (Forstner & Forstner, 1994). WNS from the cave population have higher concentrations of the acidic, neutral and mucins mixture compared with those from the house-farm population, and this probably will affect the composition of the nest. Squires (1953) revealed that the contents and varieties of salivary secretion are mostly related to the eating habits of the birds. However, it is unclear as to what extent the diet will influence the size of the glands and the secretion of the salivary glands. More than that, the different contents of secretion are due to the preference of habitat as a small change in the ambient temperature (by 2ºC) is enough to inversely affect the

flow rate of the salivary gland secretion (Kariyawasam & Dawes, 2005). Other than that, the secretion contents are also possibly related to the age of the swiftlets because the components of the salivary gland acini decrease with ageing (Drummond & Chisholm, 1984; Scott, 1986). This hypothesis remains unchallenged as there are currently no available data or studies on age determination of swiftlets. Because the nests (EBN) are abundant with glycoprotein (Wu et al., 2010), this study has proven that the sublingual glands of EBN swiftlets are full of mucin secretion, which is essential as the main source of nest production.

CONCLUSION

In conclusion, the sublingual glands appeared as the largest gland structure present in the salivary glands of the WNS. There was no significant difference in terms of the weight/relative weight of the sublingual glands between the two populations and the H0 was accepted. The only cells that could be observed under a light microscope (0µ400 magnification) were the mucous cells that were attached to a loose connective tissue forming a lobule. On the other hand, this gland holds a rich mixture of neutral and acidic mucin, which serves as the most nutritious compound in the edible nest. This study also indicated that the WNS from the cave population had a higher concentration of secretion compared to the house-farm population. However, detailed information about the types of mucin compound (glycoprotein-containing structure) in the saliva is still lacking. Further study is needed to analyse the glycoprotein content of the salivary glands, which is the source of the nutritious compound found in EBN. Other than that, it is recommended to increase the sample size for future studies concerning sexual dimorphism of the species.

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Pertanika J. Trop. Agric. Sci. 40 (1): 35 - 52 (2017)

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TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Physical, Chemical and Microbiological Properties of Different Combination of Soilless Media and Their Effect on the Vegetative Component and Nutrient Content of Hempedu Bumi (*Andrographis paniculata*)

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ABSTRACT

Soilless media (SM) is a common worldwide growing method for industrial horticultural production. It is a good growing medium that relies on the properties that benefit plant growth i.e. physical property, chemical properties and microbial activity. There are several SM with good characteristics such as empty fruit bunch compost (EFBC), coconut coir dust (CCD) and peat. EFBC is one of the organic residues of oil palm that provide beneficial microorganisms a good source of bacteria-rich, high nutrient content. The composting process reduces the pH of EFB by 6.86-7.20 to 4.5-6.0. CCD is recommended as a substitute for other media because of it is excellent for holding water and drainage and has high air porosity due to its large surface area. Microbiologically, CCD is the absence of weeds and pathogens and has anti-fungal properties that prevent soil-borne diseases. It also maintains greater oxygen levels and is reusable after sanitisation. Despite the ideal characteristics of CCD, there are some chemical limitations of this medium, including low pH and low potassium content. Peat has good aeration characteristics that are good for root growth. An experiment was conducted to evaluate the best medium combinations for growth performance and nutrient content of hempedu bumi (*Andrographis paniculata*). Three types of SM with five different combinationa were used as growing media for the plant; they included C1=CCD (1: -) as control; C2=EFBC + CCD (7:3); C3=EFBC + CCD (3:7); C4=CCD + Peat (7:3) and C5=CCD + Peat (3:7). Prior to the experiment, the physical, chemical and microbiological

ARTICLE INFO

Article history:
Received: 05 February 2015
Accepted: 31 October 2016

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ISSN: 1511-3701

properties of the media were determined. The experiment was conducted in RCBD with five replications. In this experiment, the parameters of vegetative components (plant height, number of leaves and total leaf area), dry matter yield and partitioning [root, shoot dry weight and root to shoot ratio (R:S)] and the macronutrient nitrogen [(N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg)] of *hempedu bumi* were determined. C3 showed the highest vegetative component [plant height (39.5 cm), number of leaves (72.7 leaf plant-1), total leaf area (79.8 cm2) and shoot dry weight (3.03 g)] of *hempedu bumi*. The highest macronutrient content (1.17% N, 0.07% P, 2.45% K, 2.77% Ca and 0.58% Mg) was in the leaf tissues of hempedu bumi when grown in the C3 media. In conclusion, a combination of EFBC+CCD (3:7) is recommended as a suitable growing medium for hempedu bumi due to the greater vegetative components well as the higher macronutrient content it yielded in the leaf tissues of the plant.

Keywords: soilless media, *hempedu bumi*, empty fruit bunch, coconut coir dust, peat

INTRODUCTION

The characteristics of growing media is one of the important factors that affect the growth performance of the plants. Plants require sufficient nutrients and moisture from the medium in which they are grown. Soilless media have several physical, chemical and biological functions. The physical function of soilless media is to provide support to the plant with good root aeration, gas exchange to and from the roots and sufficient water for the roost. The chemical function of soilless media is to supply adequate oxygen and nutrients for proper root functions. Biologically, availability of soilless media is important for beneficial microorganisms as a host to release nutrients from the media. Combinations of SM may affect the production of plants in which the right combination of SM can indicate the suitability of the growing medium to fulfil the needs of the roots to grow. Several SM have been studied, such as empty fruit bunch compost (EFBC), coconut coir dust (CCD) and peat. Mixing different ratios of these SM affects the characteristics of the media and the growth of the plants that are grown in it (Suhaimi & Ong, 2001). For example, EFBC, one of the organic residues of oil palm, provides beneficial microorganism with a good source of bacteria and nutrients and has an acidity value of 4.5-6 as well as a moderately fine texture such as that of sand or dust particles of size 2 mm (Kavitha et al., 2013). The chemical properties of the media have huge influence on the ability of the media to increase the production of the plant. The EFBC medium contains 0.76% N, 0.21% P, 2.60% K, 0.58% Ca and 0.20% Mg as a single substrate.

CCD is recommended for use as a substitute for other media because it has suitable physical properties such as that it can hold and release water up to eightfold of its mass and has excellent drainage and high air porosity due to the large surface area of its particles. Coir dust is light brown

in colour with a particle size of (0.2-2.0 mm) and when compared to sedge peat and sphagnum peat, has higher CEC, superior structural stability, water absorption ability and drainage and is clear of sticks and other extraneous matter (Meerow, 1994; Noguera et al., 1998). Microbiologically, CCD is the absence of weeds and pathogens and has anti-fungal properties for a conducive environment for plant growth (Wira et al., 2011). CCD also prevents soil-borne diseases when mixed with soil (Mokhtari, 2010). Despite the ideal characteristics of CCD, there are some chemical limitation of this medium, including low pH and low potassium content.

Peat has good aeration characteristics and is suitable for root growth when the growing medium is mixed with other media (Molitor & Brückner,1997). Peat is suitable for growing plants that require more acidic conditions and is generally not suitable for growing crops if not mixed with other media. The physical and chemical properties of peat have been determined by several researchers and in each study, various results were gathered because the properties of this substrate depend on the degree of intensity of decomposition (Ismail et al., 2001). As reported by Ismail (2011), CCD and EFB alone have the lowest bulk density, with 0.92 and 0.97 g cm^{-3}, respectively. The original pH for EFB has been reported to be in the range of 6.86 (Ishak et al., 2014) to 7.20 (Kavitha et al., 2013). The addition of peat has been reported to improve bulk density of CCD and EFB. In terms of water availability, which reflexes the ability of a medium to hold water, CCD and a mixture of CCD and peat presented the highest percentage (15.18% and 13.99%). Ismail also reported that soilless media such as EFB, peat and a combination both have very poor water holding capacity and therefore, were not suitable for use as a growing medium for plants. This is because these media can easily dry up and cause water stress to the plant.

The decomposition degree of peat is positively related to the medium's carbon content and particle size fraction (<250 μm). Kala et al. (2009), who conducted a study on co-composting of different oil palm wastes with sewage sludge for use as potting media for ornamental plants, reported that EFB and peat had a particle size of 40 mm and <20 mm, respectively. Additionally, observations on the physical properties of peat by Sekhar and Sai Gopal (2013) showed that the bulk density of peat was significantly high when it was compared with other soilless substrates such as CCD. Yasmeen et al. (2009), in their study to prove the efficient conversion of empty fruit bunch of oil palm into fertiliser-enriched compost, reported that EFB exhibited higher bulk density and moisture content after the composting process than before it. Similar results were also shown by Zulkarami et al. (2010) when peat was used in the composting process. Good media must have at least a proportion of 45% of organic matter, 35% of water and 20% of aeration to allow healthy plant growth as suggested by Kumar and Kumar (2013).

With this in mind, the objectives of this study were (i) to compare the physical, chemical and microbiological properties of different compositions of soilless media of CCD as well as combinations of EFBC+CCD and CCD+peat, and (ii) to determine the vegetative components of *hempedu bumi* grown in different combinations of SM.

MATERIALS AND METHODS

The experiment was conducted in the rain shelter, Faculty of Agriculture, Universiti Putra Malaysia. Three types of SM with five different combinations were used as growing media C1=CCD (1: -) as control; C2=EFBC+CCD (7:3); C3=EFBC+CCD (3:7); C4=CCD+Peat (7:3) and C5=CCD+Peat (3:7). Physical (bulk density and available water), chemical (macronutrient content, electric conductivity and pH) and microbial (fungi, bacteria and actinomycetes population) properties were determined from each of the replicates. Seeds of *Andrographis paniculata* were obtained from the Agro Gene Bank, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The fundamental principle of this method is the removal of the seed coat using the rough surface of sand paper number 120 with size 30 cm × 30 cm. After germination, the seedlings, with two expanded cotyledons, were transferred into a tray with equal proportiona of CCD, EFBC and peat (1:1:1 w/w/w) until the plant had grown to six to eight leaves. Then, healthy seedlings were transplanted into plastic pots measuring 12 cm × 17 cm with different ratios of SM. The experiment was arranged in Randomised Complete Block Design (RCBD) with four replications. The plants were well watered twice a day and other practice management was done when necessary. No mineral fertiliser was added throughout the experimental period. The physical (available water and bulk density) as well as chemical [electric conductivity (EC), pH and nutrient content] properties were recorded prior to the beginning of the experiment. The treatment effects of different media combination on vegetative components (plant height, number of leaves and total leaf area), dry matter yield and partitioning [root, shoot dry weight and root to shoot ratio (R:S)] and microbial activity (bacteria, fungi and actinomycetes) and macronutrient content [nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg)] in the leaf tissue of *hempedu bumi* were determined 60 days after planting (DAP).

Soilless Media Analysis

Physical properties. The moisture content and water availability of the SM was determined using the modified method of De Boodt and Verdonck (1971). Each mixture of SM was added to the rubber ring and saturated with water for 24 h. After overnight incubation, the media were transferred to the pressure plate with different pressure levels of pF 0 kPa, pF 1 kPa, pF 2 kPa, pF 2.54 kPa and pF 4.19 kPa. They were allowed to remain in the pressure plate for one week. The SM were then taken

out from the pressure plate and put into aluminium dishes. The initial weight (W1) of the SM was recorded and then oven-dried for 24 h at 105°C. The dry weight (W2) was obtained; the weight of the empty dish (W3) had been measured earlier. At the end of the process, the water content percentage (%) of the SM was obtained at each of the different pressure levels. The moisture content (MC) and water availability (WA) for plant use was then calculated using the following formula:

$$MC\ (\%) = \frac{W1 - W2}{W1 - W3} \times 100$$

WA = MC at field capacity (pF 2.54 kPa) - MC at permanent wilting point (pF 4.19 kPa).

The determination of bulk density was done using the modified method of De Boodt and Verdonck (1971) by fixing two copper rings together with a tape and a mesh filter at the bottom. Each composition of SM was added to the rings and saturated with water and left for 24 h. The separation of the two copper rings was conducted by removing the tape from the rings and the saturated SM was sliced to the exact level of the single ring. The water-saturated SM inside the ring was weighed (W1) and oven-dried at 105°C for 24 h. The oven-dried SM inside the ring was weighed after removing each sample from the ring (W2). The diameter and height of the single ring were also measured before the sampling for determination of ring volume (V).

$$\text{Bulk Density (g cm}^{-3}) = \frac{W1\text{-}W2}{V}$$

where, W1 = Weight before oven drying (g), W2 = Weight after oven drying (g), V = $\pi r^2 h$ (cm^3)

Chemical properties. Each mixture proportion of soilless media was oven-dried for 48 h at 60°C and ground to a size of <1 mm. Soilless media (0.5 g) was put into a digestion flask with 5.0 mL of concentrated sulfuric acid (H_2SO_4) and left for at least 2 h. Before starting the digestion, 2 mL of 50% hydrogen peroxide (H_2O_2) was added slowly down the sides of the tube while it was rotating and the heating process was allowed to proceed in the digestion block for 45 min. Another 2 mL of 50% H_2O_2 was added to the tube and left for 45 min. The process was repeated until the digestion was clear or colourless. In this study, the process of adding 2 mL of 50% H_2O_2 was repeated five times until the digestion changed to a colourless solution. Finally, the volume of the solution was made up to 100 mL with distilled water and filtered by Whatman filter paper of medium size 500 mm diameter. Nitrogen (N) and phosphorus (P) were determined using the Auto analyser (AutoAnalyzer, QuikChem FIA + 8000 Series, USA). Potassium (K), calcium (Ca) and magnesium (Mg) were determined using the Atomic Absorption Spectrophotometer (Perkin Elemer, 310, PC, California, USA). The results of the nutrient analysis were presented as percentage (%) per gram of dry weight.

The pH value was determined by adding the SM into 0.01 M $CaCl_2$ in a ratio of 1:5 v/v and left to stand for 1 hour, and then the acidity was determined using the Digital pH meter (GLP 21, Crison, Barcelona, Spain). Electrical conductivity (EC) was determined by adding 100 mL of distilled water to 10 g of SM (1:10 v/v). The mixture was then agitated using a mechanical shaker for 30 min and incubated for 24 h. After incubation, the sample was filtered and the EC was determined using an EC meter (GLP 31, Crison, Barcelona, Spain). The reading was expressed as ds m^{-3}.

Microbiological properties. The microbial population of the soilless media was determined by serial dilution. A total of 10 g of each sample was suspended in 100 mL of distilled water and incubated in a mechanical shaker for 30 min. An agar plate containing a nutrient agar, Rose Bengal streptomycin agar, and actinomycetes was prepared for the determination of bacteria, fungi and actinomycetes, respectively, and a serial tenfold dilution of each sample was prepared. All of this and 0.1 mL aliquots and were spread on each plate. The inoculated petri plates were incubated in an incubator at 28°C for three days to count the number of bacteria and five days to the count number of fungi and actinomycetes. After the incubation period, the colony forming units were counted and expressed as log10 CFU g^{-1} of soilless media on a moisture-free basis.

Plant growth analysis. Plant height (cm) was measured using a standard ruler from the growth medium surface to the tip of the main stem. The number of leaves produced or maintained by each plant was also counted numerically. After harvesting, the leaves were individually separated from the stem, and the total leaf area (TLA) per plant was estimated using the Automatic Leaf Area Meter (LI-3100C Area Meter, Lincoln, Nebraska). Four plants were harvested from each treatment and separated into root and shoots. The stem and leaves were considered part of the shoots. After separation of the plant parts, the plants were dried individually under sunlight for one day and then oven-dried at 40°C for 48 h, and the dry shoot was recorded using a digital balance (QC 35EDE-S Sartorius, Germany). The roots were washed gently and the dry weight of the root was weighed using a digital balance (QC 35EDE-S Sartorius, Germany). The root and shoot dry weight was presented as gram (g). The ratio of root to shoot was calculated based on shoot and root dry weight using the following formula:

$$\text{Root: Shoot Ratio (R:S)} = \frac{\text{Total Root Dry Weight (g)}}{\text{Total Shoot Dry Weight (g)}}$$

Macronutrient content of leaf tissue. The dried leaves (50 g) of *Andrographis paniculata* were ground to powder and stored in well-stoppered polyethylene vials and kept at room temperature (24°C). The ground leaf (0.25 g) tissue was put into a digestion flask with 5.0 mL of concentrated sulfuric acid (H_2SO_4) and left for 2 h. Before starting the digestion, 2 mL of 50%

hydrogen peroxide (H_2O_2) was slowly added down the sides of the tube while it was rotating. The tube was then left in the digestion block for 45 min for heating. Another 2 mL of 50% H_2O_2 was added to the tube and left for another 45 min. The process was repeated until the solution was clear or colourless. Finally, the solution was brought up to 100 mL with distilled water. The nutrient element of N and P were determined using the Auto analyser (Lachat instruments, QuikChem® FIA + 8000 Series). The determination of K, Ca and Mg was done using the Atomic Absorption Spectrophotometer (Perkin Elmer Model 310, California, USA).

Statistical analysis. Statistical analysis was determined using a one-way ANOVA using Statistical Analysis System (SAS) (release 9.3, SAS Institute Inc., Cary, NC, USA). Fisher's Least Significant Differences (LSD) was used for comparison of treatments mean where the F values were significant at $P \leq 0.05$. All the assumptions of ANOVA were checked by carefully analysing the results of the statistical analysis. Sigma Plot (version 12.5, Systat Software, San Jose, California USA) was used for the data that were presented in a graph.

RESULTS AND DISCUSSION

Physical Property of Soilless Media

The capacity of the media to supply sufficient amounts of water for plant growth can be indicated by the percentage of available water present in the field capacity and wilting point. There was a significant difference in the content of available water (Figure 1A) between each composition of the SM. The lowest water content was recorded in C5, containing 70% of peat, which has low water-holding capacity. A similar observation was confirmed by Iberahim (2001), who noted available water content of a single proportion of peat in his study. The available water for the plant and aeration in the growth medium were estimated by the physical criteria derived from the physical properties (Sharma & Kumawat, 2012).

The highest bulk density (Figure 1B) among different compositions of SM was found in C5 followed by C2, C3 and C4 as compared to the control media (C1). A study showed that C5, C2, C3 and C4 had significantly higher bulk density by 161%, 125%, 99.9% and 65%, respectively than their control. Higher amounts of peat increase bulk density of a soilless mixture; this was reported by Noorhanin et al. (2013) and Zulkarami et al. (2010). This is probably due to the characteristic of peat itself, which has higher bulk density than CCD (Abad et al., 2005). The bulk density of compost is very important where it comprises a larger proportion of the growing medium. Meanwhile, C1, which comprised a single composition of CCD, had the lowest bulk density; a similar result was confirmed in the findings of Al Rawahy et al. (2009), Iberahim (2001) and Riaz et al. (2008). Increasing and decreasing the bulk density of SM is attributed to the medium components, particularly particle size and

presence of particles of different size, which lends to higher bulk density than a medium with particles of only one size.

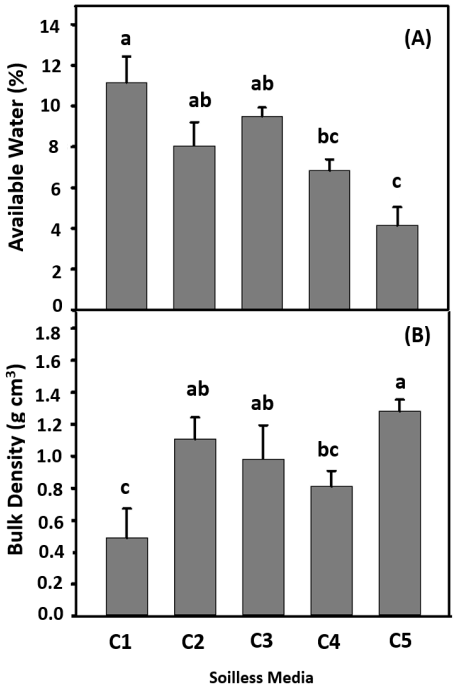

Figure 1. The physical properties of soilless media: available water (A), bulk density (B) prior the beginning of the experiment. Vertical bars represent S.E. and means value with the same letter are not significantly different at P = 0.05

trend of the changes shows that the control (C1) had the highest content of moisture followed by C3, C5, C4 and C2 throughout the pF levels. The highest percentage of moisture content at field capacity (pF 2.54) was observed in 100% CCD (C1) followed by C3 medium comprising EFBC and CCD (7:3, v/v). According to the results, both media probably had a sufficient amount of water to support growth performance of *Andrographis paniculata* even under water-

The moisture content of each composition of SM was determined under different pressure levels (Figure 2). The

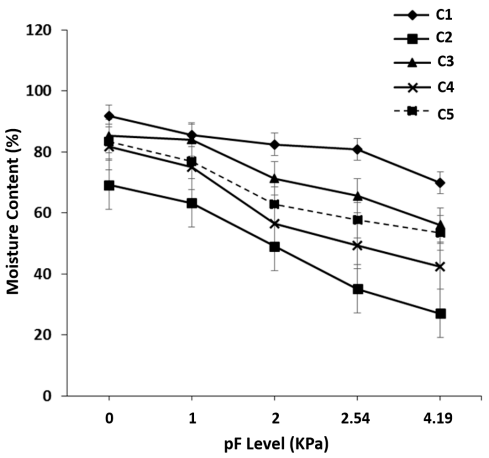

Figure 2. The moisture content of the soilless media prior the beginning of the experiment. Vertical bars represent S.E. of mean and are invisible when the values are smaller than the symbol

stress conditions. In other words, plants grown in these media will not be prone to severe wilting if the plant undergoes a sudden increase in the transpiration rate (Raviv & Blom, 2001). Similar findings have been reported by Phurailatpam et al. (2013), who found that CCD and EFB were successfully used as growing media for the production of cauliflower. It has been reported that the water-holding capacity of CCD is high because it can hold and release water eightfold of its mass (Mokhtari, 2010).

Chemical Properties of Soilless Media

Results of the macronutrient contents N, P, K, Ca and Mg of the soilless media are shown in Table 1. The highest N content was

Table 1
Nutrient Contents of the Soilless Media (% on Dry Weight Basis) Prior to the Beginning of the Experiment

SM	N	P	K	Ca	Mg
C1	0.11 ± 0.004e	0.10 ± 0.002c	0.22 ± 0.008a	0.12 ± 0.007c	0.09 ± 0.004d
C2	0.57 ± 0.008a	0.45 ± 0.010a	0.24 ± 0.003a	0.22 ± 0.006ab	0.14 ± 0.002a
C3	0.27 ± 0.004d	0.10 ± 0.002c	0.08 ± 0.004b	0.18 ± 0.011bc	0.11 ± 0.002bc
C4	0.34 ± 0.008c	0.30 ± 0.011b	0.23 ± 0.007a	0.21 ± 0.009ab	0.13 ± 0002ab
C5	0.42 ± 0.007b	0.13 ± 0.002c	0.03 ± 0.002c	0.28 ± 0.025a	0.1 ± 0.008dc

Mean values ± S. E followed by the same lower case within columns are not significantly different at P=0.05

observed in C2 (0.57%), which contained EFBC and CCD (7:3, v/v). High proportions of EFBC increased the N content of the C2 mixture of SM. On the contrary, the lowest value of N was observed in C1 (0.11%), which contained a single composition of CCD. However, Wira et al. (2011) reported that the N content of CCD did not differ significantly from the mixture of CCD with EFBC. Smith et al. (1989) suggested that CCD requires mixing with a nutrient-rich material in order to become suitable for use as a SM. Furthermore, Tang et al. (2006) indicated that N usually increased during the composting process. C5 exhibited higher N content (0.42%) than C3 (0.27%) and C4 (0.34%). Higher N content in C5 was probably due to the fact that that medium contained 70% of peat compared to C3 and C4, which contained 70% of CCD.

The results also showed that C5 and C1 (control) exhibited the highest (0.28%) and the lowest (0.12%) content of Ca, respectively (Table 1). C2 contained significantly higher Mg (0.14%) than the control (C1) and the other mixtures. Reduction of Mg content was probably due to an increase in the pH value of the substrate used. However, an earlier study by Wira et al. (2011) claimed that there were no specific high macronutrient (N, P, K, Ca and Mg) contents in EFBC and the CCD mixture and in the single composition of CCD. In this study, the K content was found to be higher in C2 (0.24%), followed by C4 (0.23%), C1 (0.22%) and C5 (0.03%), with no significant difference among them. Davies et al. (2000) observed that increases in the K content are expected when a combination of EFB and CCD is used as the growing medium compared to a medium with single CCD components.

Acidity (pH) of the media, which is a measure of the acidity or alkalinity of the media, is one of the most important chemical properties of growing media. The pH value for each SM composition is shown in Figure 3A. The results of this study showed that C2, C5, C3 and C4 have significantly higher pH by 36%, 19.4%, 10.9% and 8.4%, respectively, than the control. The high pH value of C2 enhanced the availability of essential nutrients in the growing medium (Griffiths et al., 2003). The increases of the pH value of the EFBC might be due to the active microbial activities that caused the

release of ammonia (NH_3). CCD has been reported to have a high pH value (Mokhtari, 2010), and this could also be the reason the C4 medium, which consisted of CCD and peat (7:3, v/v), exhibited a higher pH value than the control (C1). However, in this study, the C2 SM gave a low pH value, even though it had higher proportions of CCD content. In general, the pH of the growing medium played an essential role in nutrient availability, which is essential for good plant growth (Ismail et al., 2013).

The salinity or electrical conductivity (EC) value was significantly different among the SM samples (Figure 3B). The highest value of EC was shown in C2, followed by C3, while the lowest value was found in C5. Electrical conductivity (EC) is a measurement of dissolved salt concentration in a growing substrate and is an indicator of the available amount of fertiliser for plant growth (Mafakheri et al., 2010). The high EC of C2 and C3 was also probably due to the loss of EFB weight and release of other mineral salt and organic matter throughout the decomposition process of the EFB (Zarrouk et al., 2005).

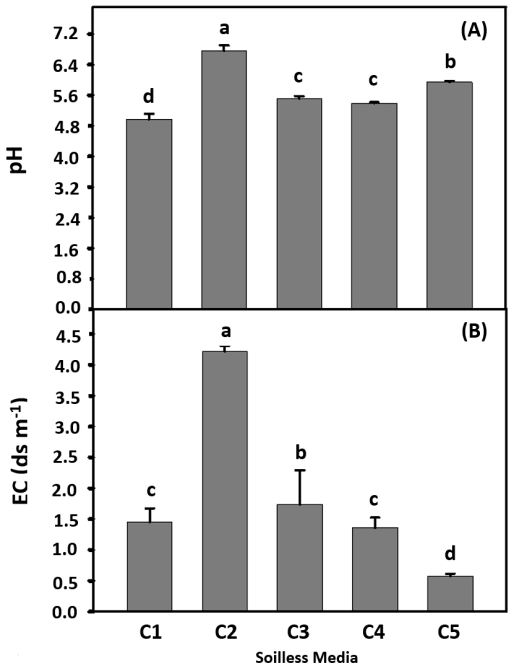

Figure 3. The pH value (A) and EC value (B) of soilless media prior the beginning of the experiment. Vertical bars represent S.E and means value with the same letter are not significantly different at P = 0.05

Microbiological Property of Soilless Media

The population of bacteria was significantly higher in C2 [$\log_{10}$CFU g^{-1}=6.9 or CPU=$10^{6.9}$ colonies g^{-1}] than in the other treatments (Figure 4A). On the contrary, there was no significant difference among the bacterial population of C1 [CFU=$10^{4.7}$ colonies g^{-1}], C3 [CFU=$10^{5.4}$ colonies g^{-1}], C4 [CFU=$10^{4.5}$ colonies g^{-1}] and C5 [CFU=$10^{5.2}$ colonies g^{-1}]. According to the results shown in Figure 4B, medium C2 gave the highest population of fungi [CFU=$10^{7.2}$ colonies g^{-1}] followed by C3 [CFU=$10^{6.4}$ colonies g^{-1}]. However, the fungi population of medium C5 [CFU=$10^{5.6}$ colonies g^{-1}] and medium C4 [CFU=$10^{5.2}$ colonies g^{-1}] and medium C1 [CFU=$10^{4.4}$ colonies g^{-1}] was not significant and relatively lower than in C2 and C3. The actinomycete population of the SM is represented in Figure 4C. The highest population of actinomycetes was detected in C2 [CFU=$10^{7.1}$ colonies g^{-1}] followed by C3 [CFU=$10^{5.8}$ colonies

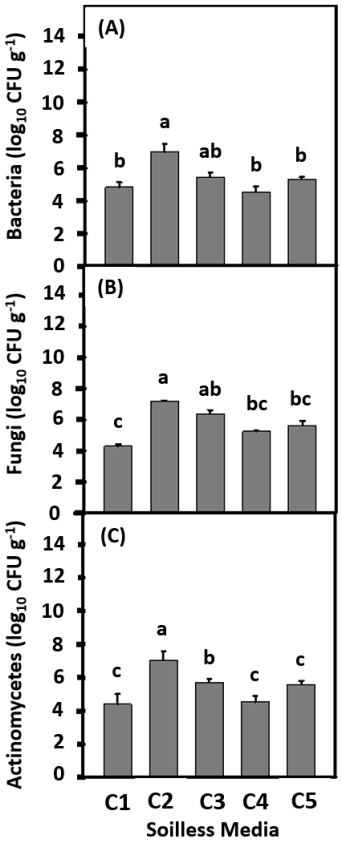

Figure 4. The bacteria (A), fungi (B) and actinomycetes (C) population of soilless media on 60 DAP. Vertical bars represent S.E. and means value with the same letter are not significantly different at P = 0.05

g^{-1}], while the lowest was recorded in C5 [CFU=$10^{5.6}$ colonies g^{-1}], C4 [CFU=$10^{4.6}$ colonies g^{-1}] and C1 [CFU=$10^{4.5}$ colonies g^{-1}]. Increased microbial population of C2 could have been due to an increase in the pH of these media as a result of the addition of EFBC. Similar results were obtained by Saravanan et al. (2009), in whose study the microbial population of the media increased at the highest level of EFBC.

In addition, availability of inherited microorganisms in the EFBC also contributed to increase the soil's microbial population (Abdalla, 2005). The lowest microbial population of C1, which contained 100% CCD might be related to low percentage of water availability as presented in Figure 1A. Other findings claimed that low availability of microbial populations of peat correlated with the moisture content of peat (Stankovic, 2011). Kyparissis et al. (1995) also reported that microbial population of peat increased with an increase in moisture content. However, the population of microbes in peat was also influenced by pH (Stankovic, 2011). The enhancement of the microbial population of C2, which contained a high ratio of EFBC might have been due to the availability of high organic nutrient matter in the media (Douds Jr et al., 1997). The high microbial population of the media was also influenced by the moisture content, pH and EC of the media where media with high moisture altered the population of microbes (Griffiths et al., 2003). Nutrient content was another factor that affected the microbial population of the media where the availability of beneficial microbes in the compost converted unavailable forms of nutrient to available forms. Despite the high moisture content of CCD, the fungal population of CCD was low and this was attributed to the anti-fungal properties of the CCD (Mokhtari, 2010). Addition of beneficial microbes was also previously recommended due to the cleanness of the CCD (Prabhu & Thomas, 2002).

Plant Vegetative Component

The plant height of *Andrographis paniculata* was significantly affected by different

combinations of SM (Figure 5A). The highest mean of plant height was observed in C3 (39.50 cm) followed by C4 (29.83 cm) and C5 (26.33 cm), while the lowest was observed in C2 (15.00 cm). Plant height varied significantly due to different proportion of SM. This finding obviously varied from the previous study done by Noorhanin (2013). They found that the highest *Andrographis paniculata* plant was observed when the plant grown in 100% CCD was fed inorganic fertiliser. Plant height varied significantly due to different proportion of SM at different growth stages. The increased plant height in C3 compared to other SM treatments was probably due to the perfect combination and balance between nutrient availability, suitable pH and EC provided by the medium. A similar observation was recorded by Zulkarami et al. (2010); in their study the highest rock melon plant was observed in the EFB medium with an EC value between 1 and 2.5 ds m-1. Bloom et al. (1985) stated that plants responded to their environment in such a way as to optimise their resource use. Good results in plant height can also have been due to the response of the shoots

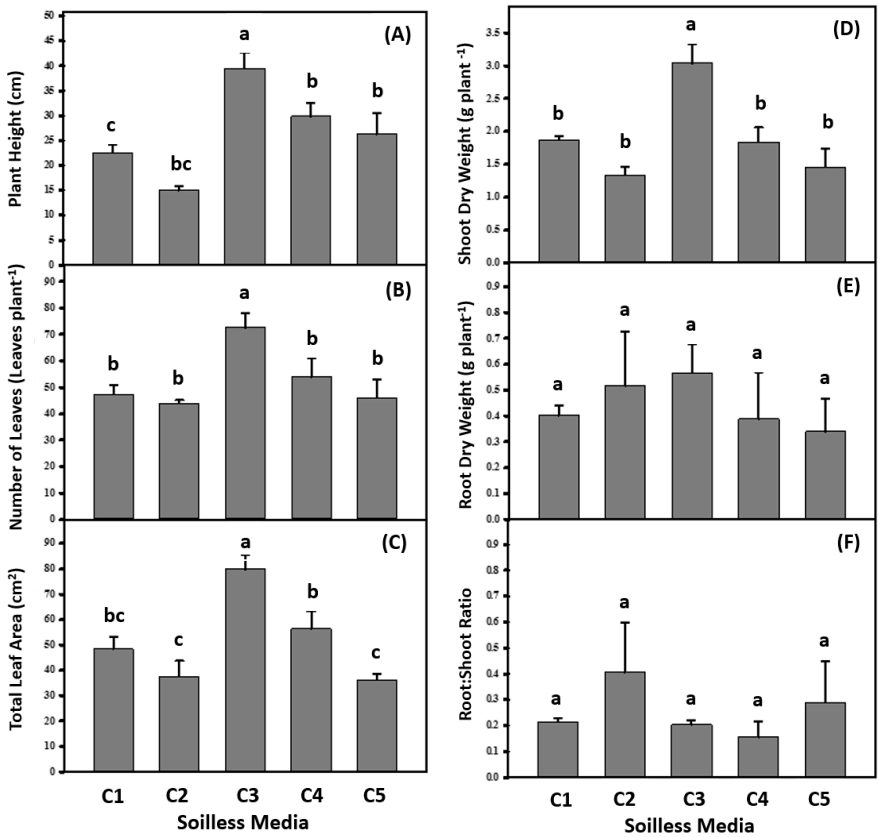

Figure 5. The plant height (A), number of leaves (B) and total leaf area (C), shoot dry weight (D), root dry weight (E) and root to shoot ratio (F) of hempedu bumi on 60 DAP grown under different combination of soilless media of soilless media. Vertical bars represent S.E. and means value with the same letter are not significantly different at P = 0.05

and roots to optimal nutrient availability based on the carbon balance between both parts (Cannell & Dewar, 1994). In this study, healthy roots easily grew when the *hempedu bumi* plants were grown in C3 and the nutrients were absorbed well, thus aiding the growth in plant height. Results from another study have revealed that EFB enhanced the production of cabbage when used as its growing medium (Wahid et al., 2011).

Medium C3 and C4 produced 40.86% and 14.09% higher number of leaves per plant compared to the control (C1), respectively (Figure 5B). Similarly, as reported earlier, using EFB as a growing medium resulted in the highest number of rock melon leaves compared to using the CCD medium (Zulkarami et al., 2010). The result of this study illustrated that the increase in the number of leaves of plants grown in C3 and C5 media was probably due to increased nutrient release from C3 and C5 media; this correlates with the results of a study done by Abdul Mutalib et al. (2009). On the reverse side, the plant grown in medium C2 and medium C5 exhibited 7.04% and 2.81% lower number of leaves than in the control.

The total leaf area (TLA) of *Andrographis paniculata* was significantly affected by different combinations of SM (Figure 5C). The mean of the TLA was higher (64.95% and 50.57%) in the plants grown in medium C3 and medium C4 and lower (22.52% and 25.11%) in plants grown in medium C2 and medium C5 as compared to the control medium (C1). A similar result was obtained by Wira et al. (2011), who recorded that the highest TLA of rock melon was observed in combinations of EFBC and CCD (3:7, v/v). In general, CCD was associated with high water-holding capacity but it was poor in aeration, causing decreases in oxygen diffusion to the roots (Abad et al., 2005). The combination of both substrates can increase the diffusion of oxygen to the roots, which leads to good plant growth. In addition, at the vegetative stage, the leaves and roots scramble for carbohydrate assimilation. To gain an increase in leaf area, the growth of the leaves and roots should be in good balance (Noorhanin et al., 2013). The increase in the TLA when the plant was grown in the C3 medium might be attributed to the availability of beneficial microorganisms in the EFBC media. These results are similar to those of a study done by Zydlik and Zydlik (2008) in which the beneficial microorganisms also significantly resulted in releasing nutrients from the soil to an available form for the growth of apple trees. Increase in the TLA of *Vigna mungo* was also observed with application of beneficial effective microorganisms (Karthick Raja, 2012).

The significant and highest shoot dry weight was found in the plant grown in the C3 medium (2.37 g plant-1) compared to the plant grown in the control (1.87 g plant-1) and other treatments. However, there were no significant differences between the C4, C5, C2 and C1 plants (Figure 5D). These results were in agreement with the findings of Wira et al. (2011). They recorded that application of EFBC with CCD (3:7, v/v)

enhanced the biomass production of rock melon. Results of this study were somewhat similar to results of Iberahim (2001), who suggested that the application of EFBC as SM increased the biomass production of cauliflower. Even though the nutrient content of C2 was higher, the nutrients available in C2 were not efficiently absorbed by the plants. This was probably due to the antagonistic influence of other nutrients that were available in the medium. In addition to this, toxicity to plants will also occur whenever a high amount of essential nutrients is supplied. An optimum ratio of EFBC enhanced the productivity of dry weight of shoot biomass of *Andrographis paniculata*, a result similar to that recorded by Zulkarami et al. (2010), who noted that application of EFBC significantly increased the production of rock melon.

Figure 5E showed there was no significant difference between treatments as influenced by different combinations of SM. The highest mean of root dry weight was obtained by C3 (0.57 g plant^{-1}) followed by C2 (0.52 g plant^{-1}), C1 (0.40 g plant^{-1}) and C4 (0.39 g plant^{-1}), while the lowest (0.34 g plant^{-1}) root dry weight was obtained by the plant grown in medium C5. According to Awang et al. (2009), media that have a good balance of air and water-holding capacity would increase root dry weight of *C. cristata*. In addition, EFBC has moderately fine texture-like sand or dust particle of size 2 mm. A compact medium was probably attained, which led to low favourable conditions for root growth.

Unlike other parameters, the highest R:S (0.24) was obtained in plants grown in the C2 medium (Figure 5F). There were no significant differences in the R:S between different compositions of SM. These results are in conformity with the reports by Wira et al. (2011), who reported that applications of EFBC and CCD as a growing medium for rock melon decreased the R:S of the plant. What influenced the R:S ration was probably the physical conditions and chemical composition of the plant itself, which may have experienced various stresses during the growth process such as lack of nutrients or water for the root. Decrease in the R:S of the plant grown in the C3 medium indicated that the roots were able to supply the shoots of the plant with water, nutrients, stored carbohydrates and certain growth regulators (Harris, 1992). Increase in the R:S of C2 could be an indication of a healthier plant, provided the increase came from greater root size and not from a decrease in shoot weight. This was also due to the highest population of bacteria (A), fungi (B) and actinomycetes (C) in the SM, which may have helped the root achieve healthier growth while the shoot too was able to absorb nutrients and water. This result also has a relationship with the nutrient content accumulation in the SM that is presented in the next section.

Macronutrient Content of Leaf Tissue

The macronutrient content of leaf tissue of *Andrographis paniculata* is shown in Table 2. The highest N, P, K, Ca and Mg content (1.17%, 0.07%, 2.45%, 2.77%

Table 2
The Leaf Tissue Nutrient Content (% on Dry Weight Basis) on 60 DAP

SM	N	P	K	Ca	Mg
C1	0.87 ± 0.022b	0.04 ± 0.005ab	1.85 ±0.106ab	2.00 ± 0.11 b	0.33 ± 0.037c
C2	0.53 ± 0.054 c	0.06 ± 0.010 a	2.00 ± 0.137ab	2.12 ± 0.122 ab	0.44 ± 0.016abc
C3	1.17 ± 0.037a	0.07 ± 0.009a	2.45 ± 0.218a	2.77 ± 0.118a	0.58 ± 0.010 a
C4	1.11 ± 0.036a	0.04 ± 0.007ab	2.15 ± 0.104ab	2.34 ± 0.154ab	0.49 ± 0.013ab
C5	0.63 ± 0.038c	0.01 ± 0.001 b	1.62 ± 0.095b	1.91 ± 0.051b	0.42 ± 0.042bc

Mean values ± S. E followed by the same lower case within columns are not significantly different at P=0.05

and 0.58%) of leaf tissue was observed in the plants grown in C3 compared to C1 (control). The medium that yielded the next highest amounts of N, K, Ca and Mg in leaf tissue was C4 (1.11%, 2.15%, 2.34% and 0.49%), followed by C2 in P content (0.06%). The lowest N content (0.53%) was obtained from leaf tissue of plants grown in the C2 medium, while the lowest value of P, K and Ca (0.01%, 1.62%, and 1.91%, respectively) was found in plants grown in the C5 medium. The minimum content of Mg (0.33%) was observed in C1. Increases in the macronutrient content of leaf tissue of plants grown in the C3 medium associated with the availability of several microorganisms in the medium. These results were supported by Shen et al. (2004), whose study found that a growth medium with beneficial microorganisms significantly increased the nutrient content of plant tissue.

CONCLUSION

The results of this study showed that the biomass production and leaf tissue nutrient content of *Andrographis paniculata* could be significantly affected by growing the plant in different combinations of SM. Growing the plant in EFBC and CCD (3:7, v/v) improved plant vegetative growth, dry matter yield and leaf tissue nutrient content. Application of EFBC in high or low volume released nutrients, increased pH and enhanced the microbial population of the potting medium. This suggests that EFBC can be used as an alternative to peat and CCD as a substrate in a soilless culture system.

ACKNOWLEDGEMENT

The author is grateful to Universiti Putra Malaysia, Serdang, Selangor for providing a conducive environment and good facilities and scientific equipment that proved indispensible in conducting this study.

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Pertanika J. Trop. Agric. Sci. 40 (1): 53 - 72 (2017)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

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Identification and factors affecting *in-vitro* growth of an indigenous mushroom, *Boletus* sp. from Bachok, Kelantan, Malaysia

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ABSTRACT

Wild mushrooms including *Boletus* are among edible mushrooms consumed by local populations. However, the species identity for many types of wild edible mushroom in Malaysia is poorly known. The present study was conducted to identify and to determine factors affecting *in-vitro* growth of an edible *Boletus* sp. (*kulat gelam*) commonly found in peat swamp forests in Bachok, Kelantan. Identification was initially done using macroscopic and microscopic characteristics of the fruiting bodies. However, due to overlapping chemical colour reactions, morphological and anatomical characteristics of the fruiting body with other *Boletus* species, species identity was confirmed using ITS region, and the mushroom was identified as *Boletus griseipurpureus*. A toxicity test indicated that *B. griseipurpureus* is an edible mushroom with low toxic levels (LC_{50}=4.33 mg/mL). From growth studies, the results suggested that potato dextrose agar (PDA), cassava dextrose agar (CDA) and yeast malt extract (YME) were the most suitable artificial media for mycelial growth of *B. griseipurpureus* at pH 6.0 and 30°C. To our knowledge, this is the first documented report on wild edible mushroom *B. griseipurpureus* in Malaysia.

Keywords: wild mushroom, *Boletus griseipurpureus, in-vitro* growth, toxicity, solid media, liquid media

ARTICLE INFO

Article history:
Received: 11 July 2015
Accepted: 10 November 2016

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INTRODUCTION

In Malaysia, collecting wild mushrooms for food and medicine is a well-known activity among local communities (Lee & Chang, 2004). According to Lee et al. (2009), a total of 45 species of macrofungi have been reported by Bateq, Che Wong, Jakun, Semai and Temuan communities, in which 31 species were consumed as food

ISSN: 1511-3701

and 14 species were utilised as medicine including *Auricularia* spp., *Cantharellus* spp., *Clavulina* spp., *Ganoderma* spp. *Lignosus* spp., *Russula* spp., *Schizophyllum* spp. and *Termitomyce*s spp.

Although *Boletus* is widely distributed throughout Malaysia, the consumption of *Boletus* is not common and is confined to a particular species, *B. aureomycelinus* (Lee et al., 2009) as knowledge on the edibility of unknown *Boletus* species is lacking. Moreover, cases of mushroom poisoning due to the consumption of *Boletus* species have been reported in Malaysia. The victims had confused the mushroom with a synonymous species that was inedible and poisonous (Chew et al., 2008). Thus, identification of wild *Boletus* is required to avoid poisoning from mushroom consumption.

Based on personal communication with the locals, there is an edible *Boletus* sold in local wet markets seasonally in Bachok, Kelantan, locally known as '*kulat gelam*' and recognised by its brown-grey cap and lilac-grey stipe. The fruiting bodies are commonly found in peat swamp forests where *Melaleuca cajuputi* ('*pokok gelam*') is the dominant vegetation. Due to its pleasant odour and distinctive flavour, this mushroom is a favourite dish in Malay cuisine. However, detailed information such as species identity, toxicity and growth conditions of the *Boletus* is lacking.

Therefore, the objectives of the present study were to identify the edible *Boletus* mushroom (*kulat gelam*) from peat swamp forests in Bachok, Kelantan using morphological and molecular characteristics; to determine fungal toxicity based on the brine shrimp lethality test; and to determine *in vitro* mycelial growth of the *Boletus* sp. in different media, pH and temperature.

MATERIALS AND METHODS

Sampling Area

Boletus samples were collected from an area of peat swamp forest located at 7 m above mean sea level with latitude 05° 58' 00" and longitude 102° 25' 1" in the district of Bachok, Kelantan. The forest floor was partly waterlogged and covered with a thick layer of decomposed plant litter. Soil analysis showed that the peat soil from which the *Boletus* fruiting bodies were collected was acidic (pH 3.0-4.1), having a high carbon content and low nitrogen content with sufficient levels of phosphorus, aluminium, calcium, ferrum, magnesium, potassium, sodium, manganese and zinc. Heavy metals, namely cadmium, copper, mercury, plumbum and nickel were also detected in the peat soil.

Sampling and Isolation of *Boletus*

The mushroom was seasonally fruiting from June to September every year. The first crop of fruiting bodies can be observed after a long dry season at the end of June. For this study, a sharp knife was used to lift whole fruiting bodies from the ground. The fruiting bodies were then kept in a flat basket and taken to the lab for further processing. Surface sterilisation was done by rinsing the fruiting bodies with hypochlorite solution (1% v/v)

for 10 s. After drying, a piece of tissue from the internal central stem tissue was torn off, inoculated onto a potato dextrose agar (PDA) and incubated at 27±10°C for 24 h. Sub-cultures were performed until a pure culture was obtained. The pure culture was then maintained in PDA slants and kept at 2-9°C. Seed culture was grown on a PDA containing 20 g glucose.

Morphological Identification

For preliminary identification, chemical colour reactions were performed using ammonia solution (10% v/v), potassium oxide (5% v/v), ferrous sulphate (10% v/v) and Melzer's reagent in the field as described by Smith and Smith (1973) and Moser (1983).

The macroscopic and microscopic evaluations were based on taxonomic keys and descriptions of Corner (1972), Smith and Smith (1973) and Moser (1983). The colour and grid designations were standardised based on Kornerup and Wanscher (1978). The descriptions of morphological characteristics were based on fresh mushroom samples.

Macromorphological features observed were the pileus, tube layer, stipe, context layer and any veils that were present. For pileus description, the colour, margin, shape, surface texture and size were recorded, followed by an examination of the tube attachment, pore shape and pore surface colour. For stipe description, the colour, shape, surface texture and size as well as the context colour were recorded. All observations were recorded in annotation worksheets as outlined by Lodge et al. (2004). Spore print was obtained by putting the cap with the tubes downwards on white paper, covering it with a glass bowl and leaving it overnight. The spore deposit was then observed to determine its colour.

For micromorphological observation, slides were prepared in the Histology Lab, School of Biological Sciences, Universiti Sains Malaysia, Penang. The anatomy of the hymenium, context texture, spore size and spore shape were observed. The presence or absence of the hyphal clamp was also determined.

Molecular Identification

Five *Boletus* isolates from five fruiting bodies (USMBo1, USMBo2, USMBo3, USMBo4 and USMBo5) were used for molecular identification. About 25 mg of mycelium from the seed cultures was homogenised using liquid nitrogen in a mortar. Genomic DNA was extracted using the Invisorb® Spin Plant Mini Kit (Stratec Molecular GmbH, Germany) according to the manufacturer's protocol.

For amplification of the internal transcribed spacer (ITS) region, the universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') were used (White et al., 1990). A PCR reaction was prepared in 25 µL containing, 5.0 µL PCR buffer (Promega), 4.0 µL magnesium chloride (25 mM), 0.5 µL dNTP mix (Promega), 2.5 µL each primer, 0.125 µL *Taq* polymerase (5 unit/µL, Promega),

0.5 µL genomic DNA and deionised water made up to 25 µL.

The PCR was performed using MyCycler™ thermal cycle system (Bio-Rad Laboratories Incorporation, United States) with the following cycles: An initial denaturation at 95°C for 1 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 30 s and extension at 72°C for 1 min, followed by the final extension step at 72°C for 10 min.

The PCR product was then purified using the FavorPrep™ PCR Purification Kit (Favorgen® Biotech Corporation, Taiwan) according to the manufacturer's instructions. The purified PCR product was sent to a service provider for sequencing.

For detection of the PCR product, agarose gel (1%) electrophoresis was run at 90 V and 400 mA for 90 min with the gel tank containing a 1x Tris-borate-EDTA (TBE) buffer. One kb and 100 bp DNA markers (Gene Ruler™, Fermentas) were used to estimate the size of the bands. Five µl of the PCR product and 1 µL of 6x loading dye (Fermentas) were loaded in the well of the agarose gel and stained with 0.04 µL ethidium bromide. After electrophoresis, the gel was viewed using the Molecular Imager® Gel Doc™ XR system connected to the Discovery Series™ Quantity One® 1-D analysis software version 4.6.5 (Bio-Rad Laboratories Incorporation, United States).

After sequencing, pair-wise alignment was performed using Cluster W integrated in Molecular Evolution Genetic Analysis (MEGA) version 5.1 (Tamura et al., 2011). The aligned DNA sequence was BLAST against sequences in GenBank. The Neighbour Joining (NJ) tree was applied in a phylogenetic analysis. Three *Boletus* species, namely *B. edulis* (GQ900593), *B. griseipurpureus* (JQ726594) and *B. reticulatus* (GU198973), were included for comparison with *Leccinum scabrum* (AF454585) as the out-group.

Toxicity Test

The potential of *Boletus* to be poisonous was determined by brine shrimp bioassay (Meyer et al., 1982). A total of 20 fruiting bodies from two sampling locations were rinsed with distilled water for 1 min and dried overnight in an oven at 60°C. The oven-dried mushroom was ground into fine powder and kept in a universal bottle until used.

Extraction Procedure

Five g of the mushroom fine powder was dissolved with 100 mL methanol (70% v/v) in a 250 mL conical flask. The mixture was agitated by a sonicator for 20 min. The suspension was filtered and dried in an oven at 60°C for 48 h to obtain a crude extract.

Brine Shrimp Hatching and Lethality Test

The brine shrimp eggs, *Artemia salina*, were hatched in artificial seawater prepared by dissolving 38 g sea salt in 1000 mL distilled water. After 24 h of incubation at 27±10°C with constant light, the nauplii of brine shrimp were collected.

For the lethality test, the crude extract was prepared in triplicate. An amount of 600 mg of the crude extract was dissolved in 4 mL dimethyl sulfoxide (DMSO, 4% v/v) and then, 1 mL of the extract and 2 mL of artificial seawater were pipetted into the first well of a microplate. The extract's initial concentration was 50 mg/mL. A two-fold dilution was carried out to obtain different concentrations from 50 mg/mL to 0.195 mg/mL. At the last well, a negative control was set up with 3 mL artificial seawater. Ten nauplii of brine shrimp were loaded into each concentration and the control and incubated at 27±1°C for 24 h. The number of dead nauplii was counted and recorded at the 6th and 24th hours. For positive control, the same procedure was repeated using potassium dichromate.

The percentage of brine shrimp mortality was calculated and plotted against the logarithms of concentration. The regression equation was applied to determine the lethal concentration value, LC_{50}. Using Abbott's formula, the calculation was as follows:

percentage of brine shrimp mortality (%) = $[1- (N_t / N_c)] \times 100$,

where, N_t=number of live brine shrimp in treatment, N_c=number of live brine shrimp in negative control

A linear regression analysis was carried out using IBM SPSS Statistics 20 program. The lethal concentration value, LC_{50}, was determined at 95% confidence interval using a one-way Analysis of Variance (ANOVA).

Assessment of Mycelial Growth on Solid Media

Five different solid media were chosen to assess the mycelial growth of *Boletus* isolate, namely PDA, cassava dextrose agar (CDA), corn meal agar (CMA), malt extract agar (MEA) and czapek agar (CZA). All the media (1 L) were prepared manually. Preparation of PDA, CDA and CMA was adapted from Wang and Lu (2005), while that of MEA and CZA was from Sung et al. (2011). The media was sterilised at 121°C and 15 psi for 20 min; 0.05 g thiamine (vitamin B1) was added prior to use.

Before pouring into Petri plates, the media were adjusted to different pH values, pH 5.0, pH 6.0, pH 7.0 and pH 8.0. The inoculum was prepared by inoculating a mycelium disc (10 mm) from the seed culture onto each plate. The plates were then incubated at four different temperatures: 22°C, 25°C, 28°C and 30°C. Measurement of the colony diameter was carried out every 2 weeks for three months and the mean value was recorded. The experiment was conducted in a factorial design of 4x4x5 with 80 treatments, and each treatment was set up in triplicate.

Assessment of Mycelial Growth in Liquid Media

Four different liquid media, namely malt extract peptone (MEP), yeast extract peptone (YEP), yeast malt extract (YME) and potato dextrose broth (PDB) were used. All media (1 L) were prepared manually as described by Kim et al. (2002) and Xu et al. (2003) with some modifications. The liquid media

were sterilised at 121°C and 15 psi for 20 min, and 0.05 g thiamine (vitamin B1) was added prior to use.

Before pouring into 250 mL conical flasks, each medium was adjusted to different pH values, pH 5.0, pH 6.0, pH 7.0 and pH 8.0. The study was conducted in a factorial design of 4x4 with 16 treatments, and each treatment was prepared in triplicate.

A mycelium disc (1 cm) was inoculated into each conical flask containing a 100 ml culture medium. The flasks were then incubated in an incubator shaker at 27±1°C and 150 rpm. After 12 days, the mycelium was harvested using pre-weighted filter paper and dried in an oven overnight to a constant weight at 60°C. The mycelial biomass was determined and expressed as mycelial dry weight (g) per 100 mL of culture medium.

For both mycelia growth assessments, the data was analysed by applying the Univariate Analysis of Variance using IBM SPSS Statistics 20 programme. Post hoc comparison of means were performed by Duncan Multiple Range Test (DMRT) at the significant level, α=0.05.

RESULTS

Macromorphological Characteristics

Boletes fruiting bodies were found to grow singly under *Melaleuca cajuputi* trees in the peat swamp forest (Figure 1A). The pileus was 4.0 cm wide and 1.0 cm thick on average, hemispherical to broadly convex with a decurved margin. The cuticle was velvety, subtomentose, dry but slightly viscid when wet and had no scale. The pileal colour was violet white (18A2) to light violet (18A5) when young (Figure 1B) and it turned to dull lilac (15C3) with age. The white context measured 0.7 mm and was thick at the centre as well as soft and spongy. When exposed to air, the white pileus context turned to brownish orange (6C3-4). The odour was indistinctly pleasant but the taste was slightly bitter.

From the chemical tests conducted, ferrous sulphate gave a greyish yellow (4B5-6) colour on the pileus surface whereas an ammonia solution produced a mouse grey (5E3) colour on the pileus margin. No colour reactions were observed when the pileus was tested with potassium oxide and Melzer's reagent.

The hymenophore was white when young and turned pinkish white (8A2), dull red (8B3-4) or reddish brown (8E4-5) at maturity (Figures 1C and 1D) and comprised masses of adnexed or sinuate notched tubes (Figures 1E and 1F), which were soft, moist and easily detached from the context. When handled, the colour turned to bruising clay (5D5). The tubes were up to 0.5 cm deep at the centre, longitudinal and ended with tubular or angular pores that radially elongated near the stipe. On the surface, ferrous sulphate gave olive brown (4E4) and Melzer's reagent gave reddish brown (8E4) colours, respectively. No colour reactions were produced with ammonia solution and potassium oxide.

The stipe on average was 6.0 cm long, 1.0 cm wide at the apex, 1.5 cm wide in the middle and base, centric, straight

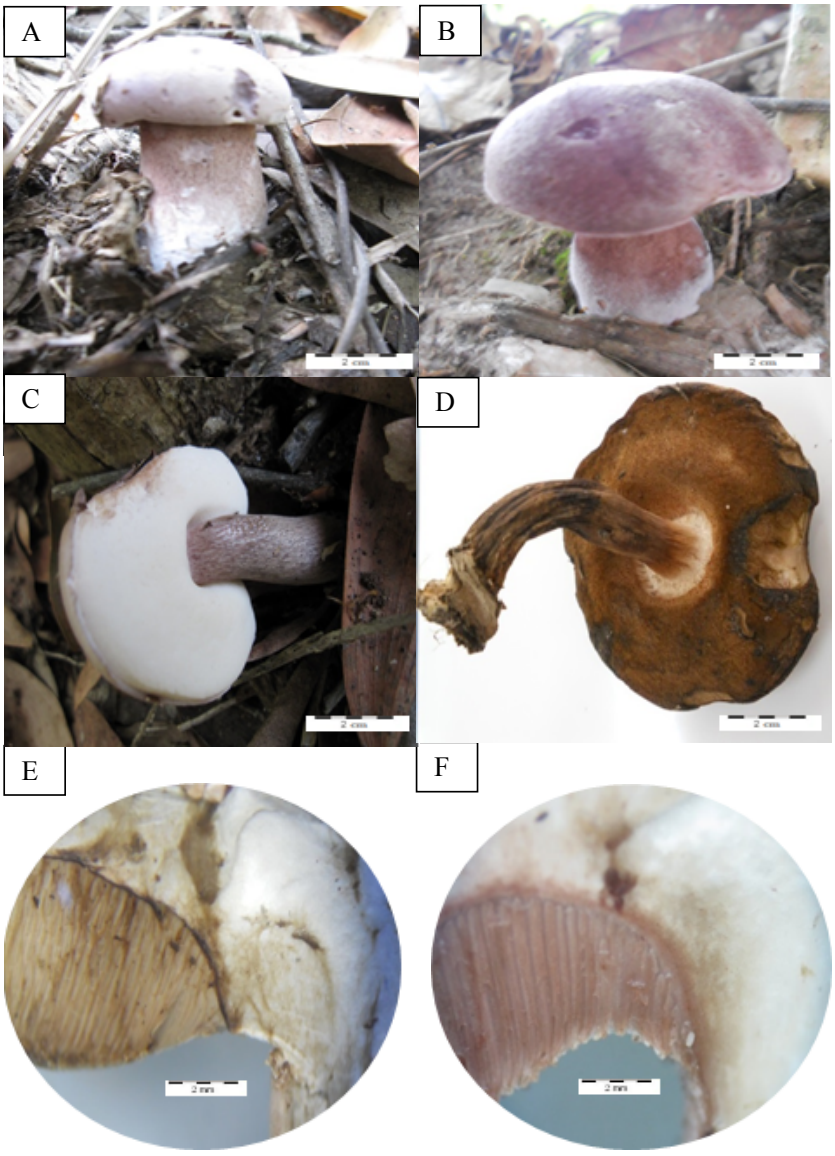

Figure 1. Macromorphological characteristics of *Boletus* sp. (A) Fruiting body of *Boletus* sp. on the forest litter. (B) Light violet young fruiting body. (C) White hymenophore when young. (D) Reddish brown (8E4-5) at maturity. (E) Adnexed tube arrangement. (F) Sinuate notched tube arrangement

or flexuous, equal or clavate with white rhizoids at the base. The annulus and volva were absent. The cuticle was smooth, glabrous, reticulated with a fine net pattern at the apex, dry and easily detached from the context. It was concolourous with the pileus when young and then turned to dull violet (15E3-4) with age, paler at the upper stipe. The white context was soft, fibrous and spongy and turned to brownish orange (6C3-4) when dried. The stipe had a pleasant odour and bitter taste as did the pileus. On the sliced context, ferrous sulphate gave olive brown (4B4-5) and Melzer's reagent gave Chinese yellow (4B7) colours, respectively. No colour reactions were observed when tested with ammonia solution and potassium oxide.

Micromorphological Characteristics

The spore print was olive brown (4E8) on white paper. The individual spore was symmetrical and ellipsoid (7-9 µm long and 2-3 µm wide) and had a smooth surface with 1 or 2 guttae (Figure 2A).

The hymenium had a row of palisade cells, some of which were basidia. The basidia were 20-40 µm long and 3-5 µm wide, fusoid-shaped with 3-4 sterigmata at the apex (Figure 2B). An abundance of pleurocystidia was found along the hymenium layer. The pleurocystidia were ventricose with a prolonged, obtuse or subcapitate apex (Figure 2C). The clavate-shaped cheilocystidia were distributed scarcely on the spore edge (Figure 2D).

The pileus surface was covered by a pile of cutis, composed of slightly erect or decumbent hyphae with cylindrical or subfusiform end cells (Figure 2E). The end cells were mostly thin-walled with vacuoles. The pileus trama was made up by interwoven hyphae without clamp connections. The tube trama showed a boletoid structure with the hyphae diverging towards the hymenium layer and the pore edge.

An abundance of caulocystidia was observed at the stipe surface. The caulocystidia were subclavate or ventricose in shape and was thin-walled with vacuoles (Figure 2F). Similar to the pileus, the stipe trama was composed of interwoven hyphae without clamps. The interwoven hyphae were more compact near the stipe surface compared to the middle.

Molecular Identification

The ITS region was successfully amplified from the five *Boletus* isolates and it showed a single band of approximately 700 bp. Based on a BLAST search, all the isolates showed 100% similarity with *Boletus griseipurpureus* (JQ726594). The nucleotide sequences of the isolates were deposited in GenBank with accession numbers from KF442405 to KF442409.

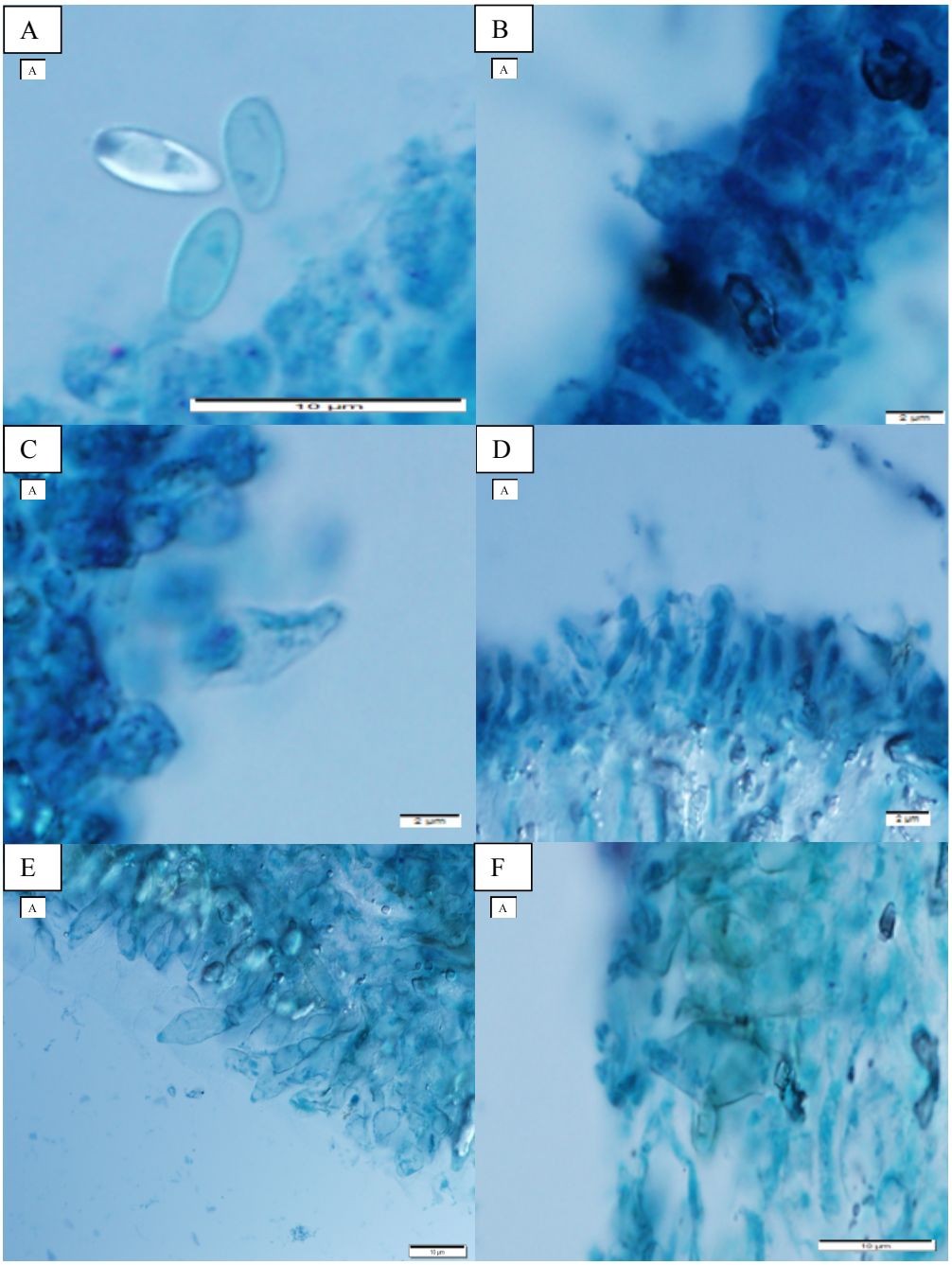

Figure 2. Micromorphological characteristics of *Boletus* sp. (A) Ellipsoid spore. (B) Fusoid basidium. (C) Subcapitate apex pleurocystidia. (D) Clavate cheilocystidia. (E) Subfusiform end pileocystidia. (F) Ventricose caulocystidia

The NJ tree generated is shown in Figure 3, and can be divided into two main clades, I and II. Main clade I consisted of the five *Boletus* isolates, grouped with *B. griseipurpureus* (JQ726594). *Boletus edulis* (GQ900593) and *B. reticulatus* (GU198973) were grouped in main clade II.

Figure 3. Neighbour-joining tree showing the relationship of *B. griseipurpureus* isolates (USMBo1, USMBo2, USMBo3, USMBo4 and USMBo5) and other *Boletus* spp. *Leccimum scabrum* is the out-group

Toxicity Test

In the lethality test, the maximum mortality of brine shrimps took place at a concentration of 100 mg/mL while minimum mortality was at 0.195 mg/mL. After 6 hours of incubation, the extract showed lethal concentration values, LC_{50} of 4.33 mg/mL (Figure 4). The toxicity increased twofold after 24 hours of incubation and the extract had LC_{50} at 2.38 mg/mL (Figure 5). Potassium dichromate acted as a positive control and showed the highest toxicity (LC_{50}=0.74 mg/mL) after 6

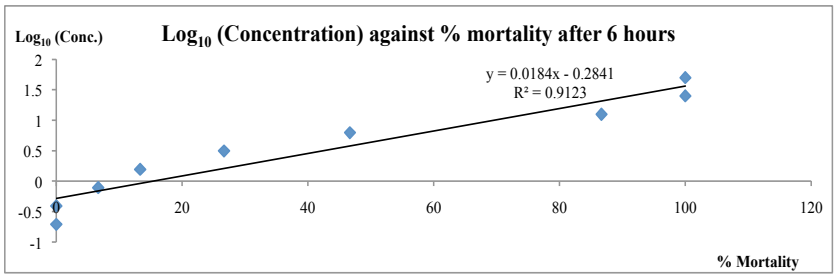

Figure 4. Toxicity of mushroom extract after 6 hours of incubation using brine shrimp bioassay. Each data point represents the mean value of three replicates per concentration level

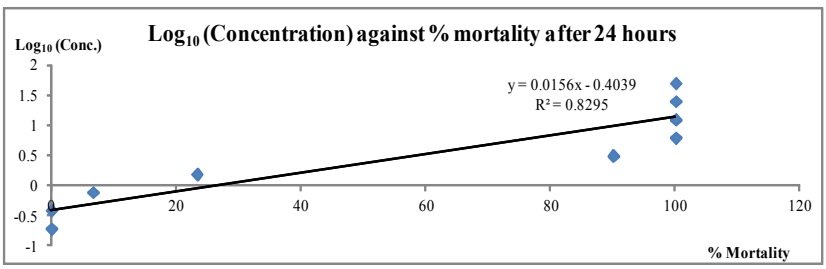

Figure 5. Toxicity of mushroom extract after 24 hours of incubation using brine shrimp bioassay. Each data point represents the mean value of three replicates per concentration level

hours of incubation. All the brine shrimps were dead after 24 hours of exposure to the solution.

Assessment of Mycelial Growths on Solid Media

Medium, pH and temperature have significant effect on the mycelial growth. There was a significant interaction between pH and incubation temperature. The mycelia grew well in acidic conditions (pH 5.0 and pH 6.0) and reached maximum growth at 30°C.

At this optimal condition (pH 6.0 and 30°C), the mycelial growth was significantly different depending on the medium (Figure 6). CZA promoted the highest mycelial diameter growth with shortest incubation time (four weeks), followed by CDA and CMA, which produced the maximum growth in the sixth week. Maximum mycelial growth on MEA and PDA was achieved after eight weeks. Full-plate growth was not observed on CDA, CMA, MEA and PDA but was present for CZA.

Assessment of Mycelial Growth in Liquid Media

The results showed that the type of liquid medium has a significant influence on the production of mycelia. There was no significant interaction between medium and initial pH. The mycelia can grow well over a wide range of pH values (pH 5.0-pH 8.0) without significant difference in biomass production.

YME and YEP tend to be the most suitable liquid media to support *Boletus* growth in acidic conditions (pH 5.0 and pH 6.0) after two weeks of incubation (Figure

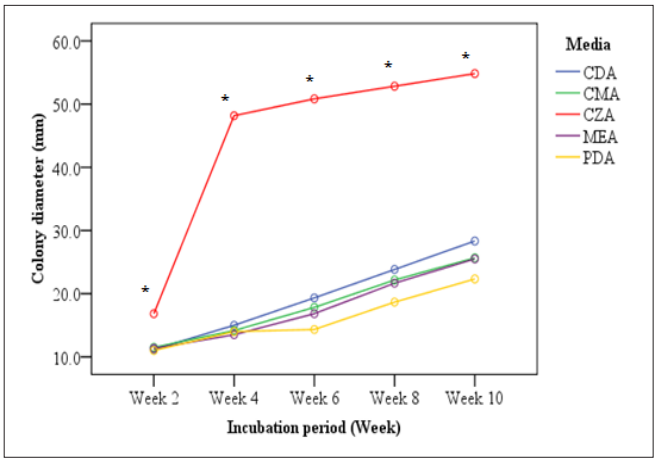

Figure 6. Mycelial growth on different solid media at pH 6.0 and 30°C, 10 weeks of incubation
* indicate significant difference (n=3, $p<0.05$) within the same incubation period

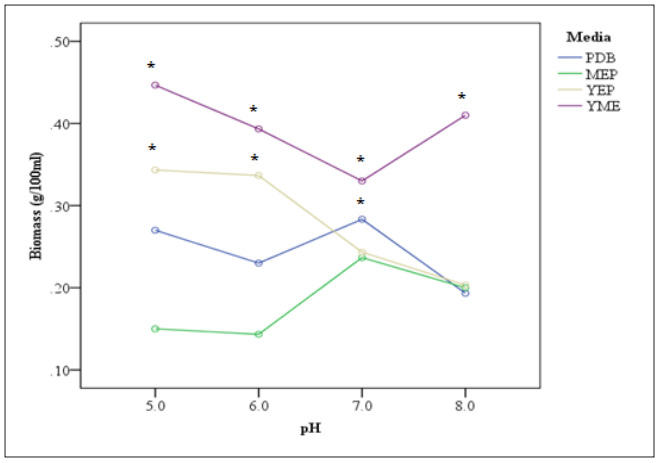

Figure 7. Effect of liquid media on mycelial biomass after 12 days of incubation
* indicate significant difference (n=3, p<0.05) within the same pH

7). YME produced the highest biomass (0.45 g/100 mL), followed by YEP with an average biomass of 0.34 g/100 mL at pH 5.0. The mycelial biomass was the lowest (about 0.20 g/100 mL) in MEP, PDB and YEP at pH 8.0.

DISCUSSION

Colour reactions on the fruiting bodies acted as a preliminary identification in the field. Among the four chemical reagents, potassium hydroxide produced the most consistent result since all the mushroom samples gave no colour changes. Similar to the present observation, the majority of *Boletus* species described by Bessette et al. (2007) reacted to ferrous sulphate and gave olive brown on the stipe contexts. The stipe context also responded to Melzer's reagent, but the yellow colour produced did not match the green-blue colour of *B. rhodopurpureus*, *B. torosus*, *B. xanthocyaneus* and *B. xanthopurpureus* (Hills, 1997). On the pileal surface, the mushroom samples showed a greyish yellow with ferrous sulphate and the result was similar to those in the studies conducted by Bessette et al. (2007) and Chantorn et al. (2007), in which the colour changes ranged from olive green to olive grey.

According to Chantorn et al. (2007), ammonia solution produced orange red on the pileal surfaces of *B. subvelutipes*, *B. laetissimus* and *B. obscureumbrinus*. It differed from the mouse grey colour of the *Boletus* pileal surface in the present study. For the temperate *Boletus* species, the colour reactions can be green or blue on *B. badius*, olive or yellowish on *B. carminiporus* and green or orange-red on *B. innixus* (Bessette et al., 2000; Bessette et al., 2007). These observations suggested that the *Boletus* species responded inconsistently in an ammonia solution to give distinctive colour changes. Melzer's reagent gave a reddish brown on the hymenophore; this

dextrinoid reaction can be attributed to the high lipid content of the spores (Watling, 1971). Similar results were also reported on *B. rhodopurpureus* and *B. edulis* (Smith & Thiers, 1971; Hills, 1997). However, there were no comparable reports on *Boletus* species of olive brown hymenophore by ferrous sulphate.

The overlapping morphological characteristics shown among related *Boletus* species have led to the difficulties in identification and classification. For instance, *Boletus* in the present study showed a purplish pileus, which resembled *B. kluzakii*, *B. xanthopurpureus* and *B. regius* (Thiers, 1975; Hills, 1997; Šutara & Špinar, 2006). However, these three species showed diverse colours of the stipe and hymenophore. Several species such as *B. appendiculatus*, *B. badius*, *B. inedulis* and *B. luridiformis* have contexts that stained blue when exposed to air (Bessette et al., 2000; Bessette et al., 2007; Læssoe, 2010) whereas *Boletus* in this study showed brownish orange. Besides that, most *Boletus* species did not show a pinkish pore surface even though olive brown spores had been reported by Corner (1972).

Because of these overlapping morphological characteristics, the ITS region was used to assist in species identification. Based on the ITS region, the *Boletus* isolates were identified as *B. griseipurpureus*. From the phylogenetic tree, all the *B. griseipurpureus* isolates (USMBo1, USMBo2, USMBo3, USMBo4 and USMBo5) were grouped in the same clade and separated from other *Boletus* species with a high bootstrap value (100%). In Peninsular Malaysia, Corner (1972) was the first to record the occurrence of *B. griseipurpureus* (Mycobank: 309689)

Based on Corner (1972) descriptions of *B. griseipurpureus*, the pileus is convex to plane-shaped, measuring 3.5-10 cm wide, is fleshy and its colour ranges from purple-mauve to purple-grey. The stipe is subclavate, measuring 6-15 cm long and 5-9 mm wide and it is around 7-15 mm at the base. It is finely reticulated, purple-grey but white at the apex of the stipe. However, the white to pale yellow-green pore surface of *B. griseipurpureus* contrasted with pinkish white to reddish brown colour as observed in this study. Corner (1972) further described the basidia of *B. griseipurpureus* to have 3-4 sterigmata, with each bearing a smooth and boletoid spore. The pleurocystidia are ventricose with a prolonged, obtuse or subcapitate apex, often thin-walled. Although several characteristics such as ventricose pileocystidia and clavate cheilocystidia conformed well to the descriptions by Corner (1972), pale red spores in potassium oxide and red-brown pleurocystidia in alcohol formalin were not observed in the present study.

The morphological characteristics of *B. griseipurpureus* in the present study were very similar to the *B. griseipurpureus* reported by Seehanan and Petcharat (2008) in Thailand. The morphological similarities included convex to plane pileus, light purple subtomentose cuticle, white context, pinkish or pale brown tubes, purple cylindrical stipe, clavate basidia and pale brown smooth spores.

According to Meyer et al. (1982), a crude extract that shows LC_{50} lower than 1.0 mg/mL may possess cytotoxic effects. In this study, the mushroom extract exhibited LC_{50} of 4.33 mg/mL. The toxic levels increased twofold after 24 hours of incubation with LC_{50} of 2.38 mg/mL. LC_{50} higher than 1.0 mg/mL implied that the *B. griseipurpureus* collected from the sampling areas were non-toxic. Similar results have been reported for *Agaricus* sp. and *Termitomyces letestui* (Nyigo et al., 2005). In contrast, several edible mushrooms such as *Cantharellus* spp., *Hypsizygus tessulatus* and *Pleurotus ostreatu*s showed LC_{50} below 1.0 mg/mL (Faridur et al., 2010; Kidukuli et al., 2010; Monira et al., 2012). These comparative data suggested that edible mushrooms normally are low in toxicity.

The edibility of *B. griseipurpureus* can be affected by its growing habitat. Studies on mushroom toxicity showed a correlation between fungal metal content and sources of metal pollution, in which the metal content of edible mushrooms sometimes exceed poisonous levels (Carvalho et al., 2005; Stihi et al., 2011). This can be attributed to the accumulation of heavy metals in fruiting bodies (Li et al., 2011). In the present study, the peat soils were slightly contaminated with copper, mercury and plumbum, which can pose risk of mushroom poisoning. In addition to heavy metals, dispersal of toxic pesticides on the growing site can also cause hazardous effects on mushrooms (Pacioni & Lincoff, 1982). The residue of pesticides may persist on the mushroom surfaces for a long time and consequently, pose a health risk for humans if consumed.

Wild *Boletus* produced a high level of secondary metabolites including ascorbic acids, phenolic acid, terpenes, tocophenols and steroids (Tsai et al., 2007; Grangeia et al., 2011; Heleno et al., 2011). These antioxidant substances may also be present in *B. griseipurpureus*, resulting in the bitter taste of the mushroom. However, some edible *Boletus* such as *B. aereus*, *B. borrowsii*, *B. edulis* and *B. reticulatus* are well known for their sweet taste and pleasant smell (Dentinger et al., 2010). This aromatic property may be due to the volatile substances of arabitol, myo-inositol, mannitol and trehalose (Tsai et al., 2008). Any form of processing of fresh mushrooms is expected to alter the composition of these volatile substances, which can be chemically reactive (Manning, 1987). Therefore, new compounds may be formed and this may be the cause of the sweet taste of the mushrooms after cooking.

Among the five solid media tested, CDA promoted the highest colony diameter (about 30 mm), suggesting high soluble starch-induced mass production of mycelium. CMA and MEA contain moderate amounts of starch and thus, allow good mycelial growth. Dung et al. (2012) reported that fungal strains that actively degrade starch always grow well on starch-containing media. Although full-plate growth was observed on CZA, poor utilisation of sucrose by *B. griseipurpureu*s resulted in a sparse appearance of the mycelium.

A similar result was reported by Hughes and Mitchell (1995) and Hatakeyama and Ohmasa (2004).

CMA and MEA contained yeast extract and peptone, respectively. Since the mycelia took a relatively longer time (8 weeks of incubation) to achieve maximum growth, media enriched with high nitrogenous nutrients are not recommended. Mycelial biomass of *Boletus* spp. is also reduced in high nitrogen content (Hatakeyama & Ohmasa, 2004). Contrasting results were shown by *Lentinus subnudus*, which grew well in CMA and MEA without the presence of yeast extract and peptone (Gbolagade et al., 2006). According to Koike et al. (2001), C:N ratio in the range of 15 to 20 provides a balance between carbon and nitrogen sources, which encourages mycelial growth and maintains stable metabolite production. When nitrogen sources became the limiting factor, *B. griseipurpureus* grew better in CDA and PDA in response to the high consumption of carbohydrates (Paustian & Schnürer, 1987).

Temperature and pH are important factors determining fungal growth on synthetic media. In the present study, the mycelia reached optimum growth at 30°C, which suggested that high temperature is required for the enzyme activities of *B. griseipurpureus*. The results contrasted with the optimal temperature (20°C) for the vegetative growth of *B. reticulatus* (Yamanaka et al., 2000). The mycelial growth of the *B. griseipurpureus* was enhanced at pH 6.0. Several single-factor designed experiments showed that temperature ranging from 25°C to 30°C and pH value from pH 3 to pH 6 are the most suitable cultural conditions for mycelial growth of some cultivated mushrooms such as *Volvariella volvacea*, *Agaricus brasiliensis* and *Pleurotus* sp. (Akinyele & Adetuyi, 2006; Calauto et al., 2008; Zharare et al., 2010).

Regardless of the type of liquid media tested, *B. griseipurpureus* grew well over a wide range of pH from pH 5 to pH8. Although most edible mushrooms are found to tolerate a broad pH range in a submerged culture, the optimal pH can be varied depending on the mushroom species. Kim et al. (2005) obtained the highest mycelial biomass of *Agrocybe cylindracea* at pH 4. Xu and Yun (2003) reported that *Auricularia polytricha* achieved the maximum growth at pH 5. The optimal pH of *Agaricus bisporus* and *B. edulis* was pH 6 (Kurbanoglu et al., 2004; Baptista & Nogueira, 2009). These observations suggested that different species prefer different pH values from acidic to neutral.

Effects of different carbon sources on the biomass production in a submerged culture have been studied (Papaspyridi et al., 2010; Smiderle et al., 2012) and it has been suggested that glucose is an excellent monosaccharide for mycelial growth. In the present study, all the liquid media were provided with the same amount of glucose, and thus the variation of mycelial biomass might be attributed to the nitrogen sources. Among the four liquid media, YME significantly produced the highest yield of mycelium, followed by YEP. A similar result

was reported by Lin and Chen (2007), who showed that liquid media supplemented with peptone and yeast extract enhanced mycelial growth of *Antrodia cinnamomea*. Several studies have also found that media containing yeast extract gave the highest mycelial yield when glucose was used as the main carbohydrate source (Feng et al., 2010; Lee et al., 2013).

MEP produced lower mycelial biomass indicating that *B. griseipurpureus* grew slowly in the absence of yeast extract. Pokhrel and Ohga (2007) reported that 1% yeast extract in liquid media stimulated maximum mycelial growth of *Lyophyllum decastes*. This stimulatory effect of yeast extract may be due to its high protein, amino acid and vitamin content. The mycelial biomass was the lowest in basal medium PDB, suggesting the deficiency of nitrogenous compounds in the medium as complex organic nitrogen generally increases the biomass production for the submerged culture of higher fungi (Papaspyridi et al., 2010). Corn steep powder, soybean meal, malt extract, tryptone and casein were found to be good nitrogen sources in submerged culturing of wild edible mushrooms such as *Agrocybe cylindracea*, *Lentinus subnudus* and *Cordyceps militaris* (Kim et al., 2005; Gbolagade et al., 2006; Shih et al., 2007) and thus, may also be suitable for the mycelial growth of *B. griseipurpureus*. However, there are many other factors that can influence mycelial growth in a submerged culture including inoculum volume, agitation rate, aeration rate, incubation duration, salinity and light intensity (Cho et al., 2006; Miao et al., 2006; Chen et al., 2008; Vargas-Isla & Ishikawa, 2008). The combined effect of these factors on the growth of *B. griseipurpureus* needs further investigation.

CONCLUSION

The mushroom locally known as '*kulat gelam*' from a peat swamp area in Bachok, Kelantan was identified as *B. griseipurpureus*. A scientific name of wild mushroom is important as it can provide information on the edibility as well as the nutritional and medicinal properties of the mushroom. A toxicity test suggested that this species is an edible mushroom with a low toxic level (LC_{50}=4.33mg/mL). At optimal cultural conditions (30°C and pH 6.0), CDA promoted the highest colony diameter on plates while YME was the most suitable medium to obtain a good biomass production for submerged culturing of *B. griseipurpureus*. The results obtained from the present study provide the basic information of substrate formulation to cultivate wild edible *B. griseipurpureus*.

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Pertanika J. Trop. Agric. Sci. 40 (1): 73 - 86 (2017)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Evaluation of Nutritive Value of Seven Kenaf (*Hibiscus cannabinus* L.) Varieties Harvested Depending on Stubble Height

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ABSTRACT

Protein feed shortage is one of the most important factors that limit the development of animal husbandry in China. Kenaf (*Hibiscus cannabinus* L.) can be used as a high-quality protein feed for livestock. The aims of this study were to evaluate the yield and quality characters of seven kenaf varieties harvested on the basis of stubble height and to screen the varieties with high nutritive value. A reasonable stubble height was selected, and then kenaf varieties were cut leaving the selected stubble height and their nutritive value was determined. The results showed that stubble height of 90 cm was the most appropriate for improving the biomass content and nutritive value of kenaf. The dry matter yield of the whole plants, crude protein content and crude fibre content of the seven kenaf varieties ranged from 15,359.31 kg/ha to 18,502.18 kg/ha, 8.99% to 16.23% and 16.71% to 37.43%, respectively. The nutritive value of the seven kenaf varieties was the same for the first two harvests, but was slightly different for the third harvest due to the cold climate during this harvest time. Variety SZHP35 had the highest nutritive value, dry matter yield (18,502.18 kg/ha) and crude protein yield content (2,027 kg/ha), while variety 4A-4B had higher leaf proportion and yield and significantly higher crude protein content ($P<0.05$) than that of other varieties. Correspondence analysis suggested that 4A-4B was closely related to leaf yield and crude protein content, while SZHP35 was closely related to high yield. Our findings suggested that4A-4B and SZHP35 could be used as forage material in China. .

ARTICLE INFO

Article history:
Received: 23 October 2015
Accepted: 30 November 2016

Keywords: correspondence analysis, kenaf, nutritive value, stubble height

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ISSN: 1511-3701

INTRODUCTION

Protein feed shortage is one of the most important factors that limit the development of animal husbandry in China, especially in south China. The high-quality forage crops grown in the northern region of China such as alfalfa cannot be planted in south China because of the hot and humid climate in summer.

Kenaf (*Hibiscus cannabinus* L.), an annual herbaceous bast fibre crop, has an ability to adapt to varying climate and environmental conditions; it is an important raw material for the traditional textile industry and has been described as a multi-use crop because of its extensive industrial applications (Falasca et al., 2014; Killinger, 1969). It can even grow in saline-alkali soil (Bai et al., 2015; Reta-Sánchez et al., 2010). Many studies have shown that kenaf has a high nutritional value: the crude protein content of the whole plant ranges from 6% to 23% and that of the leaf ranges from 14% to 34% (Swingle et al., 1978; Webber, 1993; Weber, 1993). The tender stem and leaves of the kenaf have good palatability and can be used as feed for livestock and poultry (Ning-Fang, 2006; Rajashekher et al., 1993). Unlike alfalfa, kenaf can be fed to lambs (Phillips et al., 2002); Further, the existing cultivated varieties have high resistance to anthracnose and root knot nematode disease in China, and no pesticides and herbicides are used in the growth period of kenaf. Thus, chemical pollution is very low; hence, studies on kenaf as forage have obvious ecological and economic benefits. Because of the high yield, easy defoliation and reduced branching, the stalks of kenaf are considered more favourable than its leaves, which have lower yield and percentage. However, leaves are very important with regards to the nutritive value of forage, and selection of mature harvest and specific cultivar is important to obtain a high protein and dry matter yield (Chantiratikul et al., 2009; Kipriotis et al., 2015). The harvest date and materials can influence the crude protein content, and the leaf yield and crude protein yield are significantly different across different varieties of kenaf (Webber, 1993; Weber, 1993). Chantiratikul et al. (2009) evaluated the potential yield and chemical composition of kenaf and determined a suitable harvest time. Reta-Sánchez et al. (2010) preliminarily determined the suitable planting density for kenaf as forage. JiYeon et al. (2012) compared the nutritive value of different kenaf cultivars and successfully identified a potential forage cultivar. In Malaysia, Saba et al. (2015) conducted a systematic research on harvest and post-processing of kenaf as forage based on the local ecological and economic conditions. However, studies on kenaf have mainly focussed on the effect of plant maturity or different harvest intervals on its use as forage (Chantiratikul et al., 2009; Danalatos & Archontoulis, 2010; Gonzàlez-Valenzuela et al., 2008; Phillips, 1999; Webber & Bledsoe, 2002; Xiccato et al., 1998), fertilisation measures, field management techniques and cultivation methods (Anfinrud et al., 2013; Bañuelos et al., 2002; Danalatos & Archontoulis, 2010; Reta-Sánchez et al., 2010). However, studies

involving multiple harvests of kenaf over the period of one year similar to most studies on forage grass are rare. In this study, kenaf varieties were harvested three times in one growing season.

Kenaf, a tropical crop with high regeneration ability, was thought to adapt very well to the hot and humid climate of south China and it was believed it could be used as a non-grain-based protein-rich feed resource for livestock. It was seen to be a resource for alleviating the food crisis in south China. In this study, kenaf was harvested at different stubble heights, and kenaf varieties with high nutritive value at the appropriate stubble height selected were identified.

MATERIALS AND METHODS

The test material was planted at the Innovation Experiment Base (112°42′E, 28°12′N) of the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Science. The sowing time was 16 May to 20 October for both years of the two-year study. The soil used was clay and it had a pH of 5.1. The following seven representative materials were selected from the existing varieties: Fuhong992, SZHP35-44, H1301, SZHP35, 14-514, 4A-4B and 991FN3; they are marked A, B, C, D, E, F and G in Tables 3 and 4 and Figures 4 to 7.

In the first part of the study, Fuhong992 was used for the trial and planted in a plot (25 m × 35 m) with a row space of 35 cm and planting density of 18,000 plants/ha; one stubble height was selected from three stubble heights (60, 90 and 120 cm). The first harvest was on the 65th day after planting; the second harvest, on the 45th day after the first harvest; and the third harvest, on the 45th day after the second harvest (Figure 1). The crops were harvested three times in a growing season of 155 days. The sowing times and harvest mode of the second part of the study were the same as those in the first part; the stubble height was selected in the first part. Each material was planted in a plot (2.5 m × 35 m) with a row space of 35 cm and planting density of 18,000 plants/ha. In the two parts of the study, about 600 kg/ha compound fertiliser (N:P:K=15:15:15) as base fertiliser was applied at sowing and 225 kg/ha compound fertiliser (N:P:K=15:15:15) as top application of fertiliser was applied after each harvest, and the land was irrigated with sufficient water after fertilisation.

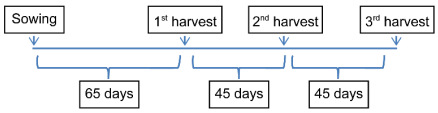

Figure 1. The harvest mode

YIELD DETERMINATION

In our field investigation, the survival rate was above 99%. The kenaf varieties were cut leaving the selected stubble height, and the fresh yield was determined. An area of 4 m2 (2 m × 2 m) was selected randomly from the plot and samples were collected from this plot; the kenaf plants were separated into stems and leaves, and the dry matter (DM) weight of each part was calculated

after de-enzyming (dried at 65°C to constant weight after drying at 105°C for 30 min). The DM yield of leaves, stems and whole plants per plot was estimated from the DM of each plant part.

Nutritive Component Determination

The plants sampled to determine the DM yield were also used to analyse the main nutritive components, including crude protein (CP; AOAC 1995), ether extract (EE; ISO 6492:1999), crude fibre (CF; ISO 6865: 2000), ash (AOAC 1995), calcium (Ca; AOAC 1995) and total phosphorus (TP; AOAC 1995).

CP Yield Determination

CP yield of each harvest was the product of the CP content and DM yield; the CP yield of the whole growth period was the sum of the CP yield of the three harvests. The values were converted to a unified measuring unit, kg/ha.

All data were analysed using the SAS 9.2 statistical software. The nutritive components and DM yield, which were classified according to harvest time, were analysed using Duncan's multiple range test. Statistical significance was set at the 5% level. The correspondence analysis for nine traits and seven varieties of kenaf as the subject was analysed using the SAS 9.2 statistical software. The average linkage clustering based on yield and nutritive traits was also analysed using SAS 9.2.

RESULTS AND DISCUSSION

Selection of Stubble Height

First, an appropriate stubble height was selected. Previous studies have shown that the nutritive value of kenaf varies depending on the numbers of days after planting, and the highest nutritive value was obtained at 8-12 weeks (about 55-80 days) after planting (Chantiratikul et al., 2009; Phillips, 1999); however, these studies were based on a yearly harvest. One study suggested that kenaf can be harvested when the stubble height is 12 cm and plant height is 90 cm; the researchers showed that when the harvest interval was 40 days, kenaf could be harvested three times in one growing season, whereas when the harvest interval was 60 days, kenaf could be harvested only twice in one growing season. Nonetheless, this study did not provide information for the optimum stubble height for kenaf (Gonzàlez-Valenzuela et al., 2008). Stubble height has a significant effect on the yield and quality of forage grass (Jinghui et al., 2005; Zhaorong et al., 2013). In our study, almost no leaves remained on the stem below 60 cm after the kenaf was grown for two months after planting; at our study site, the kenaf could be harvested three times in a growing season. On the basis of the findings of previous studies and our observation, three stubble heights (60, 90 and 120 cm) were selected for preliminary analysis, and the harvest intervals were 65, 110 and 155 days, respectively.

Yield Traits of Different Stubble Heights

Table 1 shows the DM yield of kenaf at different stubble heights. The yield of each part and that of the whole plant of the 90-cm and 120-cm stubble height trial groups was significantly higher during the second harvest than during the other two harvest times. The yield of the 60-cm stubble height trial group successively and significantly decreased during the three harvest times (Figures 2a, 2b, 2c). The yields of each part of the three trial groups were the lowest during the third harvest, owing to the cold climate that retarded the growth of the plants. Because fewer leaves remained on the stem after each harvest, the regeneration of the stubble height of the 60-cm trial group was restricted, in particular during the third harvest when the climate was cold.

Plant yield is an important index to evaluate the regeneration ability of plants (Jin-Hong et al. 2011). During the three harvest times, the yield of stems and leaves of the different treatments during the same harvest time were significantly different, indicating that stubble height affected the regeneration capacity and yield of plants, which was consistent with the findings of previous studies (Wang, 2012; Wang et al., 2005). The DM yield of the three trial groups (12,529.16, 15,943.89 and 12,232 kg/ha) during the trial phase (Table 1) was higher than that reported previously (Chantiratikul et al., 2009; Hui-Juan et al., 2012; Reta-Sánchez et al., 2010). This suggests that the DM yield of kenaf is higher when it is harvested more than once a year.

Leaf output is an important factor in evaluating the nutritive value of forage crops: the higher the percentage of leaves, the higher the nutritional value (Webber, 1993; Jinghui et al., 2005; Zhaorong et al., 2013; Zhiguo, 2009). The proportion of leaves, especially that of the stubble height of the 60-cm group (44.8%), was higher than that reported previously (Reta-Sánchez et al., 2010). Since the yield of the whole plants was high, the leaf yield was high as well (Table 1). This result suggested that

Table 1
Dry Matter Yield of Plants Harvested at Different Stubble Heights (Fuhong992)

Date	Treatment	L-Y (kg/ha)	S-Y (kg/ha)	T-Y (kg/ha)
20/7	60	2381.64[a]	3163.44[a]	5545.08[a]
	90	1679.05[b]	2892.50[b]	4571.55[b]
	120	1173.98[c]	2182.59[c]	3356.57[c]
5/9	60	1681.33[c]	2285.98[c]	3967.31[b]
	90	2764.63[a]	4735.94[a]	7500.57[a]
	120	2066.82[b]	3712.09[b]	5778.91[c]
20/10	60	1551.84[b]	1464.93[c]	3016.77[b]
	90	1658.18[a]	2213.59[a]	3871.77[a]
	120	1223.65[c]	1873.22[b]	3096.87[b]

Leaf yield (LY), stem yield (SY), total yield (TY)
[a, b, c] Significant difference at $P<0.05$

harvesting according to stubble height did not reduce the nutritional value of kenaf. Thus, kenaf can be harvested according to stubble height.

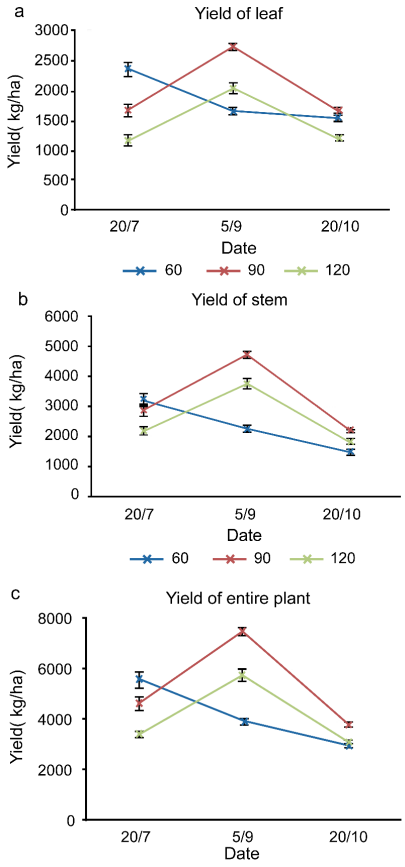

Figure 2. Dry matter yield of leaves (a), stems (b) and the entire plant (c) during the three treatments

Quality Traits of Plants Harvested at Different Stubble Heights

The nutritive components of the whole plants are shown in Table 2. Except for CF (Liu et al., 2014), the nutritive components of the different treatments were significantly different, indicating that stubble height was significantly correlated with the nutritive value of kenaf; this finding is similar to that of previous studies (Huo et al., 2001; Wang & Zhao, 2008).

For the same treatment, the content of CF, CP and EE were different during the first two harvests; the CP and EE content of the third harvest were higher than those of the first two harvests, whereas the CF content was lower than that of the first two harvests (Figures 3 a, b, c). These findings can be attributed to the retarded growth of plants during the third harvest, when the climate was cold. The content of ash, Ca and P were almost the same during the three harvest times, indicating that climate or plant maturity mainly had a significant effect on the CP, EE and CF content, consistent with the findings of previous studies (Chantiratikul et al., 2009; Reta-Sánchez et al., 2010).

Thus, different stubble heights and multiple harvesting times have a significant effect on the nutritive components of kenaf.

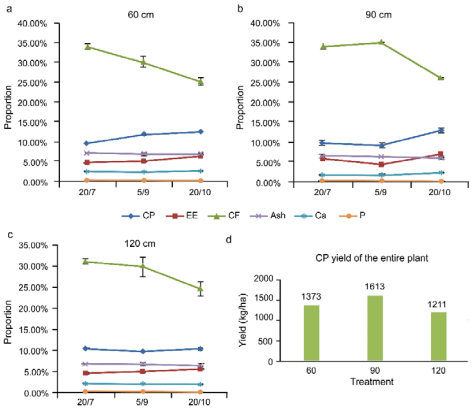

Figure 3. Nutrient content of the different harvest times (a, b and c) for the three trial groups. (d) Crude protein yield of the three treatments

Table 2
Nutrient Content of the Whole Plant of Kenaf Harvested at Different Stubble Heights (Fuhong992)

Date	Treatment	CP (g/kg)	EE (%)	CF (%)	Ash (%)	Ca (%)	P (%)
20/7	60	95.70^{b}	4.58^{b}	34.06^{a}	7.01^{a}	2.29^{a}	0.18^{c}
	90	97.00^{b}	5.58^{a}	33.25^{a}	6.44^{b}	1.76^{b}	0.17^{a}
	120	101.30^{a}	4.28^{c}	30.91^{a}	6.53^{b}	1.88^{c}	0.14^{b}
5/9	60	117.70^{a}	5.03^{a}	30.14^{a}	6.86^{a}	2.21^{a}	0.15^{a}
	90	90.00^{b}	4.38^{c}	30.28^{a}	6.23^{b}	1.79^{b}	0.14^{a}
	120	95.90^{c}	4.76^{b}	29.88^{a}	6.49^{ab}	1.88^{b}	0.12^{a}
20/10	60	124.30^{a}	6.34^{b}	25.26^{a}	6.85^{a}	2.57^{a}	0.08^{c}
	90	127.90^{a}	6.85^{a}	25.55^{a}	6.01^{b}	2.33^{b}	0.09^{a}
	120	102.30^{b}	5.50^{c}	24.56^{a}	6.32^{b}	1.77^{c}	0.09^{b}

Crude protein (CP), ether extract (EE), crude fibre (CF), calcium (Ca) and total phosphorus (P).
$^{a, b, c}$ Significant difference at $P<0.05$.

Previous studies used the DM yield and CP content as the reference to evaluate the nutritive value of forage grass (Wang & Zhao, 2008; Wang et al., 2015). In our study, the stubble height of the 90-cm trial group had the highest DM yield (15,943.89 kg/ha) and CP content (1,613 kg/ha; Table 1 and 3d). The DM yield and CP content obtained in this study were higher than those reported previously (Chantiratikul et al., 2009; Reta-Sánchez et al., 2010).

Thus, the stubble harvest method can improve the biomass and nutritive value of kenaf as forage. For further analysis, the stubble height of 90 cm was selected as the most appropriate height for harvest.

The Nutritive Value of Seven Kenaf Varieties

Yield traits of the seven varieties. The DM yield of the different varieties was significantly different at each harvest. The DM yield in the two varieties containing entire leaves was not significantly different from that of the other varieties containing divided leaves (Table 3). Further, the DM yield was not significantly different across different leaf shapes. The DM yield of all seven varieties was the highest during the second harvest, indicating that the climate of the second growth stage was very suitable for the regrowth of kenaf. Except for varieties SZHP35-44 (B) and SZHP35 (D), all the other varieties had the lowest DM yield in the third harvest, which was mainly because of the cold climate.

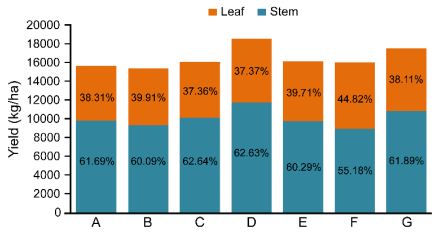

Figure 4. Yield partitioning of the leaves and stems of the seven varieties; Fuhong992, SZHP35-44, H1301, SZHP35, 14-514, 4A-4B and 991FN3 marked as A, B, C, D, E, F and G, respectively

Generally, high DM yield indicates a high proportion of stem. The SZHP35 (D) variety had the highest DM yield (18,502.18 kg/ha) because it had the highest stem yield (11,587.96 kg/ha); however, this finding was not consistent across all the varieties that had a high stem proportion probably because of the differences in germplasms of the different materials (Figure 4).

Leaf yield and biomass are important factors for selecting kenaf cultivars for forage production because leaves are the primary source of protein (Webber, 1993). Variety 4A-4B (F) had the highest leaf output and yield (44.82%), indicating that this variety had higher nutritive value per unit mass. The second-highest proportion and yield of leaves was noted in variety SZHP35 (D) (Figure 4).

The total yield for the seven varieties was more than 15,000 kg/ha (Table 3), which was higher than that reported in previous studies (Chantiratikul et al., 2009; Liang et al., 2003; Reta-Sánchez et al., 2010). Thus, the seven varieties used in this study have the highest yield potential.

Quality traits of the seven varieties. The nutritive components of all the varieties during the first two harvest times were different (Table 4). The CP and Ca content was higher, CF and TP content was lower and ash and EE content was unchanged during the third harvest time than during the first two harvest times owing to the cold climate during the third harvest.

The nutritive components of the seven varieties were significantly different during the same harvest time. Variety 4A-4B (F) had significantly higher CP and lower CF content than the other varieties during the three harvest times. Generally, forage grass with high nutritive value has higher CP content and lower CF content (Chen et al., 2012a; Xiang et al., 2008; Zhu et al., 2014); thus, variety 4A-4B (F) has high potential for use as forage grass.

Table 3
Dry Matter Yield of the Seven Varieties of Kenaf

	L-S (D/E)	1st (kg/ha)	2nd (kg/ha)	3rd (kg/ha)	T-Y (kg/ha)
A	Divided	4,312.63^{c}	7,536.28^{c}	3,905.29^{d}	15,754.20^{d}
B	Divided	3,219.53^{e}	8,613.30^{b}	3,526.48^{e}	15,359.31^{e}
C	Divided	5,513.85^{a}	5,943.80^{e}	4,660.53^{a}	16,118.19^{c}
D	Divided	3,687.11^{d}	10,282.01^{a}	4,533.06^{b}	18,502.18^{a}
E	Entire	4,920.18^{b}	7,280.93^{d}	3,905.83^{d}	16,106.94^{c}
F	Entire	4,383.83^{c}	7,608.90^{c}	4,056.90^{c}	16,049.63^{c}
G	Divided	4,891.89^{b}	8,648.64^{b}	3,912.11^{d}	17,452.64^{b}

Leaf shape (LS), Leaf yield (LY), Stem yield (SY), Total yield (TY).
*$^{a, b, c, d, e}$ Significant difference at $P<0.05$.
Fuhong992, SZHP35-44, H1301, SZHP35, 14-514, 4A-4B, and 991FN3 marked as A, B, C, D, E, F, and G, respectively

Table 4
Nutritive Components of the Seven Varieties

Variety	CP (k/kg)			EE (%)			CF (%)		
	1	2	3	1	2	3	1	2	3
A	91.40^{f}	89.90^{c}	127.90^{b}	5.58^{b}	4.38^{bc}	6.85^{a}	33.25^{c}	34.28^{ab}	25.55^{a}
B	110.80^{b}	111.70^{a}	128.30^{b}	4.75^{d}	4.24^{c}	5.80^{b}	31.56^{e}	29.72^{c}	20.93^{b}
C	101.20^{c}	105.90^{b}	120.50^{c}	5.12^{c}	5.26^{a}	6.45^{a}	34.13^{b}	33.49^{ab}	20.88^{b}
D	110.30^{b}	104.90^{b}	119.30^{c}	5.59^{b}	5.00^{ab}	4.57^{c}	32.75^{d}	31.82^{bc}	20.13^{b}
E	97.20^{d}	95.00^{c}	158.90^{a}	5.43^{bc}	5.3^{1a}	6.70^{a}	34.42^{b}	34.18^{ab}	21.04^{b}
F	115.20^{a}	111.60^{a}	162.30^{a}	7.15^{a}	4.40^{bc}	5.64^{b}	30.50^{f}	29.71^{c}	16.71^{c}
G	94.30^{e}	94.50^{c}	160.10^{a}	4.23^{e}	5.57^{a}	5.88^{b}	37.43^{a}	36.06^{a}	20.41^{b}
Variety	Ash (%)			Ca (%)			P (%)		
	1	2	3	1	2	3	1	2	3
A	6.44^{ab}	6.23^{a}	6.01^{d}	1.76^{c}	1.79^{d}	2.33^{e}	0.17^{a}	0.14^{b}	0.09^{b}
B	6.16^{c}	6.19^{a}	6.64^{c}	2.03^{b}	2.11^{c}	2.35^{e}	0.16^{ab}	0.13^{c}	0.10^{a}
C	6.45^{ab}	6.26^{a}	6.71^{c}	2.18^{b}	2.13^{c}	3.29^{b}	0.13^{d}	0.12^{cd}	0.09^{b}
D	6.56^{a}	6.46^{a}	6.13^{d}	2.19^{b}	2.14^{c}	3.38^{ab}	0.16^{ab}	0.12^{a}	0.09^{b}
E	6.35^{b}	6.37^{a}	7.88^{b}	2.54^{a}	2.62^{a}	3.39^{a}	0.10^{e}	0.12^{cd}	0.09^{b}
F	6.51^{a}	6.54^{a}	8.66^{a}	2.43^{a}	2.38^{b}	3.16^{c}	0.13^{cd}	0.13^{bc}	0.09^{b}
G	5.58^{d}	5.68^{b}	7.85^{b}	2.44^{a}	2.46^{ab}	2.44^{d}	0.15^{bc}	0.11^{d}	0.08^{c}

Please refer to Table 2 for abbreviations
*$^{a, b, c, d, e}$ Significant difference at P<0.05.
Fuhong992, SZHP35-44, H1301, SZHP35, 14-514, 4A-4B and 991FN3 marked as A, B, C, D, E, F, and G, respectively

The EE and ash content of the varieties were at an average level compared with the corresponding content reported previously (Chantiratikul et al., 2009), whereas Ca and P content was lower than the normal level; these findings could be attributed to the method of de-enzyming (Zhu et al., 2014). Future studies need to focus on improving the de-enzyming method to reduce the loss of mineral elements.

The CP content of the seven varieties of kenaf ranged from 1,571 kg/ha to 2,026 kg/ha and was significantly different across the varieties, while variety SZHP35(D) had the highest CP yield (2,026 kg/ha) (Figure 4).

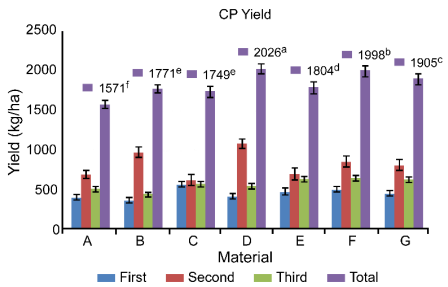

Figure 5. The total crude protein yield of the seven varieties during the three harvest times; Fuhong992, SZHP35-44, H1301, SZHP35, 14-514, 4A-4B and 991FN3 marked as A, B, C, D, E, F and G, respectively

Correspondence Analysis for Yield and Quality Traits

Generally, leaf, stem and entire plant yields are considered as yield traits, while nutritive components are considered as quality traits. Correspondence analysis is used to determine the relationship between trait points and varieties (Liu et al., 2011). In this study, nine traits and seven varieties of kenaf were subjected to correspondence analysis using the SAS 9.2 statistical software.

The results showed that the contribution of the first two Eigen values was 99.85%, while the contribution rate of the first Eigen value was 99.29%, indicating that the first dimension can explain 99.29% of the total information; thus, the data analysis was mainly based on the first dimension (Table 5).

The factor loading of LY was larger than that of SY and TY in the first dimension, suggesting that LY had a higher impact on the yield traits. The factor loading of CP, EE and Ca were larger than those of the other nutritive traits; therefore, these three traits were considered to have an important impact on the nutritive content of kenaf varieties, as has been revealed previously (Wang et al., 2010) (Table 6).

Table 5
Inertia and Chi-Square Decomposition of the Correspondence Analysis

Inertia and Chi-Square Decomposition				
Singular value	Principal inertia	Chi-square	Percent	Cumulative percent
0.03497	0.00122	280.949	99.29	99.29
0.00261	0.00001	1.567	0.55	99.85
0.00110	0.00000	0.280	0.10	99.95
0.00068	0.00000	0.107	0.04	99.98
0.00042	0.00000	0.041	0.01	100.00
0.00009	0.00000	0.002	0.00	100.00
Total	0.00123	280.947	100.00	

Table 6
R Factor Loading of the Correspondence Analysis

Row coordinates		
	Dim1	Dim2
LY	0.0615	-0.0001
SY	-0.0397	-0.0001
TY	0.0000	-0.0001
CP	0.0828	0.0257
EE	0.0482	0.0793
CF	-0.0225	0.0762
Ash	0.0351	0.0437
Ca	0.0705	0.0190
P	-0.0003	-0.0598

The closer the distance between the variety point and trait point, the higher the possibility that the variety has the trait, which can be an important characteristic (Chen et al. 2012b). The point of F variety

was closer to those of LY and CP, suggesting that F variety had higher CP and LY than the other varieties. The points for Fuhong992 (A), SZHP35-44 (B), H1301 (C), SZHP35 (D), 14-514 (E), 4A-4B (F) and 991FN3 (G) varieties were closer to those of SY and TY, suggesting that these varieties had higher biomass than variety 4A-4B(F). The CF point was distant from all the variety points, indicating that these varieties did not have sufficient CF content (Figure 6), but this result needs to be considered with caution, as no other crops were used as negative controls.

The points for EE, Ca, CP and ash were closer and formed a character group, suggesting that these traits were significantly correlated. The point for P was far from the points for the four above-mentioned traits, which was slightly different from the findings of a previous study (Wang et al., 2010); this discrepancy in findings could be attributed to the difference in the sample processing and measurement methods (Figure 6).

A dendrogram obtained using the average linkage clustering based on nutritive and yield traits is shown in Figure 7. Varieties Fuhong992 (A) and SZHP35-44 (B) can be classified into one cluster, and H1301 (C), 14-514 (E) and 991FN3 (G), into another cluster. The distance between the two clusters is small because all of the five varieties can take the stem yield and total yield as their representative traits compared with other nutritive and yield traits. Variety SZHP35 (D) can be classified into one cluster, because its total yield and CP yield were the highest among the seven varieties. Variety 4A-4B (F) was classified into one cluster because it had the highest leaf yield, and its representative traits included CP, which was different from that of the other six varieties. These findings are consistent with the analysis results shown in Tables 3, 4 and 6 and Figure 4.

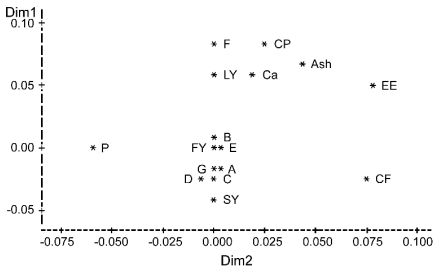

Figure 6. Correspondence analysis chart based on yield and quality traits; Fuhong992, SZHP35-44, H1301, SZHP35, 14-514, 4A-4B and 991FN3 marked as A, B, C, D, E, F and G, respectively

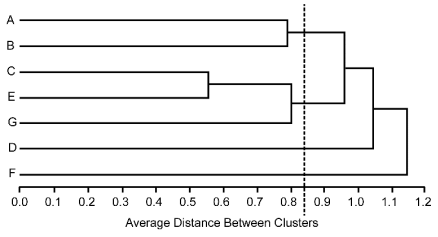

Figure 7. Dendrogram developed using average linkage clustering based on the nutritive traits of the seven varieties; Fuhong992, SZHP35-44, H1301, SZHP35, 14-514, 4A-4B and 991FN3 marked as A, B, C, D, E, F and G, respectively

CONCLUSION

The results of our trial analysis revealed that the stubble height of 90 cm was the most suitable for harvesting kenaf because this trial group had higher DM yield and CP yield than the other two treatments. Variety SZHP35 had the highest CP content. Variety 4A-4B was found to have great potential to be cultured as a forage crop because of its high leaf yield, high CP content and low CF content. SZHP35 can be considered forage material based on its total yield and nutritive value, while 4A-4B can also be good forage material owing to its high leaf yield. Therefore, both the varieties are good for feed utilisation.

Kenaf can be harvested multiple times in one growing season depending on stubble height to ensure sustainable yield. The kenaf varieties had average yield equal to that of alfalfa, which is a high biomass material; therefore, the high nutritive value of kenaf allows it to be used as an excellent non-grain-based protein-rich feed resource.

Although tender plants have high CP content, their DM yield is low. This suggests that stubble height and harvest time need to be further optimised. Since leaf proportion is an important factor in improving leaf yield, future research should focus on the relationship between leaf shape and leaf yield.

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Pertanika J. Trop. Agric. Sci. 40 (1): 87 - 98 (2017)

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Quantification of Total Phenolic compounds in Papaya fruit peel

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ABSTRACT

Phenolics are widely distributed in many plants and are known to play a major role in the plant and animal kingdom. Phenolics exhibit strong antioxidant properties and have been used as antitumor, anticarcinogenic, antiviral and hypotensive agents. Plant by-products contain a variety of these phenolic compounds and can therefore be used as an alternative source of phenolics due to their higher antioxidant capacity and low toxicity compared to those of synthetically derived phenolics. In this study, *Sekaki* papaya (*Carica papaya*) peel was used as an alternative source of phenolics. Response Surface Methodology (RSM) was employed to optimise process conditions to achieve the highest phenolic content from the fruit peel. Total Phenolic Content was analysed using the Folin-Ciocalteau method and the total phenolic content (TPC) was expressed as Gallic Acid Equivalent (GAE). The highest TPC i.e. 1735.1 mg/L GAE was obtained at a temperature of 1200C and a time of 5 h in a solid-solvent ratio of 1:40 g/mL while the lowest TPC of 616.57 mg/L GAE was obtained at a temperature of 900C and a time of 3 h at a solid-solvent ratio of 1:20 g/mL. With such a high phenolic content, *Sekaki* papaya (*Carica papaya*) peel can be used as a natural antioxidant and can protect the human body from various free-radical-associated diseases.

Keywords: phenolics, antioxidant, Total Phenolic Content (TPC), Folin-Ciocalteau method

INTRODUCTION

By-products derived from plants contain a variety of phytochemicals and phenolic compounds besides carotenoids such as flavonoids, phenolic acids, lignin, tannins and others. These compounds represent antioxidant as well as antiradical activities (Shahidi & Naczk, 2004) and possess

ARTICLE INFO

Article history:
Received: 01 April 2016
Accepted: 27 September 2016

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ISSN: 1511-3701

anticarcinogenic, antimutagenic and antiproliferative properties (Yang et al., 2005; Alasalvar et al., 2006). The antioxidant activity of phenolics is mainly attributed to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers.

'*Sekaki*' papaya (*Carica papaya*) is a popular tropical fruit widely used for its fruit flesh as well as processing activities. Malaysia is a predominant producer as well as exporter of this fruit; two main varieties of papaya (*Carica papaya*) are grown in the country, with *Sekaki*, also known as 'Hong Kong' variety, being the second most cultivated variety after *Eksotika*. The *Sekaki* has red flesh and a bright yellow, even-coloured skin without freckles. Papaya (*Carica papaya*) peel is often considered waste and can pose an environmental threat in excessive production. It does not receive much attention in terms of utilisation or recycling and is usually discarded. This could result from a lack of application for commercial purposes (Soong & Barlow, 2004). Interestingly, by-products of fruit such as peel and seed fractions may contain many valuable compounds such as carotenoids, flavonoids and phenolics and like the fruit pulp itself, may exhibit strong antioxidant capacity (Jayaprakasha et al., 2001). Research has been conducted on many fruit products such as tomato pomace, the seeds and skin of which accounted for a high level of lycopene (Choudhari & Ananthanarayan, 2007); pomegranate peel, which exhibited higher antioxidant activity than its pulp (Li et al., 2006); grape seed, which is a stronger antioxidant than its pulp, contains a high level of proanthocyanidin and can scavenge various reactive oxygen free-radical species (Guo et al., 2003). While papaya (*Carica papaya*) fruit pulp has nature's most concentrated source of carotenoids, especially lycopene, which shows strong antioxidant activity (Jamal et al., 2016), the peel of the fruit also exhibits strong antioxidant as well as phenolic content.

Recently, there has been increasing interest in the antioxidant properties exhibited by phenolic compounds extracted from certain vegetables and fruit due to their strong antioxidant activity and low toxicity compared to synthetic phenolic antioxidants such as BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole) and propyl gallate. There are various methods to determine the total phenolic content of plants and their fruit extracts. Phenols can be assessed by a variety of methods; however, results obtained from each test are difficult to analyse as sometimes, only the phenolic content of the final extracts are reported and not the total yield. Previously, total phenolic content was determined colorimetrically and measured as grams of Gallic acid (Singleton & Rossi, 1965) but the Folin-Ciocalteau method is slightly modified (Sato et al., 2004). Total Phenolic Content of the antioxidants is expressed as the Gallic acid equivalent concentration (mg/L) (Tachakittirungrod et al., 2007).

Multiple reaction characteristics and mechanisms are responsible for strong antioxidant activity of plant extracts. Several

methods including FRAP assay, DPPH radical scavenging activity, ABTS or the Trolox equivalent test, ORAC, can be employed to give an estimation of a plant's antioxidant capacity. All these methods may be subjective to conditions and the various reagents used (Chandrasekara & Shahidi, 2011). Coupling DPPH and FRAP assay together helps provide a complete vindication of antioxidant capacity of the lycopene extract from papaya fruit peel (Sanchez -Moreno et al., 2003; Shi & Le Maguer, 2000; Liu et al., 2007; Dewanto et al., 2002). Also, phenolic compounds account for a major portion of the antioxidants in many fruit extracts (Duthie & Crozier, 2000; Gertenbach, 2001; Vuong et al., 2010). Plant-derived phenolics are reported to have better antioxidant activity than those of vitamin C (Wang et al., 1996) and elucidate their activity by several mechanisms in action such as free radical scavenging, lipid peroxidation and metal ion chelation (Nilar & Harrison, 2002). Notwithstanding the criticisms of the chemical assays, numerous papers have reported good to strong correlation between the total phenolic content and the antioxidant capacity of extracts; thus, these compounds are considered major contributors to the antioxidant activity of fruit extracts. Total phenolic content is generally determined using the Folin-Ciocalteau method which, as pointed out by Prior et al. (2005), has an oxidation/reduction reaction as its basic mechanism in determining the antioxidant activity of compounds.

Therefore, the objective of this study is to demonstrate the potentiality of a new source of antioxidant that can give a new dimension to the usage of *Sekaki* papaya (*Carica papaya*) peel. After suitable heat treatment, which aided in full extraction of the carotenoids from the fruit peel due to cell disruption and release of the desired compounds (Shi et al., 2000; Chang et al., 2006), the effects of reaction parameters (temperature, time and solid-solvent ratio) on Total Phenolic Content using the Folin-Ciocalteau method to optimise the highest phenolics using response surface methodology (RSM) were studied.

MATERIALS AND METHODS

Plant Materials

Papaya (*Carica papaya*) peel of the papaya variety *Sekaki* was collected from a local fruit shop located in the International Islamic University Malaysia (IIUM) and the separated peel was freeze-dried until a constant weight was obtained. The dried samples were then ground using a grinder machine to get a uniform size of sample. Samples were kept in airtight containers and stored at -20°C until further analysis. All the experimental procedures were carried out under dim light.

Plant Extraction

A sample (1 gm) of the *Sekaki* papaya (*Carica papaya*) peel was extracted using a mixture of solvents (hexane, acetone and alcohol in the ratio of 2:1:1) containing

0.05% (w/v) butylatedhydroxytoluene (BHT) (Shi & Le Maguer, 2000). The samples were subjected to heat treatment for set time periods (Honest et al., 2010; Kaur et al., 2007; Fish et al., 2002; Choudhari & Ananthanarayan, 2007). Antioxidants such as butylated hydroxytoluene (BHT) were employed in solvents used for extraction so as to avoid oxidation and isomerisation reactions. Cold distilled water (15 ml) was added to the mixture and the suspension was agitated at 200 rpm for 8 min. The solution was then allowed to stand at room temperature for 15 min for separation of polar and non-polar layers. The nonpolar supernatant hexane layer containing the desired carotenoid compounds as well as phenolics were separated and further analysed for quantification of its lycopene content (Jamal et al., 2016). The oleoresin was then subjected to saponification and purification to separate the carotenoids, mainly lycopene and β-carotene from the phenolics. The presence of phenols was then investigated using the Folin-Ciocalteau Method.

Chemicals

Ethanol (HmbG Chemicals), hexane (EMSURE, Merck KGaA, Germany) and acetone (Bendosen Lab Chemicals) were used to extract the antioxidant compounds from the fruit peel. For the total phenolic test hydrochloric acid, Gallic acid (Fluka), Folin-Ciocalteau's reagent (Merck) and sodium carbonate (HmbG Chemicals) were used.

Determination of the Total Phenolic Content: Folin-Ciocalteau Method

Total phenolic content (TPC) of all the extracts and fractions was determined following the Folin-Ciocalteau method as described by Khoo (2009) with slight modification. In a 100-mL volumetric flask, 0.5 grams of dry Gallic acid was dissolved in 10 mL of ethanol and diluted to volume with water. For the preparation of standard curve, 0, 1, 2, 3, 5 and 10 mL of phenol stock was taken into 100-mL volumetric flasks and diluted to the required volume with water. These solutions had concentrations of 0, 50, 100, 150, 250 and 500 mg/mL Gallic acid.

Total phenolic content (TPC) of the *Sekaki* papaya peel extracts was determined using the Folin-Ciocalteau colorimetric method. First, appropriate dilution of the extracts was prepared (1 mg/mL). Then, 20 µL of diluted crude extract was added to 1.58 mL of deionised water and 100 µL of Folin-Ciocalteau reagent (FCR) in a 15-mL test tube wrapped in aluminium foil. After 10 min, 300 µL of (20%) sodium carbonate (Na_2CO_3) was added into the test tube. The mouth of the test tube was then covered with parafilm and aluminium foil and the test tube was vortexed for about 10 to 15 s. The mixture was then incubated for 2 h at room temperature in a dark environment for colour development. The absorbance was measured at 765 nm against the blank reagent using a UV-VIS spectrophotometer. The measurements were done in triplicate for more accurate results. Gallic acid was used for calibration of a standard curve with different concentrations as shown in Figure

1 below and the results were expressed as mg/L Gallic Acid Equivalent (GAE) (Tachakittirungrod et al., 2007).

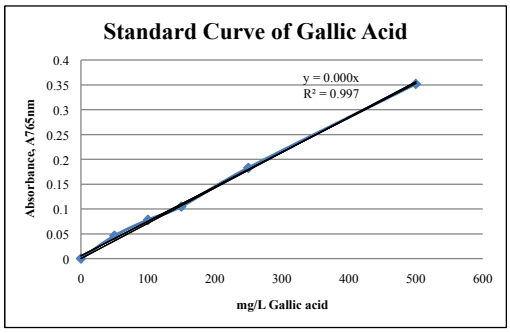

Figure 1. Standard curve for Folin-Ciocalteau test

Experimental Design

The process conditions for the total phenolic content were optimised using the Face-Centred Central Composite Design (FCCCD) under Response Surface Methodology (RSM) using the software, Design-Expert Version 8.0.8 (Stat-Ease Inc., Minneapolis, USA). Three factors were investigated in this study, namely X1 (temperature), X2 (time) and X3 (solid-solvent ratio). A total of 19 experiments were generated by FCCCD and all the experiments were replicated three times and the mean value was recorded.

The results for the phenolic content were analysed using regression analysis, where multiple equations were developed and followed by the analysis of these regression equations by statistical tools; ANOVA (analysis of variance) and p-test. The average yield of TPC was taken as the dependent variables of response (Y). A second order polynomial was developed to fit in the response as shown in Equation (1).

$$Y= \beta0+ \beta1A+ \beta2B + \beta3C + \beta11A2 + \beta22B2 + \beta33C2 + \beta12AB + \beta13AC + \beta23BC \quad (1)$$

where
A=Extraction Temperature (0C);
B=Extraction Time (h);
C=Solid to solvent ratio (g/mL);

RESULTS AND DISCUSSION

The experimental conditions allowed a fast, quantitative and maximum extraction of the antioxidant compounds and determination of the total phenolic content of the extract obtained. From the experiment results shown in Table 1, it is evident that the use of the statistical process condition optimisation approach and response surface methodology helped to locate the most significant conditions with minimum effort and time utilised. According to Toor and Savage (2006), phenolics from the extract are soluble in organic solvents such as hexane, light petroleum and methanol, and this can potentially account for some of the antioxidant activity of the extract. Therefore, as expected, different optimum operating conditions were found to yield maximum results for the assay (highlighted in Table 1).

Design of Experiment and Statistical Analysis

The polynomial regression model relating TPC (Y) with the independent variables, temperature (A), time (B) and solid-solvent ratio (C) is shown in Equation (2).

$$Y\ (mg/L\ GAE) = +1163.44+190.40*A+72.75*B+164.01*C+164.07*A*B+ 50.85\ *A*C-51.56*B*C+72.92*A2-25.72*B2-207.86*C2 \quad (2)$$

where,
A=Temperature (0C)
B=Time (h)
C=Solid to Solvent Ratio (g/mL)

The average OD of triplicate values obtained for each run was calculated as the observed TPC and summarised in Table 1 and the combination of the variables, observed and predicted are presented.

Table 1
Values of Observed and Predicted TPC Response

Run Order	Temperature (°C)	Time (h)	Solid to Solvent Ratio (g/mL)	TPC mg/L GAE	
				Observed	Predicted
1	90	3	1.20	616.57	738.9659
2	120	3	1.20	798.5	689.9425
3	90	5	1.20	780.87	659.4643
4	120	5	1.20	1135.71	1266.71
5	90	3	1.40	1218.82	1068.422
6	120	3	1.40	1120.78	1222.788
7	90	5	1.40	693.512	782.6717
8	120	5	1.40	1735.1	1593.306
9	90	4	1.30	985.71	1045.958
10	120	4	1.30	1409.42	1426.763
11	105	3	1.30	1030.42	1064.971
12	105	5	1.30	1167.44	1210.48
13	105	4	1.20	815	791.5674
14	105	4	1.40	1018.57	1119.594
15	105	4	1.30	1230	1163.442
16	105	4	1.30	1098.12	1163.442
17	105	4	1.30	1282.85	1163.442
18	105	4	1.30	1178.57	1163.442
19	105	4	1.30	1182.85	1163.442

The matching quality of the data obtained by the model proposed in Equation (1) was evaluated considering the correlation coefficient, R^2, between the experimental and modelled data. In this study, the determination coefficient (R^2=0.8734) indicated a higher correlation, 87.34%, between the observed values and the predicted values. This indicated the degree of precision with which the Total Phenolic Content was related to the three independent variables i.e. temperature, time and solid-solvent ratio.

The corresponding analysis of variance (ANOVA) is presented in Table 2. The ANOVA of the quadratic model demonstrated the significance of the model, as shown by Fisher's F-test with F-Value of 6.90 and relatively low probability (P_{model}>F)=0.0041. The P-value implied that this model was significant. It was identified that the linear term, B, was not significant. In this case A, C, AB, C^2 were significant model terms. Thus, temperature and amount of solvent were critical factors for phenol extraction of the samples.

Table 2
Analysis of Variance (ANOVA) of Response Surface Quadratic Model for TPC

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob>F	
Model	110774	9	123419.4	6.897574	0.0041	Significant
A-Temperature	362532.3	1	362532.3	20.26095	0.0015	
B-Time	52931.74	1	52931.74	2.958212	0.1196	
C-Solid-Solvent ratio	269003.3	1	269003.3	15.03387	0.0037	
AB	215344.5	1	215344.5	12.03502	0.0071	
AC	20683.54	1	20683.54	1.155947	0.3101	
BC	21269.33	1	21269.33	1.188685	0.3039	
A^2	14528.68	1	14528.68	0.811969	0.3910	
B^2	1806.951	1	1806.951	0.100986	0.7579	
C^2	118056.2	1	118056.2	6.597844	0.0303	

Analysis Using Response Surface Methodology (RSM)

The primary mode of action of various phenolic compounds is their radical scavenging activity; this assay was employed to measure that activity. Phenolic compounds are considered to be the major contributors of antioxidant activity and are determined according to the chemical structure that they possess. Antioxidant capacity generally increases when total phenolic content increases.

Figure 2 depicts that when temperature was kept constant, the TPC value varied considerably with the solvent ratio. Under certain conditions a maximum contour (1200 mg/L GAE) could be determined, meaning that the slightest change in the

amount of solvent used or time would not increase the TPC yield. These figures showed that the highest TPC was obtained at a time period of 4.33 h and a solid-solvent ratio of 1:33 g/mL while the temperature was set constant at 105°C.

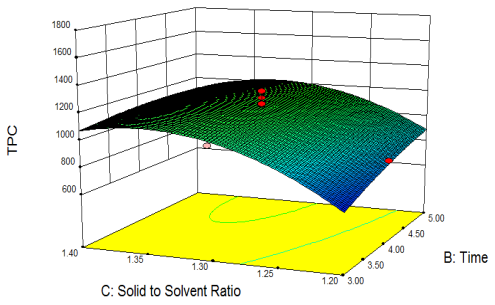

Figure 2. 3D surface plot showing the effect of Time (h) and Solid-Solvent Ratio (g/mL) to yield highest TPC value

of phenolic content, which leached into the water. Previous studies done by Lin and Tang (2007) quantified total phenolic content in powder form and lyophilised form; however, a liquid sample was used in our study.

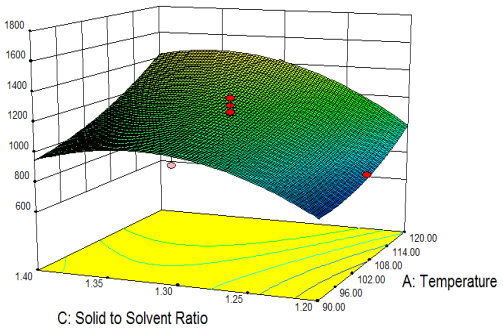

Figure 3. 3D surface plot showing the effect of Temperature (°C) and Solid to Solvent Ratio (g/mL) to yield highest TPC value

The surface plot in Figure 3 indicates that temperature was an important parameter for the TPC. However, as per a study done by Gertenbach (2001), while higher solvent temperatures typically increase mass transfer rates during extraction, in the case of polyphenols, elevated temperatures trigger competing processes such as decomposition and epimerisation (Gertenbach, 2001; Vuong et al., 2010). Temperature control during the extraction and isolation process for *Sekaki* papaya needs to be strictly controlled to minimise the loss of polyphenols as suggested by the regression model as well. According to Choo et al. (2014), the phenolic compounds in bitter gourd were sensitive to heat and the heat treatment caused a significant loss

While no previous studies regarding the optimisation of extraction conditions under heat treatment for phenolics from papaya (*Carica papaya*) plant peel were found, studies on other plant materials were found and they reported similar findings. The presence of phenolics as well as the antioxidant activity of papaya peel is highly dependent upon its maturity as stated by Prior et al. (2005). Rivera-Pastrana et al. (2010) identified ferulic acid, caffeic acid and rutin as the phenols present in the exocarp of *Maradol* papaya. Ranges of content of ferulic acid [1.33-1.62 g kg(-1) dry weight], caffeic acid [0.46-0.68 g kg(-1) dw] and rutin [0.10-0.16 g kg(-1) dw] were quantified and the presence of the phenols was found to be highly dependent

on storage temperature and ripening stage. *Sekaki* papaya peel used for this study was obtained at its maturity stage and stored at room temperature to maintain the phenolic profile of the exocarp.

The phenolic content of the heat-treated papaya peel was comparable to the study done by Lim et al. (2007) on dragonfruit (21±6 mg/100g), papaya (28±6 mg/100g), guava (138±31 mg/100g), starfruit (131±54 mg/100g) and orange (75±10 mg/100g). According to a study done by Normala and Suhaimi (2011), total phenolic compound content can be significantly influenced by the solvent and different vegetative parts. Nepote et al. (2005) reported that extraction time had a significant impact on extractability of phenolic compounds present in peanut skin, while Aludatt et al. (2011) found that with longer extraction time, both the overall yield of phenolic compounds and the antioxidant activity of the extracts from olive seeds improved. Xiaowei et al. (2011) did a polyphenolic extraction test on mango and found similar results in terms of temperature and solubility time. Also, according to Toor and Savage (2006), some phenolics are soluble in the nonpolar hexane layer of the extract and can contribute to antioxidant activity.

CONCLUSION

The distribution of phenolics was examined in this study. It gave an assessment of the diverse compounds that may be present in the *Sekaki* papaya (*Carica papaya*) peel extract. Total phenolic content was analysed using Folin-Ciocalteau's reagent and its concentration was expressed as Gallic Acid Equivalents (GAE). The results indicated that the highest amount was equal to 1735.1 mg/L GAE, which also contributed to antioxidant activity. The phenolics could be later identified. They can be utilised as potent antioxidants.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Biotechnology Engineering, International Islamic University (IIUM) for supporting and providing the laboratory facilities for this study.

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Pertanika J. Trop. Agric. Sci. 40 (1): 99 - 110 (2017)

PERTANIKA JOURNALS

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Detection of *Leptospira* spp. in Selected National Service Training Centres and Paddy Fields of Sarawak, Malaysia using Polymerase Chain Reaction Technique

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ABSTRACT

Leptospirosis is a zoonotic disease which is caused by spirochetes from the genus *Leptospira*. It can be transmitted to humans through direct contact with infected animals or indirect contact with an environment contaminated by the urine of infected animals. The objective of this study was to study the status of leptospirosis in two selected National Service Training Centres (NSTCs) and two paddy fields of Sarawak. A total of 31 captured rats, 210 soil samples and 210 water samples were collected from these study sites. All the samples were inoculated into a modified semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) broth with 5-fluorouracil. For soil and water samples, a specific polymerase chain reaction (PCR) was conducted after a one-month incubation period. Kidney and liver samples from rats were incubated and PCR was carried out monthly during the three-month incubation period. Representative PCR-positive samples which targetted LipL32, 16S rRNA and rrs genes at 423 bp, 331 bp and 240 bp in pathogenic, intermediate and saprophytic *Leptospira*, respectively, were further sequenced. From the PCR analysis, intermediate *Leptospira* was detected in one (3.2%) rat species, *Rattus exulans*, that was captured in a paddy field. A total of six (2.9%) pathogenic *Leptospira*, one (0.5%) each from intermediate and saprophytic *Leptospira*, were present in soil samples from the study sites. Six (2.9%) water samples were contaminated by pathogenic *Leptospira*, four (1.9%) by intermediate *Leptospira* and seven (3.3%) by saprophytic *Leptospira*. All the contaminated environmental samples

ARTICLE INFO

Article history:
Received: 23 February 2016
Accepted: 20 September 2016

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ISSN: 1511-3701

were collected from NSTCs except for two soil samples and four water samples from paddy fields that were infected by pathogenic *Leptospira*. Results from DNA sequencing analysis indicated that the dominant pathogenic, intermediate and saprophytic *Leptospira* species circulating in these study sites were *Leptospira noguchii*, *Leptospira wolffii* serovar Khorat and *Leptospira meyeri*, respectively. Although the prevalence of *Leptospira* is low, there is still a risk of infection to those who are involved in outdoor activities at training centres and paddy fields. Control and preventive measures are, therefore, important in tackling preventable diseases related to pathogenic *Leptospira*.

Keywords: Leptospira, rat, soil, water, National Service Training Centres, paddy fields

INTRODUCTION

Leptospirosis is a worldwide zoonotic disease caused by pathogenic *Leptospira*. This aerobic bacterium is highly motile and is observed as helical shaped under a dark-field microscope (Flores-Encarnación et al., 2014). More than 1.7 million cases of severe leptospirosis are reported worldwide annually, with an approximately 10% mortality rate (Chen et al., 2015). This disease is usually transmitted through direct contact with the urine of infected animals or indirect contact with an environment contaminated with the bacteria (Sumanta et al., 2015).

Leptospirosis has been associated with occupation and is reported to commonly occur among rice farmers, sewer workers, fishermen or military personnel (Alavi et al., 2014). However, recreational activities such as water sport have been recorded as a risk factor as well (Narita et al., 2005). In Malaysia, leptospirosis outbreaks originating from National Service Training Centres (NSTCs) and paddy fields have been documented. The National Service Training Programme was introduced by the Malaysian government with the aim of enhancing patriotism among youths and encouraging national integration and unity. In January 2010, a contaminated pond in the Junaco Park National Service Training Camp, Sibu, Sarawak was closed due to the presence of *Leptospira* (Bernama, 2010). In April 2012, a trainee died due to leptospirosis in the Terkok National Service Training Centre in Sungai Siput, Perak (Bernama, 2012). An outbreak of leptospirosis during high-risk fisheries activities in a neglected swamp was also documented in Pauh, Perlis in the same year. Further investigation showed that 87.5% of water samples collected from the swamp was contaminated with pathogenic *Leptospira*. It was discovered that this neglected swamp had previously been a paddy field that could no longer be cultivated (Baharudin et al., 2012).

To the best of our knowledge, no detection study on *Leptospira* in NSTCs and paddy fields of Sarawak have been previously published. Due to the potential risk of *Leptospira* infection in these localities, the objectives of this study were (i) to screen for *Leptospira* spp. in selected NSTCs and paddy fields of Sarawak, and

(ii) to identify the dominant *Leptospira* spp. circulating in these localities.

MATERIALS AND METHODS

Sampling

Sampling was conducted from May 2014 to January 2015. A total of 31 captured rats, 210 soil samples and 210 water samples were collected from two NSTCs and two paddy fields in Kuching and Miri, Sarawak. Sampling was conducted at NSTCs approved by the National Service Training Department and camp managers of the respective NSTCs. The two paddy fields chosen were situated in villages with most of the population engaged in agricultural activities, namely paddy field cultivation.

In the NSTCs, cage traps were placed in open fields, trainees' hostels, canteens, store rooms, jungle areas and along the lakes in the camps with salted fish and banana as bait. Soil samples were collected from the jungle areas and open field whereas water samples were collected from lakes, water streams and drain effluent from the canteens in the camps. Cage traps were set at random in the paddy fields and barns. Soil samples were collected from the paddy fields whereas water samples were collected from the paddy fields, streams or rivers nearby.

Collection of Rat Samples

Rectangular cage traps were used to capture the rats. All the trapped rats under the family Muridae were identified based on the morphological characteristics recorded by Payne and Francis (2007). They were then euthanised humanely and dissected aseptically. Kidney and liver samples were inoculated into a modified semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) broth with 100 µg/mL 5-fluorouracil. The enriched samples were incubated aerobically at room temperature for three months. PCR was conducted every month to detect the presence of *Leptospira* spp. (Houemenou et al., 2013).

Collection of Soil and Water Samples

Soil and water samples were collected using 50 mL sterile falcon tubes. About 20 g of soil samples were mixed vigorously with sterile distilled water before they were allowed to settle for 15 min. They were passed through sterile a 0.2-µm pore size membrane filter (Sartorious AG, Germany). Approximately 50 mL of water samples were filtered using the same type of membrane filter. Next, about 1 mL of the samples was inoculated into a modified semisolid EMJH broth with 100 µg/mL 5-fluorouracil. The enriched samples were incubated aerobically at room temperature for one month prior to PCR amplification (Ridzlan et al., 2010).

Detection of *Leptospira* spp.

The Wizard™ Genomic DNA Purification Kit (Promega Corporation, USA) was used to extract DNA before conducting specific PCR amplification. Different primer pairs were used to target LipL32, 16S rRNA and rrs genes in pathogenic, intermediate and saprophytic *Leptospira*, respectively (Pui

et al., 2015). The reaction mixtures (25 µL) included 5 µL of 5x PCR buffer, 0.2 mM of dNTP mix, 0.4 µM of each primer pair, 2.0 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Promega Corporation, USA) and 5 µL of DNA template. The cycling conditions were initial denaturation at 95°C for 2 min; 35 cycles each of denaturation at 95°C for 1 min, primer annealing at 55°C for 30 s and extension at 72°C for 1 min; further extension at 72°C for 5 min and indefinite holding period at 4°C. Electrophoresis was run on 2% agarose gel in 1x TBE buffer at 90V for 75 min.

Identification of *Leptospira* spp.

The amplicons from representative positive samples (one rat, five soil and nine water samples) were subjected to sequencing by commercial facility (First BASE Laboratories Sdn Bhd, Malaysia). The sequencing data were compared with GenBank database using nucleotide BLAST from the National Centre for Biotechnology Information (NCBI).

RESULTS

The prevalence of *Leptospira* spp. in rat, soil and water samples as detected by PCR is summarised in Table 1. PCR detection indicated that only one (3.2%) rat sample captured from a paddy field was infected by intermediate *Leptospira*. Among the soil samples, six (2.9%) were contaminated by pathogenic *Leptospira*, one (0.5%) by intermediate *Leptospira* and one (0.5%) by saprophytic *Leptospira*. All of these contaminated soil samples were collected from the NSTCs except for two from the paddy fields that were infected by pathogenic *Leptospira*. A total of six (2.9%) pathogenic *Leptospira*, four (1.9%) intermediate *Leptospira* and seven (3.3%) saprophytic *Leptospira* were recovered from the water samples. They were all present in the NSTCs except for four from the paddy

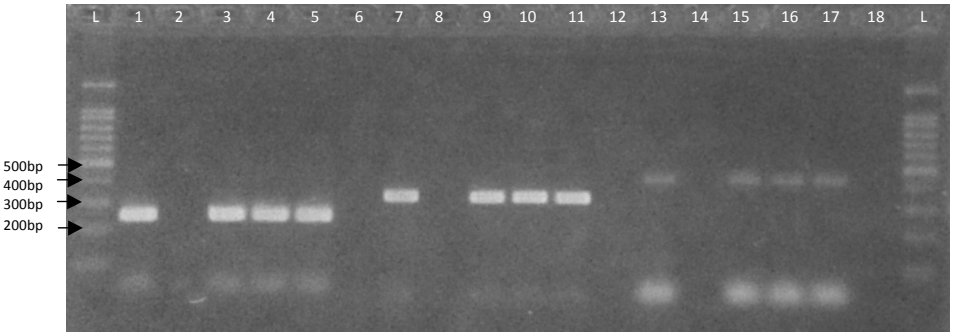

Figure 1. Representative gel image from PCR amplification. Lanes L show the 100 bp DNA ladder. *Leptospira* meyeri strain Sant-1 (lane 1), Leptospira wolffii serovar Khorat strain Khorat-H2 (lane 7) and *Leptospira noguchii* strain LT796 (lane 13) are positive controls. Lanes 2, 8 and 14 are negative controls. Lanes 3 to 5 show positive PCR amplicons specific to saprophytic *Leptospira* at 240 bp. Lanes 9 to 11 show the positive PCR amplicons specific to intermediate *Leptospira* at 331 bp. Lanes 15 to 17 show the positive PCR amplicons specific to pathogenic *Leptospira* at 423 bp. Lanes 6, 12 and 18 are negative PCR amplicons.

Table 1
Prevalence of Leptospira Spp. in Rat, Soil and Water Samples Collected from NSTCS and Paddy Fields in Sarawak

Sample	Study sites	Pathogenic		Intermediate		Saprophytic	
		No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]
Rat	National Service Training Centres	0/15	0.0	0/15	0.0	0/15	0.0
	Paddy fields	0/16	0.0	1/16	6.3	0/16	0.0
	Total	0/31	0.0	1/31	3.2	0/31	0.0
Soil	National Service Training Centres	4/150	2.7	1/150	0.7	1/150	0.7
	Paddy fields	2/60	3.3	0/60	0.0	0/60	0.0
	Total	6/210	2.9	1/210	0.5	1/210	0.5
Water	National Service Training Centres	2/150	1.3	4/150	2.7	7/150	4.7
	Paddy fields	4/60	6.7	0/60	0.0	0/60	0.0
	Total	6/210	2.9	4/210	1.9	7/210	3.3

Note: anumber of positive samples/total number of samples collected; b, prevalence (in %) of positive samples among the samples collected

fields that were contaminated by pathogenic *Leptospira*. The representative gel image is shown in Figure 1.

A total of 15 rats comprising four different rat species were captured in two NSTCs in Sarawak, where *Sundamys muelleri* and *Niviventer cremoriventer* dominated at 46.7% and 33.3%, respectively. Ten rats were male and the remaining five were female. Adult rats made up 93.3% of the captured population. Based on a PCR analysis, none of the rats were infected by *Leptospira* spp. However, it was found that the soil samples in the two NSTCs were contaminated by pathogenic, intermediate and saprophytic *Leptospira* at 2.7%, 0.7% and 0.7%, respectively. Out of the 150 water samples collected, 1.3%, 2.7% and 4.7% of them were positive for the gene specific to pathogenic, intermediate and saprophytic *Leptospira*.

In the two paddy fields of Sarawak, a total of 16 individual rats comprising eight species were captured. *Rattus rattus* and *Rattus exulans* dominated by 50.0% and 18.8%, respectively. Out of the 16 rats, 12 were male and 11 were adult rats. None of the rats was infected by pathogenic and saprophytic *Leptospira*. However, one *Rattus exulans* was positive to intermediate *Leptospira*. This infected rat was an adult male rat. Pathogenic *Leptospira* was found to have contaminated 3.3% of the soil samples. The water samples from paddy fields were contaminated by pathogenic *Leptospira* at 6.7%.

From the PCR analysis, the DNA from 12 pathogenic strains, six intermediate strains and eight saprophytic strains of *Leptospira* were amplified. At least one positive sample from each study site was sent for DNA sequencing, subject to the availability of sample sources. The representative positive samples included seven pathogenic *Leptospira*, five intermediate *Leptospira* and three saprophytic *Leptospira*. Nucleotide BLAST results (Table 2) indicated that *Leptospira noguchii* was the dominant pathogenic *Leptospira* present in these study sites. *Leptospira wolffii* serovar Khorat was the dominant intermediate *Leptospira* whereas *Leptospira meyeri* was the dominant saprophytic *Leptospira*. The results showed similarity ranging from 78% to 99%.

DISCUSSION

In this study, *Sundamys muelleri* and *Niviventer cremoriventer* dominated the rat population in the selected NSTCs of Sarawak. This was different from the rat species found in West Malaysia, where 88.1% out of the total 268 rats captured in NSTCs in Terengganu, Kelantan, Malacca and Selangor were *Rattus tiomanicus* (Mohamed-Hassan et al., 2012). In a study where 90 rodents were trapped in Cotonou, West Africa, *Rattus rattus* was the rat species with the greatest distribution as it spread inland and was present in small towns and villages (Houmenou et al., 2013). In reality, different species of rodents tend to be selective of their own habitat. Nonetheless, the rodent species diversity may change as rodent habitats are destroyed due to agricultural intensification, urbanisation or deforestation (Paramasvaran et al., 2013).

From the present results, none of the rats trapped from the two NSTCs in Sarawak were positive for *Leptospira* spp. This contradicts the finding by Mohamed-Hassan et al. (2010). In their study on 168 rats captured in NSTCs in Kelantan and Terengganu, 17.9% showed positive sera against leptospiral antibodies. For the soil and water samples from the two NSTCs, low prevalence of *Leptospira* spp. was recorded. Soil samples in NSTCs showed contamination at 2.7%, 0.7% and 0.7% by pathogenic, intermediate and saprophytic *Leptospira*, respectively while 1.3%, 2.7% and 4.7% of the water samples were contaminated by pathogenic, intermediate and saprophytic *Leptospira*, respectively. These results supported the findings of Ridzlan et al. (2010), who reported the presence of pathogenic *Leptospira* in three out of 145 (2.1%) environmental soil and water samples collected from NSTCs in Kelantan and Terengganu. Previously, two series of screening programmes for *Leptospira* were carried out in 2010 at all NSTCs in northern and eastern regions of Peninsular Malaysia by a public health laboratory in Malaysia. During the first screening programme, 18% (21/115) of water samples from 13 NSTCs were positive for pathogenic *Leptospira*. A total of 13% (16/123) of water samples from nine NSTCs were detected to contain pathogenic *Leptospira* in the second screening programme (Hasanatunnur

Table 2
Nucleotide Sequence Similarity of Leptospira spp. Isolates Targetting lipL32 and 16S Ribosomal RNA Genes Based on Leptospira spp. Reference Strains from GenBank Entries

Sample ID	Study site	Sample	Accession number	Descriptio	Query length (bp)	Query coverage (%)	Maximum identity (%)
Pathogenic *Leptospira* targetting outer membrane protein, lipL32 gene							
CFP11	NSTC	Water	KC800989.1	*Leptospira interrogans* serovar Autumnalis strain RTCC 2802	434	99	89
CFP12	NSTC	Water	KF297610.1	*Leptospira weilii* clone lipL32-122069	446	81	92
CFP16	NSTC	Soil	AY461920.1	*Leptospira noguchii* strain LT796	438	97	90
CFP22	PF	Water	AY461920.1	*Leptospira noguchii* strain LT796	447	98	88
CFP26	PF	Water	AY461920.1	*Leptospira noguchii* strain LT796	427	99	79
CFP23	PF	Soil	AY461920.1	*Leptospira noguchii* strain LT796	445	98	88
CFP27	PF	Soil	AY461920.1	*Leptospira noguchii* strain LT796	439	98	78
Intermediate *Leptospira* targetting 16S ribosomal RNA geneles collected							
CFG29	PF	Rat	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H2	342	97	95
CFG9	NSTC	Water	AB758753.1	L*eptospira* sp. MS341	338	97	98
CFG11	NSTC	Water	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H2	339	98	98
CFG21	NSTC	Water	KP031573.1	*Leptospira* sp. Neco007	361	93	97
CFG12	NSTC	Soil	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H2	340	97	99
Saprophytic *Leptospira* targetting 16S ribosomal RNA gene							
CFS3	NSTC	Water	NR_103924.1	*Leptospira biflexa* serovar Patoc strain 'Patoc 1 (Ames)' strain Patoc 1 (Ames)	256	97	98
CFS5	NSTC	Water	JQ988852.1	*Leptospira meyeri* strain Semaranga_DB49	259	98	97
CFS11	NSTC	Soil	JQ988852.1	*Leptospira meyeri* strain Semaranga_DB49	253	98	99

et al., 2011). Elsewhere, 0.5% (1/220) of stripped field mice, *Apodemus agrarius*, was detected with *Leptospira* in two Korean-operated military training sites (O'Guinn et al., 2010). In 2014, American Marines were reported to be sick due to l*eptospirosis* after attending jungle warfare training at Camp Gonsalves, Japan (Stewart, 2010).

From these results, it can be concluded that although the prevalence of *Leptospira* in these NSTCs is low, the risk is still present. Prior to 2015, there was an intake of more than 100,000 trainees annually at the 93 NSTCs in Malaysia. These trainees were exposed to physical activities like canoeing, abseiling and obstacle courses, which are conducted near the ponds in NSTCs. These activities would have put them at risk for leptospiral infection from urine-contaminated soil and water (Mohamed-Hassan et al., 2010). The National Service programme was suspended in 2015 for a year by the Malaysian government but according to the latest press release (The Star Online, 2016). Defence Minister Datuk Seri Hishammuddin Tun Hussein relaunched a new and improved National Service Training Programme (PLKN2.0) on 24 April, 2016. Therefore, the camp management of NSTCs should always take preventive measures to ensure the safety of the camp environment for any future activities in the camp.

Rodents breed rapidly in paddy fields and this may lead to uncontrolled population growth (Fadzlina et al., 2013). Paddy fields are favourable places for their survival because they can obtain sufficient food in this habitat (Vedhagiri et al., 2010). Out of the 16 rats captured from the paddy fields, only one was infected by intermediate *Leptospira*. Nonetheless, pathogenic *Leptospira* was found in two soil samples and four of the water samples collected. This highlighted the presence of *Leptospira* spp. in the selected paddy fields of Sarawak. Yasouri et al. (2013) reported the presence of 47.0% (54/115) saprophytic *Leptospira* and 33.0% (38/115) pathogenic *Leptospira* in 36 soil samples, 67 water samples and 12 faeces samples collected from a paddy field in Iran. *Leptospira* was also detected in a paddy field in Nakhornratchasrima Province in Thailand (Tangkanakul, 2000).

Leptospirosis outbreaks related to paddy farming is known to be common. Until 1960, more than 200 deaths due to leptospirosis were reported annually in Japan; most of the victims were farmers working in paddy fields (Saito et al., 2013). Consequently, occupation has been perceived as a significant risk factor of *Leptospira* spp. Most leptospirosis infection related to occupation has involved rice field workers, fish farmers, veterinarians, sewer workers and soldiers (Tansuphasiri et al., 2006). In 2011, a large leptospirosis outbreak involving traditional and full-time paddy farmers was reported in Anuradhapura district of Sri Lanka. Paddy farming is the main source of income for these farmers.

In Malaysia, a total of 7,806 cases and 92 deaths resulted from leptospirosis in 2014, while 4,457 cases with 71 deaths were reported in 2013. The distribution of leptospirosis cases by occupation in

Malaysia indicated that farmers contributed to 6% of the total cases in 2013 and 7% in 2014 (Ministry of Health Malaysia, 2015). It can be concluded that the presence of *Leptospira* spp. in the two paddy fields of Sarawak put the farmers at risk of infection to leptospirosis. Hence, the villagers must take preventive measures such as wearing protective clothing and rubber boots to minimise contact with a contaminated environment as suggested by Koay et al. (2004).

CONCLUSION

This study gave an insight into the leptospirosis status in selected NSTCs and paddy fields of Sarawak, Malaysia. Since there is a lack of information on the real status of leptospirosis in these localities, this study serves as a source of important surveillance data for the public health sector in Malaysia, especially Sarawak. Knowing that there is a risk of infection to humans, appropriate preventive measures must be taken by the authorities, the public health sector and the public. Some of the effective preventive measures include usage of protective boots by paddy field farmers and prohibition of using or drinking water from ponds in National Service training centres. Future studies on the risk of exposure to *Leptospira* spp. in other agricultural sites such as land farms and oil palm estates in relation to wet or dry seasons can be conducted for more comprehensive surveillance data.

ACKNOWLEDGEMENTS

This study was funded by the Ministry of Higher Education Malaysia under the Fundamental Research Grant Scheme FRGS/SG03(01)/970/2013(11) and a UNIMAS internal grant under the PhD Student Fund F07(DPP21)/1191/2014(21). Special acknowledgement goes to the National Service Training Department and the camp managers of selected NSTCs for approval to collect samples from the NSTCs in Sarawak. Appreciation also goes to the paddy farmers for allowing us to collect samples from their fields. The authors appreciate the assistance given by lab assistants and undergraduates of the Department of Molecular Biology in Faculty of Resource Science and Technology, Universiti Malaysia Sarawak.

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Pertanika J. Trop. Agric. Sci. 40 (1): 111 - 118 (2017)

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TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Xanthones from *Calophyllum inophyllum*

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ABSTRACT

Repeated separation and purification of fractions from the crude chloroform extract of *Calophyllum inophyllum* via column chromatography afforded five known xanthone caloxanthones A, B, C, macluraxanthone and pyranojacareubin. Structural elucidations and determination of the isolated compounds were supported by spectral analyses obtained by subjecting the compounds to various spectroscopic techniques. The chloroform extract, when tested against RAW264.7 cells for anti-inflammatory potentials, exhibited the most promising activity with an IC_{50} value of 14.81±0.04 µg/mL compared to moderate activities shown by the ethyl acetate and n-hexane extracts. Antimicrobial tests showed that the n-hexane and chloroform extracts acted moderately against *Staphylococcus epidermidis* S273 and *Bacillus subtilis* B145.

Keywords: anti-inflammatory, antimicrobial, *Calophyllum inophyllum*, xanthones

INTRODUCTION

Calophyllum is one of the 47 genera of the Clusiaceae (Guttiferae) family. This family comprises a stunning 1350 different species across the world (Daud et al., 2014). Habitat distribution of plants from this family includes tropical areas such as the Indo Pacific region. *Calophyllum* is known locally as 'bintangor' or 'tamanu' (Mah et al. 2012). Secondary metabolites from this genus are main contributors towards various biological activities reported from previous work (Mah et al. 2015). *Calophyllum* species have been reported to be rich in secondary metabolites such as xanthones, coumarins, terpenoids and chromene acids (Dharmaratne et al.,

ARTICLE INFO

Article history:
Received: 06 May 2016
Accepted: 10 November 2016

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ISSN: 1511-3701

2009). This genus has been vastly utilised as folk or traditional medicine to treat ailments. It has also been reported that this genus possesses compounds with antifungal, anti-microbial, anti-cancer and anti-HIV properties (Alkhamaiseh et al., 2011). A greenish oil extracted from the fruit kernel of *Calophyllum inophyllum* was reported to relieve joint pain, bruises and even rheumatism (Zakaria et al., 2014). Recent studies on the leaf extract of this species also exposed its anti-inflammatory potential, suggesting that it can be used in applications for inflammatory-related illnesses (Tsai et al., 2012). This paper focusses on the isolation and structural elucidation of xanthones. In addition, cell viability and toxicity of LPS stimulated RAW264.7 cells and anti-microbial activities of crude extracts will also be discussed.

MATERIALS AND METHODS

Plant Material

The stem bark sample of *Calophyllum inophyllum* was acquired from the campus grounds of Universiti Putra Malaysia (UPM), Serdang, Selangor. Sample identification was carried out by Associate Professor Dr Rusea Go, a biologist from the Biology Department, UPM. A voucher specimen (RG 5016) was deposited in the Herbarium, Biology Department, UPM.

Analysis Instrumentation

NMR spectra were generated from a JEOL FT-NMR 500 MHz spectrophotometer with tetramethylsilane (TMS) as the internal standard. EIMS data were recorded on a Shimadzu GC-MS model QP2010 Plus spectrophotometer whereas ultraviolet spectra were recorded in EtOH on a Shimadzu UV-160A, UV Visible Recording Spectrophotometer. Infrared spectra were accomplished using a universal attenuated total reflection (UATR) technique on a Perkin-Elmer 100 Series FT-IR spectrometer. A Leica Galen III microscope equipped with a Testo 720 temperature recorder was used to determine the melting points of compounds isolated.

Extraction and Isolation

A mass of 3.8 kg of *Calophyllum inophyllum* stem bark was primarily air dried and subsequently ground into fine powder. Successive extractions of the powder in a selection of organic solvents with increasing polarity, namely, n-hexane, chloroform, ethyl acetate and methanol were achieved. The filtered liquid extracts underwent the drying process under reduced pressure by rotary evaporator to afford 106.0 g, 80.3 g, 63.5 g and 67.4 g of n-hexane, chloroform, ethyl acetate and methanol dry extracts. A designated portion of the dried chloroform extract was subjected to vacuum column chromatography over silica gel 60 PF_{254} for isolation and separation process. Thirty different fractions were acquired from the elution of the sample-packed silica gel with a stepwise gradient of n-hexane-chloroform and chloroform-ethyl acetate mixtures. A series of meticulous steps in the purification process of fractions 3-8 afforded yellow needles of pyranojacareubin

(5). Caloxanthones B (2) and C (3) were also isolated from further separation of fractions 10-16 in the appearance of yellow crystals. A similar chromatographic technique was used to separate fractions 20-23 and fractions 26-28, respectively, which resulted in the isolation of two additional xanthones, namely caloxanthone A (1) and macluraxanthone (4) after the recrystallisation process.

Cell Viability and Cytotoxicity Test

The seeding process of raw cells ($2x10^6$ cells/mL) was carried out in a 96-well plate and subsequently subjected to a 24-hour incubation stage. These cells were then treated and induced with 10µg/mL lipopolysaccharide (LPS) in the presence of the crude n-hexane extract and made up to a final volume of 100µL and further incubated for 24 hours. Then, Griess reagent (50µL) was added to react with 50µL of cell-free culture supernatant, after which, incubation was carried out for 10 minutes at room temperature. These cells were introduced into the microplate reader and readings at OD=550nm were taken. A fresh culture medium was used as a blank. The results were expressed as mean ±SEM. Repetition of the following steps was carried out on the remaining crude extracts and isolated compounds.

Anti-Microbial Test

This test was carried out by placing a 6-mm diameter of paper disc containing antibiotics onto a plate in which microbes were growing. The microbe culture was standardised to 0.5 McFarland standard, which was approximately 10^8 cells. Streptomycin standard was used for bacteria, while nystatin standard was used for yeast. The plates were inverted and incubated at 30°C to 37°C for 18 to 24 h or until sufficient growth had occurred. Each plate was examined after incubation. The diameter of the zone of complete inhibition was measured, including the diameter of the disc. Zones were measured to the nearest whole millimetre (mm).

RESULTS AND DISCUSSIONS

Column chromatographic separation and purification of the fractions of the crude chloroform extracts gave rise to a total of five known xanthones. They were caloxanthone A (1), caloxanthone B (2), caloxanthone C (3), macluraxanthone (4) and pyranojacareubin (5). Their structures and spectral data are as shown below.

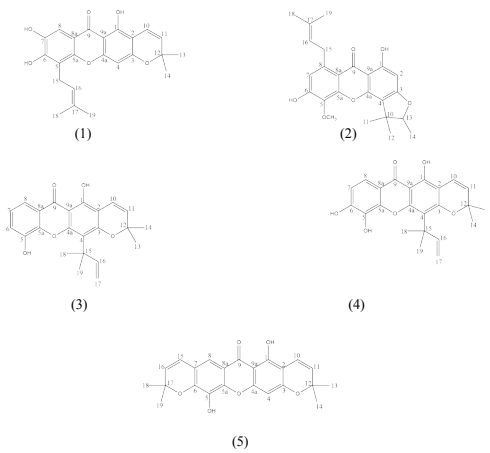

Figure 1. Structures of compounds 1-5

Caloxanthone A (1): Yellow needles; m.p. 237-240°C (Lit 238-240°C) (Iinuma et al., 1994); EIMS (m/z) : 394[M^+], $C_{23}H_{22}O_6$; UV (EtOH) λ_{max} : 388, 494nm; IR v_{max} cm^{-1} : 3393, 2941, 1611, 1457; ^{1}H NMR ($CDCl_3$, 500MHz) δ_H : 6.32(1H, s, H-4), 7.43(1H, *s*, H-8), 6.63(1H, *d, J*=10.3Hz, H-10), 5.69(1H, *d, J*=10.3Hz, H-11), 1.43(2x3H, *s*, H-13&H-14), 3.57(2H, *d, J*=8Hz, H-15), 5.27(1H, *t, J*=7.4Hz, H-16), 1.86(3H, *s*, H-18), 1.62(3H, *s*, H-19), 13.60(1H, *s*, 1-OH); ^{13}C NMR ($CDCl_3$, 125MHz) δ_C : 157.5(C-1), 104.1(C-2), 159.9(C-3), 94.5(C-4), 157.2(C-4a), 151.4(C-5a), 115.7(C-5), 142.8(C-6), 149.9(C-7), 105.5(C-8), 112.5(C-8a), 180.1(C-9), 102.7(C-9a), 115.0(C-10), 127.8(C-11), 78.0(C-12), 27.7(C-13&C-14), 22.2(C-15), 121.6(C-16), 131.7(C-17), 17.3(C-18), 25.1(C-19)

Caloxanthone B (2): Yellow needles; m.p. 157-158°C (Lit 160.5°C) (Iinuma et al., 1994); EIMS (m/z) : 410 [M^+], $C_{24}H_{26}O_6$; UV (EtOH) λ_{max} : 317, 247nm; IR v_{max} cm^{-1} : 3288, 2929, 1641; ^{1}H NMR ($CDCl_3$, 500MHz) δ_H : 6.21(1H, *s*, H-2), 6.79(1H, *s*, H-7), 1.58(3H, *s*, H-11), 1.29(3H, *s*, H-12), 4.51(1H, *q, J*=6.9Hz, H-13), 1.39(3H, *d, J*=6.9Hz, H-14), 3.98(2H, *d, J*=8Hz, H-15), 5.34(1H, *t, J*=8Hz, H-16), 1.73(3H, *s*, H-18), 1.70(3H, *s*, H-19), 13.75(1H, *s*, 1-OH), 3.98(3H, *s*, 5-OCH_3); ^{13}C NMR ($CDCl_3$, 125MHz) δ_C : 164.5(C-1), 94.1(C-2), 165.6(C-3), 112.2(C-4), 151.8(C-4a), 151.2(C-5a), 132.4(C-5), 153.6(C-6), 113.5(C-7), 142.0(C-8), 112.7(C-8a), 182.2(C-9), 104.0(C-9a), 43.7(C-10), 25.6(C-11), 21.7(C-12), 90.8(C-13), 14.2(C-14), 33.6(C-15), 122.4(C-16), 133.5(C-17), 26.0(C-18), 18.0(C-19), 61.9(5-OCH_3).

Caloxanthone C (3): Yellow needles; m.p. 212-213°C (Lit 217°C) (Iinuma et al., 1994); EIMS (m/z) : 378 [M^+], $C_{23}H_{22}O_5$; UV (EtOH) λ_{max} : 382, 290, 271, 240nm; IR v_{max} cm^{-1} : 3428, 2929, 1589, 1711; ^{1}H NMR ($CDCl_3$, 500MHz) δ_H : 7.34(1H, *d*, 8Hz, H-6), 7.24(1H, *t, J*=8Hz, H-7), 7.63(1H, *d, J*=8Hz, H-8), 6.68(1H, *d*, *J*=10.3Hz, H-10), 5.72(1H, *d, J*=10.3Hz, H-11), 1.48(2x3H,*s*, H-13&H-14), 6.48(1H, *dd, J*=10.3Hz, 17.2Hz, H-16), 4.85(1H, *d, J*=10.3Hz, H-17a), 5.00(1H, *d, J*=17.2Hz, H-17b), 1.71(2x3H, *s*, H-18&H-19), 13.67(1H, *s*, 1-OH), 8.79(1H,s, 5-OH); ^{13}C NMR ($CDCl_3$, 125MHz) δ_C : 156.4(C-1), 105.0(C-2), 159.3(C-3), 113.7(C-4), 155.1(C-4a), 145.1(C-5a), 146.4(C-5), 120.1(C-6), 124.1(C-7), 115.2(C-8), 120.9(C-8a), 181.6(C-9), 103.6(C-9a), 115.4(C-10), 127.8(C-11), 78.5(C-12), 27.2(C-13&C-14), 41.0(C-15), 151.8(C-16), 106.7(C-17), 29.2(C-18&C-19).

Macluraxanthone (4) Yellow needles; m.p. 170-172°C (Lit 170-172°C) (Iinuma et al., 1994); EIMS (m/z) : 394 [M^+], $C_{23}H_{22}O_6$; UV (EtOH) λ^{max} : 337, 270, 242nm; IR v_{max} cm^{-1} : 3348, 2965, 1589, 1434; ^{1}H NMR ($CDCl_3$, 500MHz) δ_H : 6.93(1H, *d, J*=8Hz, H-7), 7.67(1H, *d, J*=8Hz, H-8), 6.76(1H, *d, J*=9.2Hz, H-10), 5.60(1H, *d, J*=10.3Hz, H-11), 1.50(2x3H, *s*, H-13&H-14), 6.71(1H, *dd, J*=10.8Hz, 18.3Hz, H-16), 5.21(1H, *d, J*=18.3Hz, H-17a), 5.04(1H, *d, J*=10.3Hz, H-17b), 1.64(2x3H, *s*, H-18&H-19),

13.52(1H, *s*, 1-OH), 6.26(1H, *s*, 5-OH), 5.93(1H, *s*, 6-OH); ^{13}C NMR ($CDCl_3$, 125MHz) δ_C : 156.8(C-1), 105.7(C-2), 159.0(C-3), 113.1(C-4), 154.2(C-4a), 144.6(C-5a), 131.1(C-5), 149.1(C-6), 112.8(C-7), 117.6(C-8), 113.8(C-8a), 180.9(C-9), 103.1(C-9a), 116.2(C-10), 127.3(C-11), 78.3(C-12), 28.0(C-13&C-14), 41.5(C-15), 156.9(C-16), 103.4(C-17), 28.3(C-18&C-19).

Pyranojacareubin (5): Yellow needles; m.p. 259-260°C (Lit 259-261°C) (Harrison et al., 1993); EIMS (m/z) : 392 [M^+], $C_{23}H_{20}O_6$; UV (EtOH) λ_{max} : 347, 300, 291, 264nm; IR v_{max} cm^{-1} : 3627, 2924, 1612, 1458; ^{1}H NMR ($CDCl_3$, 500MHz) δ_H : 6.32(1H, *s*, H-4), 7.56(1H, *s*, H-8), 6.72(1H, *d, J*=9.1Hz, H-10), 5.58(1H, *d, J*=10.3Hz, H-11), 1.46(2x3H, *s*, H-13&H-14), 6.87(1H, *d, J*=10.3Hz, H-15), 5.74(1H, *d, J*=10.3Hz, H-16), 1.54(2x3H, *s*, H-18&H-19), 13.30(1H, *s*, 1-OH); ^{13}C NMR ($CDCl_3$, 125MHz) δ_C : 157.7(C-1), 104.6(C-2), 160.2(C-3), 94.9(C-4), 157.0(C-4a), 145.8(C-5a), 141.9(C-5), 146.5(C-6), 109.3(C-7), 108.8(C-8), 114.1(C-8a), 180.1(C-9), 103.3(C-9a), 115.7(C-10), 127.5(C-11), 78.2(C-12), 28.4(C-13&C-14), 115.53(C-15), 130.0(C-16), 79.4(C-17), 28.4(C-18&C-19).

The cell viability and cytotoxicity test of the plant crude extracts on the RAW264.7 cells found that the chloroform extract exhibited significant activity whereas the n-hexane and ethyl acetate extracts indicated moderate activity. This showed that the chloroform extract had anti-inflammatory potential. Moreover, the n-hexane and chloroform extract showed favourable activity against *Staphylococcus epidermidis S273* and *Bacillus Subtilis B145*.

Table 1
Cell Viability and Cytotoxicity in LPS Stimulated RAW264.7 Cells Treated with Crude Extracts and Compounds

Crude Extracts and Compounds	IC50 (μg/mL)
n-hexane	32.50±0.06
Chloroform	14.81±0.04
Ethyl acetate	35.65±0.09
Methanol	>100

Note: Each value of IC50 represented mean ±S.E.M.

Compound 1 was acquired as yellow needles with a melting point of 237-240°C. It had a molecular formula of $C_{23}H_{22}O_6$, which corresponded with the molecular ion peak of *m/z* 394 as displayed in the EIMS spectrum. The fragment ion peak due to the loss of a methyl group could be observed at *m/z* 379 in the spectrum, indicating the presence of a methyl side chain in the compound. Absorption bands at 3393 cm^{-1} (OH), 2941 cm^{-1} (sp^3 CH), 1611 cm-1 (C=O) and 1457 cm^{-1} (C-O) shown from the FTIR spectrum were consistent with that of the xanthone skeleton. The ^{1}H NMR spectrum indicated a one-proton triplet at δ 5.27 (J=7.4Hz) and a two-proton doublet at δ 3.57 (*J*=8.0Hz), which were represented by H-16 and H-15, respectively. The COSY NMR spectrum further suggested that H-15 and H-16 were coupled with one another. Hence, the presence of a prenyl side chain was deduced.

Table 2
Anti-Microbial Activities of Crude Extracts via Disc Diffusion Method Against Gram Positive Bacteria and Yeast

Crude Extracts	Target Microbes (Bacteria & Yeast)						
	I	II	III	IV	V	VI	VII
n-hexane	-	-	16	13	-	-	-
Chloroform	-	-	16	13	-	-	-
Ethyl acetate	-	-	7	6	-	-	-
Methanol	-	-	-	-	-	-	-

Note: Each value is in millimetres (mm)
I: Methicillin Resistant *S. aureus* (MRSA)
II: *Streptococcus sp*
III: *Staphylococcus epidermidis* S273
IV: *Bacillus Subtilis B145*
V: S*taphylococcus aureus S276*
VI: *Candida albicans C244*
VII: *Candida tropicalis A3*
-: no activity recorded

In addition, a pyrano ring was also found to be present in this compound and it was supported by a ^{2}J and ^{3}J coupling exhibited by H-10 and H-11 with C-2, C-3, and C-12. Through comparison with previous reports, compound 1 was established to be caloxanthone A (Iinuma et al., 1994).

Compound 2 was isolated as yellow needles with a melting point of 157-158°C. A molecular formula of $C_{24}H_{26}O_6$ was supported by the molecular ion peak in the EIMS spectrum at *m/z* 410. The FTIR spectrum displayed absorptions at 3288 cm^{-1} (OH), 2929 cm^{-1} (sp^3 CH) and 1641 cm^{-1} (C=O), which fundamentally fulfilled the characteristics of a xanthone. A ^{2}J correlation between H-15 and C-8 observed from the HMBC spectrum suggested the attachment of the prenyl side chain to C-8. This suggestion was further strengthened by a triplet at δ 5.34 (J=8Hz), which had a ^{3}J correlation with C-18 and C-19. A sharp and strong singlet peak at δ 3.98 representing a methoxyl (OCH3) side group was attributed to C-5 via a ^{3}J correlation between the methoxyl protons and C-5. A dihydrofuran ring was suggested from a 3J correlation between H-13 with C-11 and C-12. The attachment of the ring to C-3 and C-4 was further evidenced by ^{3}J correlation between H-12 with C-4, C-11 and C-13. By associating the spectral data with literature data, it was concluded that compound 2 was caloxanthone B (Iinuma et al., 1994).

Compound 3 was isolated as yellow needles with a melting point of 212-213°C. The molecular formula, $C_{23}H_{22}O_5$, tallied with the molecular ion peak at *m/z* 378 as displayed in the EIMS spectrum. Absorptions at 3428 cm^{-1} (OH), 2929 cm^{-1} (sp^3 CH), 1711 cm^{-1} (C=O), and 1589 cm-1 (C=C) evidently showed the presence of a xanthone skeleton. Compound 3 and compound 4 differed from each other by an additional hydroxyl (OH)

group attached to C-6 in compound 4. The presence of a pyrano ring was justified by two doublet signals at δ 6.68 (J=10.3Hz) and δ 5.72 (J=10.3Hz), which were cis-coupled. The attachment of the ring to C-2 and C-3 was supported by the ^3J correlations of H-10 with C-1 and C-3. Apart from that, a prenyl moiety was proposed due to a doublet of doublet signal observed at δ 6.48 (J=10.3, 17.2Hz), a singlet at δ 1.71 and a pair of doublets at δ 4.85 (J=10.3Hz) and δ 5.00 (J=17.2Hz), respectively. The splitting pattern for the peak at δ 6.48 was due to two non-equivalent protons at δ 4.85 (H-17a) and δ 5.00 (H-17b). ^3J correlations of H-18 and H-19 with C-4 proved that the prenyl moiety was attached to C-4. Hence, it was concluded that compound 3 was caloxanthone C (Iinuma et al., 1994).

Compound 4 appeared as yellow needles with a melting point of 170-172°C. Analysis of the EIMS spectrum revealed the molecular ion peak at *m/z* 394, which consequently supported the molecular formula, $C_{23}H_{22}O_6$. Absorptions of the FTIR spectrum at 3348 cm^{-1} (OH), 2965 cm^{-1} (sp^2 CH), 1589 cm^{-1} (C=O) and 1434 cm^{-1} (C=C) presumed the presence of a xanthone skeleton. A prenyl moiety was attached to C-4 and its presence was evidenced by the presence of a doublet of doublet at δ 6.71 (J=10.8Hz, 18.3Hz) and a pair of doublets at δ 5.21 (J=18.3Hz) and δ 5.04 (J=10.3Hz). This position of this moiety was established via two ^3J correlations between H-18 and H-19 with C-4. Apart from that, ^2J and ^3J correlations of H-10 and H-11 with C-2 proved that there was an attachment of the pyrano ring to C-2 and C-3. Comparison with provided literature data enabled us to conclude that compound 4 was macluraxanthone (Iinuma et al., 1994).

Compound 5 was isolated as fine yellow needles with a melting point of 259-260°C. The molecular ion peak at *m/z* 392 as observed from the EIMS spectrum matched with the molecular formula of $C_{23}H_{20}O_6$. The presence of the functional groups present in the xanthones was displayed in the IR spectrum with absorptions at 3627 cm^{-1} (OH), 2924 cm-1 (sp^2 CH), 1612 cm^{-1} (C=O), and 1458 cm^{-1} (C=C). From the ^1H NMR spectrum, a pair of doublets at δ 6.72 (J=9.1Hz) and δ 5.58 (J=10.3Hz) along with another pair at δ 6.87 (J=10.3Hz) and δ 5.74 (J=10.3Hz) pointed out the presence of two pyrano rings. The position of the first pyrano ring was determined via ^3J coupling between H-10 and C-3 and between H-11and C-2. The second pyrano ring position was supported by ^3J correlation between H-16 and C-7 and between H-15 and C-6. Therefore, it was concluded that compound 5 was pyranojacareubin after comparing with previous literature data (Harrison et al., 1993).

CONCLUSION

Five known xanthones, namely, caloxanthone A, caloxanthone B, caloxanthone C, macluraxanthone and pyranojacareubin were obtained from the chloroform extract of *Calophyllum inophyllum* stem bark. In addition, the chloroform extract also showed promising activity in the cell viability and cytotoxicity test of LPS-stimulated

RAW264.7 cells and acted moderately against *Staphylococcus epidermidis S273* and *Bacillus Subtillis B145*. Hence, there is a possibility for the stem bark of *Calophyllum inophyllum* to provide potential cytotoxic lead compounds that can be developed into drugs for use in cancer therapy.

ACKNOWLEDGEMENT

The authors express our utmost gratitude to the Malaysian Ministry of Higher Education for providing financial funding under the FRGS research grant and Universiti Putra Malaysia (UPM) for providing research facilities and technical support. The Sarawak Biodiversity Centre is also acknowledged.

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Pertanika J. Trop. Agric. Sci. 40 (1): 119 - 130 (2017)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Comparative Study of Antioxidant Level and Activity from Leaf Extracts of Annona muricata Linn Obtained from Different Locations

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ABSTRACT

Annona muricata Linn possesses an anti-tumorigenic effect towards cancer. Several of its bioactive components have already been assessed in previous findings. However, none of the previous studies actually addressed the important consideration of the association between cultivation area of this medicinal plant and its bioactive compounds/antioxidants. In this study, the antioxidant level and antioxidant activity of 19 *Annona muricata* collected from different locations were evaluated by phenolic and flavonoid assays together with Oxygen Radical Absorbance Capacity (ORAC), Ferric Reducing Ability of Plasma (FRAP) and 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assays. M1 was found to have an attractive antioxidant profile as it had the highest content of phenolics (73.2 µg/mL GAE) and flavonoids (191.4 µg/mL CE) and also the highest antioxidant capacity in ORAC assay (254.7 µM). Additionally, it had a favourably high ferric ion reducing capacity (15.55 µM Fe2+/µg) and the best free DPPH-radical scavenging activity (IC_{50}=143.5 µg/mL). On the contrary, R1 showed the lowest level of phenolics with a GAE value of 21.92 µg/mL, ranked second lowest in flavonoid content (65.42 µg/mL CE), and it had the least antioxidant capacity in ORAC (94.66 µM), FRAP (4.17 µM Fe2+/µg) and DPPH assays (1597 µg/mL), making it the least desirable antioxidant source. Based on this finding, it was concluded that *Annona muricata* Linn had varied antioxidant levels and activity regarding its cultivation area; hence, it would be a guide in the selection of potential candidates for natural antioxidants in phytopharmacy.

Keywords: *Annona muricata*, antioxidant, phenolic, flavonoid, ORAC, DPPH, FRAP

ARTICLE INFO

Article history:
Received: 10 March 2016
Accepted: 02 November 2016

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ISSN: 1511-3701

INTRODUCTION

Antioxidants are known for their role in stabilising or deactivating free radicals from attacking targets in biological cells (Sreeramulu et al., 2013). Free radicals like reactive oxygen species (ROS) and other oxidants induce damage to DNA and tissues, which results in the occurrence of various diseases including cancer (Franco et al., 2008). The most abundant antioxidants found in plants are polyphenolic compounds, which are the secondary plant metabolites that arise from a common intermediate, phenylalanine, or its close precursor, shikimic acid (Pandey & Rizvi, 2009). Polyphenolic compounds can be classified into four main classes, which include flavonoids, phenolic acids, stilbenes and lignans (Pandey & Rizvi, 2009). A previous study showed that the antioxidant activity of plant materials was correlated with the content of their phenolic constituents, suggesting that a high phenolic content indicates high antioxidant activity (Moein & Moein, 2010).

Annona muricata Linn, which belongs to the Annonaceae family, is commonly known as soursop, graviola or guanabana. It is native to sub-Saharan countries (Gavamukulya et al., 2014) though it is now widely cultivated in many tropical countries in the world such as India, Malaysia and Nigeria. Often, this plant is sought for its therapeutic effects. Each part of the tree i.e. the root, stem-bark, leaves, fruit and even the seed is used in traditional medicines around the world (Onyechi et al., 2012). The supposed therapeutic benefits of the soursop has attracted intensive research on the chemical composition of the leaves and seeds that has led to the finding of acetogenin compounds (Moghadamtousi et al., 2015). This molecular structure is a very potent compound against cancer as it deprives the high-energy demanding cancer cells of adenosine triphosphate (ATP) supply via the disruption of the mitochondrial electron transport system, resulting in apoptosis (Degli et al., 1994; McLaughlin, 2008)). These isolated compounds, which are secondary metabolites/antioxidants, answer the potential of the soursop for possessing anti-cancer, insecticidal, sedating as well as pain and immunosuppressing properties (Bermejo et al., 2005).

In the past, several studies focussed on antioxidant activity of extracts from pulps, leaves and peel of *Annona muricata* Linn. Akomolafe and Ajayi (2015) referred to a comparative study on antioxidant properties of the peel and pulp of ripe *Annona muricata* Linn that reported that the antioxidant potential in soursop peel was found to be significantly higher than in the pulp, as determined by ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Fe^{2+} chelation and hydroxyl scavenging tests. Furthermore, flavonoid and phenolic content was also higher in the soursop peel compared to the soursop pulp extract. Interestingly, *Annona muricata* possessed more potent in vitro antioxidant activity compared to the leaves of *Annona reticulata* and *Annona*

squamosa as revealed by Baskar et al. (2007). Additionally, *Annona muricata* Linn leaf aqueous extract was found to alleviate the pancreatic B-cells of Streptozotocin-treated diabetic rats by directly quenching lipid peroxides and indirectly enhancing production of endogenous antioxidants, thus addressing its antioxidant potential (Adewole & Caxton-Martins, 2006). Omale and Olakunle (2015) reported that the hepatoprotective and antioxidant activity of soursop stem bark extract against oxidative stress in rats induced by carbon tetrachloride (CCl_4) as determined from serum enzyme markers. According to Moghadamtousi et al. (2014), ethanol-induced gastric injury in rats could be treated by ethyl acetate extract of *Annona muricata* Linn leaves, which provide a suppressive effect against oxidative damage and a preservative effect on gastric wall mucus. Despite extensive research into the antioxidant level and activity possessed by *Annona muricata* Linn and its effectiveness in treating disease, a comparative study of the antioxidant level and activity of *Annona muricata* Linn obtained from different locations has not been reported. Nonetheless, previous studies have shown that there are different levels of antioxidant/phenolic content among plants of similar species (Lim & Quah, 2006). Therefore, the present study was initiated to determine the antioxidant level and antioxidant activity of aqueous leaf extracts of the *Annona muricata* Linn in different cultivation areas in Peninsular Malaysia.

MATERIALS AND METHODS

Plant Material and Preparation of Plant Extracts

Nineteen *Annona muricata* Linn leaf samples were collected from Peninsular Malaysia. The plant was identified and deposited with a voucher number by the Forestry Division, Forest Research Institute Malaysia (FRIM). The details of the sampling sites and voucher number of each sample are shown in Table 1. All of the 19 samples of old mature *Annona muricata* leaves were air-dried for a week before being ground to a powder using a grind mill. Later, about 10 g of each powdered sample was transferred into a Schott bottle containing 200 mL of sterile distilled water. The samples were incubated for three days with frequent agitation using an orbital shaker at room temperature. The mixture was then filtered to discard any solid material/marc. Finally, the filtrate extract was dried using the freeze dryer/lyophiliser machine to obtain the end product, *Annona muricata* crude extract (AMCE). The extract obtained was kept in sterile sample tubes and stored in a refrigerator at 4°C.

Determination of Total Phenolic Content in the Plant Extract

The total phenol content was determined using the Folin-Ciocalteau method developed by Singleton and Rossi (1965). A twofold serial dilution of positive standard (Gallic acid) was prepared from a 1 mg/mL concentration of the mother stock (i.e. 0.5 mg/mL; 0.25 mg/mL; 0.125 mg/mL;

Table 1
Sampling Sites of Annona muricata Linn in Peninsular Malaysia with the Code and Voucher Number of Each Sample

Location (State)	Code (Sampling Site)	Voucher Number
Johor	J1 (Muar)	ATCL1
Melaka	M1 (Jasin)	ATCL2
	M2 (Jasin)	ATCL3
	M3 (Bukit Beruang)	ATCL4
	M4 (Bukit Katil)	ATCL5
Negeri Sembilan	N1 (Kuala Klawang)	ATCL6
	N2 (Kuala Pilah)	ATCL7
	N3 (Kuala Pilah)	ATCL8
Selangor	B1 (Serdang)	ATCL9
	B2 (Serdang)	ATCL10
	B3 (Serdang)	ATCL11
Perak	A1 (Sungai Terap)	ATCL12
	A2 (Kg. Piandang)	ATCL13
	A3 (Kg. Batu Tujuh)	ATCL14
	A4 (Kg Bota)	ATCL15
Perlis	R1 (Kaki Bukit)	ATCL16
	R2 (Guar Jentik)	ATCL17
	R3 (Santan)	ATCL18
	R4 (Santan)	ATCL19

0.0625 mg/mL; 0.03125 mg/mL; 0.015625 mg/mL). The 19 extract samples were prepared at a concentration of 500 µg/mL. One millilitre of Gallic acid standard, extract samples and blank were transferred to separate test tubes. A total of 5 mL of diluted FC reagent was added into all the test tubes and incubated for 8 min at room temperature. Next, 4 mL of 7.5% sodium carbonate solution was added after some time and the solution was incubated for a further 2 h at room temperature before being read by a UV spectrophotometer at 765 nm wavelength.

Determination of Flavonoid Concentrations in the Plant Extract Samples

The flavonoid content was determined following the method described by Baba and Malik (2015). All extract samples were prepared at 1 mg/mL concentration. A serial dilution of standard (catechin) was prepared (i.e. 50µg/mL, 100µg/mL, 150µg/mL, 200µg/mL, 250µg/mL, 250µg/mL, 300µg/mL, 350µg/mL, 400µg/mL, 450µg/mL and 500µg/mL). A volume of 450µL of the extract samples and standard were added into different 1.5 mL tubes, after which 27µL of 5% $NaNO_2$ was added into each tube. The tubes were incubated for 6 min in a dark setting before the addition of 27µL of 10% AlCl3. An incubation period of 5 min was acquired in a dark setting. Afterwards, 180 µL of 1M NaOH was added into each tube followed by 216µL of dH_20. All the tubes were vortexed for 5 s before 300µL of the solution from each tube was transferred into a 96-well plate. The plate was read using ELISA at 430 nm wavelength.

Evaluation of Antioxidant Activity by ORAC Assay

All the samples were prepared at a concentration of 100ug/mL. Serial dilution of standard (Trolox) were prepared (i.e. 0 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM). A volume of 50 µL of the samples and Trolox was added in triplicate into a 96-well plate before a 50-µL of 60nM fluorescin was added into each well. The plate was incubated for 15 min at 37°C before 50 µL of

150mM AAPH solution was added into each well. The plate was read at 5-min intervals for 90 min (Exc=485nm and Emm=520 nm).

Evaluation of Antioxidant Activity by FRAP Assay

The FRAP assay was done according to Benzie and Strain (1996) with slight modification. The FRAP solution was prepared by mixing the 300-mM acetate buffer, 20-mM ferum trichloride and 10-mM TPTZ together at a ratio of 10:1:1. The FRAP solution was warmed at 37°C before use. All the extract samples were prepared at a concentration of 50 µg/mL. A volume of 50 µL of the extract samples and the positive control ($FeSO_4$) was added in triplicate into a 96-well plate. A volume of 250 µL FRAP solution was added into the wells containing the extract samples and positive control/ standard and incubated for 30 min in a dark setting. The plate was read with an ELISA plate reader at 593 nm wavelength.

Evaluation of Antioxidant Activity by DPPH Assay

DPPH-free radical scavenging activity was determined according to the method developed by Suja et al. (2005) with slight modification. An AMCE sample (50 µL), each with different concentrations (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.63 µg/mL, 7.81 µg/mL) was added in triplicate in a 96-well plate. Then, a volume of 250 µL of 0.02 g/L DPPH solution in methanol was added into the wells containing the extract samples and allowed to stand for 30 min in a dark setting. The plate was read with an ELISA plate reader at 517 nm wavelength. The amount of AMCE sample needed to decrease the initial DPPH concentration by 50% was calculated graphically. Radical scavenging activity was calculated using the equation as follows:

$$\text{Scavenging activity} = \frac{[Abs_{control} - Abs_{sample}]}{[Abs_{control}]} \times 100$$

Statistical Analysis

All data were represented as means ±standard deviation of means. The analysis was performed using a one-way analysis of variance (ANOVA) and the group means were compared using the Duncan test. The statistical significance was evaluated at $p<0.05$ level.

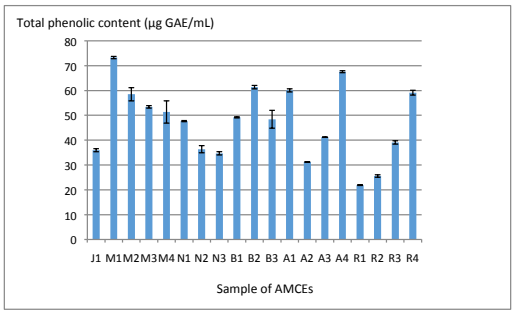

Figure 1. Total phenolic content for 19 samples of *Annona muricata* crude extract. The results obtained were equivalent to Gallic acid in µg/mL. Each value is expressed as the mean from three replicate measurements. The error bars indicate standard deviations

RESULTS AND DISCUSSION

The level of antioxidants in *Annona muricata* was determined by five antioxidant assays: total phenolic, total flavonoid, ORAC, FRAP and DPPH as depicted in Figures 1 to 5. M1 showed the richest source of phenolics (73.2 µg/mL GAE) followed by A4 (67.58 µg/mL GAE) and B2 (61.36 µg/mL GAE) samples, while R1, R2 and A2 possessed the lowest level of phenolics with

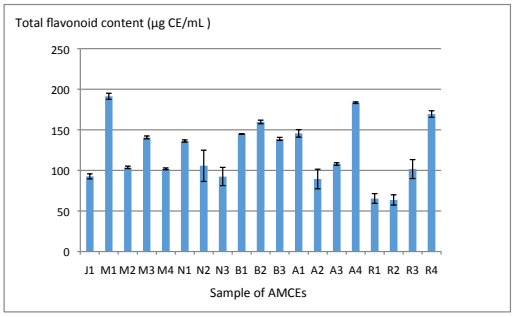

Figure 2. Total flavonoid content for 19 samples of *Annona muricata* crude extract. Antioxidant activity detected by flavonoid assay was represented as µg/mL catechin equivalent (CE). Each value is expressed as the mean from three replicate measurements. The error bars indicate standard deviations

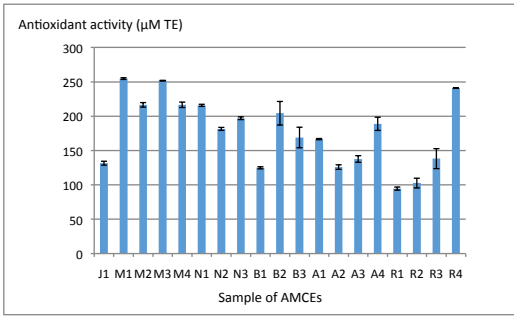

Figure 3. Oxygen radical absorbance capacity (ORAC) assay for 19 samples of *Annona muricata* crude extract equivalent to Trolox (µM). Each value is expressed as the mean from three replicate measurements. The error bars indicate standard deviations

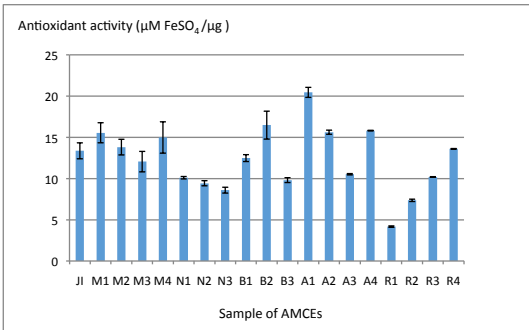

Figure 4. Ferric reducing ability of plasma assay (FRAP) for 19 samples of *Annona muricata* crude extract. Antioxidant activity detected by FRAP assay was represented as µM/µg FeSO4 equivalent. Antioxidant activity was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 µM/µg of FeSO4. Each value is expressed as the means from three replicate measurements and the error bars indicate standard deviations

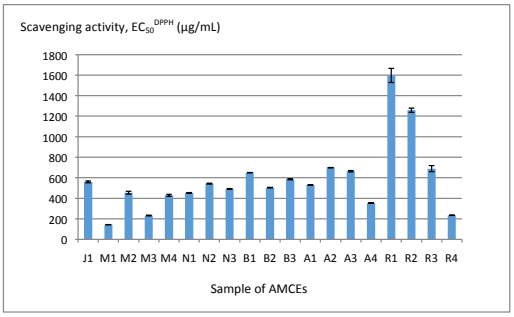

Figure 5. DPPH-free radical scavenging activity of 19 samples of *Annona muricata* crude extract. The scavenging activities of the AMCE samples were evaluated as the EC_{50}^{DPPH}, the concentration of antioxidants that reduces the DPPH-free radical by about 50%. Each value is expressed as the mean from the three replicates and the error bars indicate standard deviations

GAE value of 21.92 µg/mL, 25.56 µg/mL and 31.21 µg/mL, respectively (Figure 1). A similar order of the highest and lowest flavonoid content is recorded in Figure 2. M1 and A4 contained the highest level of the flavonoids (191.4 µg/mL and 183.5

µg/mL CE, respectively) while R2 (63.65 µg/mL CE) showed the lowest flavonoid level followed by R1 (65.42 µg/mL CE) (Figure 2). The radical scavenging capacity of AMCE was carried out by ORAC assay as depicted in Figure 3. The ORAC value varied from 94.66 to 254.7 µM Trolox equivalent per sample, which represented a variation of about threefold. The highest antioxidant capacity was found in M1 (254.7 µM), followed by M3 (251.7 µM). M2, M4 and N1 showed a similar level of ORAC values (216.5 µM, 216.6 µM and 215.8 µM, respectively). In this assay, R1 showed the lowest antioxidant potential (94.66 µM). The results of Ferric reducing capacity of AMCE are presented in Figure 4. The trend for the ferric ion reducing activities of the 19 AMCE samples tested varied approximately by fivefold. A1, B2, A4, A2 and M1 showed very strong ferric ion reducing activity (20.45, 16.48, 15.81, 15.62 and 15.55 µM Fe2+/µg, respectively). In this study, R1 and R2 extract possessed the lowest ferric reducing capacity (4.17 and 7.36 µM Fe2+/µg, respectively). The DPPH-scavenging activity of AMCE is shown in Figure 5. M1 showed the lowest EC50 value (143.5 µg/mL), indicating the highest antioxidant activity among the extract samples whereas R1 was the weakest antioxidant in reducing radical DPPH with EC_{50} value of 1597 µg/mL. In general, the DPPH-reducing power of the 19 AMCE samples varied approximately by elevenfold.

The comparative data of total phenolic and total flavonoid content presented in Figure 1 and 2, respectively, were determined primarily using the leaf aqueous extract from each *Annona muricata* Linn. sample. It is usual for antioxidant or secondary metabolite constituents to be distributed unevenly within each part of the plant and, to some extent, to not be expressed at all as it is mainly due to different morphology, anatomy and function of the plant organs (Mantle et al., 2000). However, the level of phenolic and flavonoid content varies throughout the soursop samples despite the fact that these samples were from the leaves of similar plant species. This anomaly could be related to the geographical/environmental differences that prevail where these soursop plants are cultivated (Gavamukulya et al., 2014; Scalzo et al., 2005). This is in agreement with the findings of Shams Ardekani et al. (2011), who found that pomegranates of different cultivars contained different levels of phenolics and flavonoids. The geographical difference of the cultivated plant means that each plant is exposed to a different climate and environmental stress factors such as humidity, temperature and soil composition (Gull et al. 2012). The synthesis and accumulation of secondary plant products are enhanced in stress environments such as a water-deficit condition (Selma & Kleinwachter, 2013). In a harsh environment, plants adjust their regulation of the phenylpropanoid biosynthesis pathway at multiple levels in response to the exogenous factors. For instance, the high intensity of visible light present would initiate the elevation of biosynthetic enzymes in the phenylpropanoid

pathway, which, consequently, enhances the production of anthocyanins and flavones as a means to reducing the amount of light available to the photosynthetic cells (Dixon & Paiva, 1995; Beggs et al., 1987). Moreover, flavonoids such as kaempferol derivatives are also highly induced to absorb UV rays to prevent damage that can result in cell death (Beggs et al., 1987). Previous studies have isolated and identified several polyphenol compounds from the leaves of the *Annona muricata* Linn. that include Gallic acid, epicatechine, catechine, quercetin, kaempferol, quercetin 3-O-rutinoside, quercetin 3-O-neohispredoside, quercetin 3-O-robinoside, kaempferol 3-O-rutinoside, quercetin 3-O-α-rhamnosyl-(1''→6'')-β-sophoroside and chlorogenic acid (Nawwar et al., 2012). The different levels of flavonoid and phenolic acid might be due to the level of expression of these aforementioned compounds present in the soursop samples.

Antioxidant activity of the soursop extract samples were evaluated by ORAC, FRAP and DPPH assay. At the present time, there is no single assay that is able to accurately reflect all of the radical sources or all antioxidants present in a certain food or extract sample (Dragovic-Uzelac et al., 2007; Prior et al., 2005). Therefore, it is best to analyse the antioxidant capacity of AMCE in several assays in order to generate a better antioxidant profile. The comparative data of the AMCE's antioxidant capacity in the ORAC, FRAP and DPPH assay are shown in Figures 3, 4 and 5, respectively, where the antioxidant activity of each sample varies in the assays. Only a few of the extract samples in ORAC assay had similar antioxidant capacity ranking order as those in the DPPH assay. This included M1, M3 and R4, which were observed to be the best three extract samples in scavenging the peroxyl radical and DPPH radical. The extract samples in the FRAP assay did not conform to the ranking order of both assays. M1, M3 and R4 were only ranked at 5th, 11th and 7th position, respectively, in reducing the Fe $(TPTZ)_2$ (III) in the FRAP assay. However, it is interesting that R1 and R2 were consistent in their ranking order, placed at the two lowest positions in all the assays. This event of ranking order discrepancy between assays in this present study is in accordance with the findings of Rufino et al. (2010). It is a plausible event considering the underlying reaction mechanisms of the assays employed, the source of oxidant/free radicals and method of quantitation that were different from each other (Huang et al., 2005). It is noteworthy that the three assays employed in this study were based on the capacity of the antioxidants possessed in the extract samples to break the radical chain sequences rather than inhibit the formation of the reactive oxygen species (chain-breaking antioxidants vs preventive antioxidants) (Ou et al., 2002). DPPH and FRAP assays, which are based on electron transfer, measure the antioxidant's capacity to reduce an oxidant that changes colour when reduced (Dudonne et al., 2009; Prior et al., 2005). ORAC assay, on the other hand, is based on the hydrogen atom transfer reaction, which involves the competition between antioxidant and

substrate for peroxyl radicals that are generated through the decomposition of azo compounds (Payne et al., 2013; Prior et al., 2005). DPPH and FRAP assay are simple, rapid and inexpensive but are subject to certain limitations. For instance, steric accessibility is a major concern in the DPPH assay (Prior et al., 2005) whereas in the FRAP assay, not all antioxidants possess the capability to reduce Fe(III) at a fast rate (or within a fixed time frame) as some polyphenols such as tannic acid, ferulic acid and quercetin might require a longer reaction time (Ou et al., 2002). To that extent, the 30-min reaction time in the FRAP assay is rendered insufficient for certain soursop extract samples; this affects the ranking order of the samples. On the other hand, an ORAC assay that uses the area under curve (AUC) approach for its kinetic reaction measurement allows the quantification of antioxidants that exhibit a distinct lag phase and those that have no lag phases, giving a more accurate result for antioxidant capacity (Huang et al., 2005). Above all, it is inappropriate to compare the antioxidant capacity ranking order of the extract samples between ORAC, FRAP and DPPH assays as they adopt different principles and none of the mentioned methods indicate the total antioxidant capacity of each extract. However, it is safe to assume that M1 could be considered the best antioxidant with regards to its high capacity (ranking order) across the assays.

CONCLUSION

This study has shown that Malaysia's *Annona muricata* Linn contains varied antioxidant levels and activity among the cultivars. Among the samples tested, M1 showed promising attributes to serve as an anti-oxidative agent due to possessing the highest phenolic and flavonoid content, as well as exhibiting the desirable antioxidant activity in the ORAC, FRAP and DPPH assays. On the other hand, R1 exhibited the lowest level of phenolic and flavonoid content; this explains their lowest rank in each of the antioxidant-activity assays. Based on the results presented, it is safe to make the assumption that *Annona muricata* Linn possesses different antioxidant levels and activity with regards to their cultivation areas. As it is anticipated that the soursop plant would be a useful pharmaceutical material in treating diseases associated with oxidative stress or cancer, the results provide a good platform for further evaluation and analysis of this plant. An extended study such as HPLC fingerprinting and other assays are needed to identify the specific compounds responsible for the varying antioxidant level and activity among the soursop samples.

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Pertanika J. Trop. Agric. Sci. 40 (1): 131 - 142 (2017)

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TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Supplementation of Antioxidant BHT to Different Bull Semen Extenders Enhances Semen Quality after Chilling

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ABSTRACT

The effects of adding various concentrations of antioxidant, butylated hydroxytoluene (BHT) on chilled bull semen for 72 h at 4°C in Bioxcell® (BX), Tris egg-yolk- (TEY) and citrate egg-yolk- (CEY) based semen extenders were studied. Twenty-two ejaculates collected from three bulls, each extended using three extenders with BHT at 0 (control), 0.5, 1.0, 1.5, 2.0 and 3.0 mM/mL, were evaluated for sperm quality parameters. Extended semen was packaged into 0.25 mL straws containing 20 x 10^6 spermatozoa and chilled for 72 h. Four random straws each from the control and treatment groups were warmed at 37°C, pooled and evaluated using a computer-assisted semen analyser (IVOS Hamilton Thorne Biosciences) for general and progressive motilities, and for morphology, viability and acrosome damage using eosin-nigrosin stain under phase-contrast microscope. Results showed that sperm morphology, viability and protection of acrosome damage were significantly improved ($p<0.05$) at BHT concentrations of 0.5 in BX and 1.0 mM/mL in TEY and CEY compared to the controls. The BHT also showed a potential positive effect on progressive motility at 0.5 mM/mL in BX and 1.0 mM/mL in TEY and CEY. High concentrations of BHT (2.0 and 3.0 mM/mL), however, produced deteriorative effects on the sperm parameters in all the extenders. In conclusion, BHT when added at 0.5 mM/mL in BX and 1.0 mM/mL concentration to TEY and CEY extenders

ARTICLE INFO

Article history:
Received: 12 May 2016
Accepted: 10 November 2016

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ISSN: 1511-3701

improved the quality parameters of bull chilled semen.

Keywords: antioxidant, bull, butylated hydroxytoluene, extender, oxidation, semen

INTRODUCTION

Reproductive specialists have tried using fresh or chilled semen for artificial insemination (AI) with the view of preserving the quality of spermatozoa. Research revealed that while fresh bull semen was viable at body temperature for a few hours, semen chilled between 4°C and 8°C was viable for up to 24 h at best, without significant decrease in fertilisation rate. The low temperature is needed to reduce sperm metabolism, minimising substrate utilisation by the cell, thereby prolonging its survival time. Though semen has been kept successfully in the short-term by chilling, some adverse effects have been identified, which include depressed motility, decreased viability and structural integrity as well as reduced conception rate (Lemma, 2011).

In an effort to improve semen storage standards and the fertility potential of fresh bull semen, current studies are now focussed on the effects of adding antioxidants such as butylated hydroxytoluene (BHT) to extended semen. Antioxidants primarily protect spermatozoa from debilitation as a result of oxidation, which is caused by higher concentrations of oxidising agents than is needed within the spermatozoa. Oxidising agents such as reactive oxygen species (ROS) are produced by normal cells and small quantities are required for the fertilisation process. The production of ROS is usually increased by injured, dead and phagocytic cells. Low temperature such as experienced during cooling leads to physical injuries to spermatozoa, affecting its structural integrity and resulting in an increase in ROS production and impairment of sperm quality parameters (Medeiros et al., 2002). Natural antioxidants such as superoxide dismutase (SOD) are usually secreted within the sperm plasma to control the excess ROS production but their effect becomes minimal with sperm extension (Ijaz et al., 2009). Butylated hydroxytoluene is a lipid soluble antioxidant and synthetic analogue of Vitamin E. Studies have reported effects on semen quality parameters after addition of BHT into chilled different extenders compared to untreated controls in species such as turkey (Donoghue & Donoghue, 1997), goat (Khalifa et al., 2008) and dog (Sahasha et al., 2011). Being lipophilic gives BHT unique properties that allow it to dissolve easily in sperm cytoplasm, increase intra-cytoplasmic fluidity and exert its effects both from within and outside the sperm cell (Asadpour & Tayefi-Nasrabadi, 2012), thereby improving the viability of spermatozoa by protecting the sperm cell membrane. So far, there has been little information on antioxidant effects of BHT on chilled bull semen. Therefore, the aim of this study was to evaluate the effects of various concentrations of BHT on bull semen's quality such as general motility, progressive motility, morphology, viability and acrosome integrity extended in three different semen extenders.

MATERIALS AND METHODS

Animals

Semen samples were collected from three sexually mature and fertile crossbred bulls: Simmental-Brangus, Brangus-Hereford and Kedah-Kelantan-Brangus at the Universiti Putra Malaysia (UPM) farm. The bulls were 5.3±0.3 years old and 649.3±9.7 kg in body weight. All the bulls were maintained under uniform management, fed with Surinam grass (*Brachiaria decumbens*) *ad libitum*, and supplemented with commercial cattle concentrate containing approximately 16% crude protein and 2.6% crude fat, given at a rate of 3 kg/bull/day. They were also given mineral licks and water *ad libitum*.

Semen Collection and Preparation of Extenders

A total of 24 ejaculates were collected during the experiment; two did not pass the inclusion criterion. Therefore, the result representing data from 22 ejaculates were collected using electroejaculation (Electrojac 5. NEOGEN®, Lexington USA), with seven ejaculates from two bulls each and eight from the other. Two ejaculates were collected at four-day intervals. The samples were kept in a Coleman cooler box containing warm water at 37°C for transportation to the laboratory and pre-chilling evaluation. Each ejaculate was processed and treated separately. To minimise a possible effect of individual bull differences, a minimum semen quality criterion as described by Khumran et al. (2015) was followed before processing. Samples were then extended with Bioxcell® (BX), Tris egg yolk (TEY) and citrate egg yolk (CEY), respectively, each of which was further subdivided into six groups. The respective extended semen groups were poured into dried, pre-warmed test tubes containing BHT antioxidant (prepared in ethanol) to make up 0, 0.5, 1.0, 1.5, 2.0 and 3.0 mM/mL BHT concentrations. These mixtures were then left in a water bath at 37°C for 5 min to allow for proper uptake of BHT by spermatozoa before cooling (Ijaz et al., 2009).

Bioxcell®, a commercial semen extender (IMV, France), was diluted at 1:4 in distilled water according to the manufacturer's instructions, while TEY and CEY extenders were prepared according to Bearden et al. (2004) with a penicillin-streptomycin mixture (BP2959-50) as antibiotic at 0.01 mL/mL of extender. All the extenders were adjusted to pH 6.7 using the SevenEasy pH meter (Mettler Toledo Ltd., England). Semen was extended to adjust the concentration of spermatozoa to $20x10^6$ per 0.25 mL mini straws at 37°C, and was then slowly chilled to 4°C over a two-hour period. First, the test tubes containing extended semen were held for 30 min in a beaker of water at 37°C while loaded into the chiller. Secondly, straws were packed with the chilled semen under a 4°C working environment and kept at the same temperature until analysis.

Evaluation of Fresh Semen Quality

Motility and concentration were determined by CASA (Computer Assisted Semen Analyser, IVOS Hamilton Thorne Biosciences). Ten µL of diluted samples in

0.85% normal saline were placed on 20 µm Hamilton Thorne research 2X-cell-glass slides and loaded on the CASA for analysis (Yimer et al., 2011). At least 200 sperm cells from an average of 10 fields were counted per reading.

Sperm morphology was determined by eosin-nigrosin (E & N) stain as described by Memon et al. (2011) and then air dried. Stained slides were viewed under a phase-contrast microscope at 1000x magnification (oil immersion). Two hundred spermatozoa were counted from an average of four microscopic fields. Spermatozoa showing complete normal structures having a smooth-oval head with clearly defined acrosome joined to the tail by the mid-piece without any visible defect were considered normal. Large/small head sizes, abnormal mid-piece, presence of droplets, broken parts, double head and/or tail were considered abnormalities and therefore, sperm cells showing any of these characteristics were considered abnormal (Nagy et al., 2013).

Post-Chilling Evaluation

Samples were evaluated after 24, 48 and 72 h of chilling by randomly selecting four straws from each BHT concentrations treatment and control once each time. The straws were warmed in a water bath at 37°C for 30 s, pooled together and analysed using CASA for general and progressive motilities. The E & N stain was used to determined morphology, viability and acrosome damage. Spermatozoa with clear white heads that did not take up the stain were identified as viable, whereas those that showed partially/completely purple-coloured heads were non-viable. The percentage of acrosome damage was counted according to the method proposed by Yildiz et al. (2000) in order to assess acrosomal morphology.

Statistical Analysis

The statistical analysis system (SAS V 9.1, SAS Inst. Inc., Cary, North Carolina) was used for data analysis. The means of the effects of various concentrations of BHT and control on the chilled semen parameters were compared by factorial ANOVA (using a 3x3x6 design). Duncan's test was used as the post hoc test. The data were checked for normality using the UNIVARIATE procedure of SAS software and the results were presented as means and standard error of the mean. Analyses were conducted at 95% confidence level; therefore, p values<0.05 were considered significant.

RESULTS

Interaction among BHT treatment concentrations, days of chilling and extenders used on sperm parameters (p-values) are presented in Table 1. The table shows that treatments, days and extender all independently influenced general and progressive sperm motility. Significant interaction between treatment, days and extender was noted, however, with regards to morphology, viability and acrosome damage.

Table 2 shows the effects of BHT on chilled bull semen quality parameters in the BX extender after three days of

Table 1
Interaction Effects Among Treatments, Days and Extenders (P Values)

Parameter (%)	Treatment (Trt)	Day (D)	Extender (Ext)	Trt*D	Trt*Ext	D*Ext	Trt*D*Ext
General motility	<.0001	<.0001	<.0001	0.9959	0.5036	0.7169	0.9763
Progressive motility	0.0001	0.0024	<.0001	0.9305	0.2289	0.2791	0.8909
Normal morphology	<.0001	<.0001	<.0001	0.0295	<.0001	0.0025	<.0001
Viability	<.0001	<.0001	<.0001	0.7513	<.0001	0.0674	<.0001
Acrosome damage	<.0001	<.0001	<.0001	0.2090	<.0001	0.0901	<.0001

Values less than 0.05 are considered significant interaction between factors

chilling. General motility was significantly higher ($p<0.05$) in the control and BHT concentrations of 0.5, 1.0 mM/mL compared to 3.0 mM/mL on days 1 and 2. In the control, BHT concentrations of 0.5, 1.0 and 1.5 mM/mL were significantly higher than 3.0 mM/mL on day 3. Progressive motility was best at BHT concentration of 0.5 mM/mL, significantly different with 3.0 mM/mL on day 1. Progressive motility at 0.5 mM/mL was also the same as 1.0, 1.5 mM/mL and control, significantly higher than 2.0, 3.0 mM/mL on days 2 and 3. Normal morphology was also the best in a BHT concentration of 0.5, same as 1.0 mM/mL, and significantly better compared to the control, 1.5, 2.0 and 3.0 mM/mL treatments during quality evaluation over three days. Similarly, BHT concentration of 0.5 was best for viability and acrosome damage, significantly higher compared to the controls and the rest of the treatments during the three days except for 1.0 mM/mL in viability on day 3 and 1.0 mM/mL in acrosome damage on day 1 where they were the same.

Table 2 also indicates that all parameters (except for general motility) were maintained without significant difference in the BX extender for the three days' quality evaluation at BHT concentration of 0.5 mM/mL. This was in opposition to BHT concentration of 3.0 mM/mL, in which all parameters (with the exception of viability) deteriorated daily in quality evaluation over the three days. Progressive motility, viability and acrosome damage were maintained in the control, while general motility and viability deteriorated daily in quality evaluation over the three days.

Table 3 shows the effects of BHT on chilled bull semen quality using a TEY extender after quality evaluation of the semen over the three days. Normal morphology, viability and acrosome damage were better at a BHT concentration of 1.0 mM/mL, significantly higher compared to the controls and BHT concentrations

Table 2
Effect of BHT on Chilled Bull Semen Quality in Bioxcell® Extender Kept for 3 Days

Parameter %	Day	Control	BHT concentrations (mM/mL)				
			0.5	1.0	1.5	2.0	3.0
General motility	D1	68.87±4.28^{a,y}	68.80±4.40^{a,y}	67.27±4.38^{a,y}	62.80±5.19^{ab}	56.80±6.48^{ab,y}	49.40±6.16^{b,x}
	D2	61.40±3.70^{a,xy}	61.73±5.10^{a,xy}	62.27±5.10^{a,xy}	54.13±5.15^{ab}	43.40±5.18^{bc,xy}	35.20±5.38^{c,xy}
	D3	54.13±4.93^{a,z}	52.07±4.90^{a,z}	51.00±4.93^{ab,z}	47.73±5.36^{ab}	36.47±4.74^{bc,z}	29.53±4.78^{c,z}
Progressive motility	D1	23.89±2.84^{ab}	27.78±4.29^{a}	27.00±3.54^{a}	25.44±3.59^{ab}	17.56±2.85^{ab}	16.11±2.22^{b,x}
	D2	20.56±2.80^{abc}	28.78±7.01^{a}	22.78±2.81^{ab}	18.56±3.02^{abc}	14.11±2.14^{bc}	10.33±1.83^{c,xy}
	D3	19.22±2.72^{a}	20.89±2.65^{a}	20.56±2.76^{a}	17.67±2.90^{ab}	11.22±2.34^{bc}	7.11±1.98^{c,y}
Normal morphology	D1	84.33±0.57^{b,x}	92.67±1.19^{a}	89.33±0.71^{ab,x}	86.67±1.07^{b,x}	78.67±2.79^{c,x}	77.83±2.63^{c,x}
	D2	82.50±0.90^{b,xy}	90.67±1.18^{a}	87.00±0.92^{ab,xy}	84.33±0.81^{b,xy}	72.33±2.67^{c,xy}	70.00±2.89^{c,y}
	D3	81.50±0.93^{b,y}	90.50±0.88^{a}	86.17±0.86^{ab,y}	83.67±0.81^{b,y}	69.17±2.85^{c,y}	66.83±2.33^{c,y}
Viability	D1	76.83±0.81^{cb}	85.67±1.02^{a}	79.83±1.21^{b}	78.33±1.93^{b}	72.33±2.80^{cd}	71.17±2.38^{d}
	D2	76.50±0.87^{b}	85.83±0.93^{a}	79.67±1.38^{b}	76.17±1.88^{b}	68.33±3.06^{c}	66.83±3.24^{c}
	D3	76.17±0.98^{b}	83.67±1.81^{a}	78.83±1.49^{ab}	76.83±1.53^{b}	65.83±3.10^{c}	64.00±3.30^{c}
Acrosome damage	D1	16.17±1.02^{a}	3.17±0.93^{c}	5.17±1.15^{bc,x}	8.67±1.29^{b,x}	17.00±1.55^{a,x}	19.33±1.61^{a,x}
	D2	17.33±0.92^{b}	3.50±1.00^{d}	7.00±0.97^{d,xy}	11.17±1.43^{c,xy}	21.50±2.02^{a,xy}	21.83±1.34^{a,y}
	D3	17.50±1.11^{c}	5.00±1.11^{f}	9.17±0.77^{e,y}	13.50±1.14^{d,y}	24.83±1.50^{b,y}	28.33±1.10^{a,y}

Data are expressed as Mean ±Standard Error (SE) n=22. Different superscripts a-f within rows and x-z within columns denote significant differences ($p<0.05$)

Table 3
Effect of BHT on Chilled Bull Semen Quality in Tris Egg Yolk Extender Kept for 3 Days

Parameter %	Day	Control	BHT concentrations (mM/mL)				
			0.5	1.0	1.5	2.0	3.0
General motility	D1	58.47±4.00^{x}	61.93±3.81^{x}	61.07±4.22^{x}	55.87±4.16^{x}	53.33±4.38^{x}	51.73±4.69^{x}
	D2	44.60±3.34^{y}	50.47±4.21^{xy}	50.53±4.41^{xy}	41.67±5.48^{xy}	38.07±5.54^{y}	35.40±5.17^{y}
	D3	42.00±4.22^{y}	42.87±4.54^{y}	43.93±4.67^{y}	38.73±5.32^{y}	32.87±5.61^{y}	29.67±5.65^{y}
Progressive motility	D1	23.00±2.61	25.33±2.52	29.00±2.06	28.22±2.61	25.11±2.80	20.78±2.73
	D2	24.11±2.84	23.22±2.77	27.44±2.41	25.89±3.41	20.56±2.56	18.56±2.65
	D3	21.11±3.12	21.56±2.64	27.11±2.53	24.33±3.03	19.33±2.32	37.67±22.81
Normal Morphology	D1	91.67±0.77^{b,x}	90.83±0.68^{b}	94.50±0.54^{a}	91.33±1.15^{b}	87.83±0.82^{c,x}	86.83±0.59^{c,x}
	D2	89.00±1.14^{bc,xy}	89.50±1.40^{b}	93.17±0.71^{a}	90.67±1.08^{ab}	86.33±0.83^{cd,x}	84.83±0.51^{dy}
	D3	86.83±1.26^{b,y}	87.50±1.18^{b}	93.17±0.68^{a}	90.50±0.90^{a}	82.50±0.79^{c,y}	81.50±0.69^{c,z}
Viability	D1	59.50±2.73^{bc}	66.83±2.59^{a}	67.83±1.26^{a}	65.00±2.58^{ab}	54.67±0.93^{cd,x}	49.67±0.51^{d,x}
	D2	59.17±2.46^{b}	65.83±2.41^{a}	68.17±1.74^{a}	63.33±2.85^{ab}	51.00±0.65^{c,y}	47.00±0.65^{c,y}
	D3	56.83±2.36^{b}	63.17±1.97^{a}	67.00±1.71^{a}	64.67±1.75^{a}	47.67±0.75^{c,x}	43.00±0.43^{d,z}
Acrosome damage	D1	9.83±2.28^{bc}	12.67±2.44^{ab}	4.50±0.97^{c}	9.17±2.13^{bc}	16.67±1.45^{a,x}	17.17±1.60^{a,x}
	D2	12.67±2.04^{b}	13.50±2.27^{b}	5.33±1.48^{c}	10.33±2.39^{cb}	20.67±1.64^{a,xy}	21.83±1.51^{a,xy}
	D3	15.83±1.98^{b}	15.00±2.16^{b}	7.50±2.00^{c}	13.17±1.97^{cb}	24.50±2.03^{a,y}	26.33±1.99^{a,y}

Data are expressed as Mean ±Standard Error (SE) n=22. Different superscripts a-f within rows and x-z within columns denote significant differences ($P<0.05$)

of 2.0 and 3.0 mM/mL for all three days. Even though there was no significant difference between treatments and controls for general and progressive motilities, BHT concentration of 1.0 gave the highest percentage during the three days of quality evaluation. All semen quality parameters deteriorated daily with BHT concentrations of 2.0 and 3.0 mM/mL, as well as all treatments in general motility over the three days of evaluation.

Table 4 shows the effects of BHT on chilled bull semen quality using the CEY extender after three days of quality evaluation. Progressive motility was not significantly affected by either treatment or days but the BHT concentration of 1.0 mM/mL gave the highest percentage among the groups in the entire duration of semen quality evaluation. Higher viability and less acrosome damage were obtained at the BHT concentration of 1.0 mM/mL, significantly different when compared to the controls, BHT concentrations of 2.0 and 3.0 mM/mL during the entire duration of semen quality evaluation. This was followed by general motility on day 3.

DISCUSSION

In this study, the effects of BHT added to BX, TEY and CEY on chilled bull semen were examined. It was found that optimum results were achieved when a certain amount of BHT was applied to a particular extender. Based on the results, 0.5 mM in BX and 1.0 mM/mL in TEY and CEY concentrations were found to increase sperm plasma membrane protection by improving the cells' normal morphology and viability and prevent acrosome damage when compared with the control groups. On the other hand, high BHT concentrations tended to produce opposing effects on the spermatozoa parameters in all the extenders. These findings (that BHT is useful at lower and detrimental at higher concentrations) are consistent with a number of previous studies done on different animals. BHT has been found to protect chilled semen in goats (Khalifa et al., 2008). Specifically, addition of BHT or other exogenous antioxidants have been found useful in chilled semen of various species of animals. Chilled turkey sperm viability was improved and loss of motility was reduced by addition of BHT (Donoghue & Donoghue, 1997). It was also reported that vitamin E, which is a natural analogue of BHT, improved viability of chilled canine spermatozoa (Michael et al., 2009). Moreover, selected antioxidants such as glutathione, cysteine and hypotaurine have been reported to improve viability and functional status in chilled boar semen (Funahashi & Sano, 2005).

Some researchers reported significantly increased motility in chilled semen of other animal species such as goat (Khalifa et al., 2008) and turkey (Donoghue & Donoghue, 1997) using BHT compared to the controls. In the present study, there was no significant difference between the treated groups and the control for general motility. However, the fact that BHT maintained consistently higher values of progressive motility at 0.5 mM in BX and 1.0 mM in TEY and CEY than the control may imply its potential

Table 4
Effect of BHT on Chilled Bull Semen Quality in Citrate Egg Yolk Extender Kept for 3 Days

Parameter %	Day	Control	BHT concentrations (mM/mL)				
			0.5	1.0	1.5	2.0	3.0
General motility	D1	49.80±5.46^{ab,x}	60.87±5.13^{a,x}	57.60±5.65^{ab,x}	52.33±4.67^{ab,x}	45.20±5.99^{ab,x}	42.47±5.65^{b,x}
	D2	36.27±3.94^{ab,y}	43.07±3.99^{a,y}	40.00±4.41^{ab,y}	33.80±3.78^{abc,y}	29.33±2.74^{bc,y}	23.33±3.18^{c,y}
	D3	22.33±3.30^{abc,z}	27.60±3.52^{ab,z}	29.87±3.08^{a,y}	24.27±3.44^{ab,y}	19.27±2.23^{bc,y}	14.20±2.54^{c,y}
Progressive motility	D1	23.00±3.46	25.67±2.66	27.44±3.16	25.11±3.60	18.78±3.32	17.00±3.73
	D2	18.11±3.43	18.89±3.16	21.67±4.35	18.11±4.15	13.00±3.31	11.44±3.51
	D3	15.44±4.02	17.89±3.89	18.89±3.95	15.78±4.21	11.89±3.45	10.22±3.33
Normal morphology	D1	89.17±0.21^{a,x}	90.00±0.25^{a}	89.00±0.43^{a}	88.33±0.71^{a}	69.17±2.35^{b,x}	64.17±1.28^{c,x}
	D2	86.00±0.98^{b,y}	89.83±1.35^{a}	87.67±0.57^{ab}	88.83±0.41^{ab}	63.33±1.50^{c,y}	58.67±0.38^{d,y}
	D3	83.33±1.44^{b,y}	86.33±1.85^{ab}	87.00±1.40^{a}	88.33±0.38^{a}	56.33±0.57^{c,z}	52.00±0.85^{d,z}
Viability	D1	58.00±1.50^{d,x}	67.00±0.65^{e,x}	78.33±1.88^{a}	73.00±1.50^{b,x}	57.33±0.99^{d,x}	54.83±1.17^{d,x}
	D2	50.67±0.28^{d,y}	65.33±0.62^{c,x}	74.83±1.24^{a}	69.00±1.76^{b,xy}	53.67±0.99^{d,y}	50.67±1.81^{d,x}
	D3	51.67±0.71^{d,y}	62.67±0.57^{c,y}	74.00±1.09^{a}	67.33±1.73^{b,y}	49.33±1.36^{d,z}	44.17±1.83^{e,y}
Acrosome damage	D1	17.33±1.58^{bc,x}	14.33±0.99^{cd,x}	9.17±0.77^{e,x}	11.67±0.14^{de,x}	19.33±1.26^{b,x}	24.33±1.48^{a,x}
	D2	20.33±1.78^{bc,y}	17.33±1.68^{c,y}	10.83±0.37^{d,y}	13.17±0.51^{d,y}	24.00±1.48^{ab,y}	27.33±1.36^{a,y}
	D3	25.67±1.86^{bc,y}	22.67±1.36^{c,y}	12.67±0.54^{e,y}	18.33±0.75^{d,y}	28.67±1.36^{b,z}	33.67±1.44^{a,y}

Data are expressed as Mean±Standard Error (SE) n=22. Different superscripts a-f within rows and x-z within column denote significant differences ($P<0.05$)

positive effect on progressive motility. Nevertheless, it is worth noting that bull semen is specifically more sensitive to refrigeration temperatures (between 4 and 8oC) compared with turkey and goat semen and, even though egg yolk is an excellent cold shock protectant, it cannot completely prevent cold damage in bulls (Parks, 2013) due to its high density lipoproteins. Therefore, we theorised that the damage caused by the low temperature might have affected sperm motility in this study, thereby masking the effect of BHT. Similarly, the measure of acidity or alkalinity (pH) is another factor that determines extracellular sperm motility (Carr & Acott, 1984). Even though decreases in sperm motility due to pH can be reversed by controlling the pH, it is unlikely that pH changes affected the differences observed in the current study since all the semen extenders were set at the common pH of 6.7 (De Pauw et al., 2003 ; Brannigan & Lipshultz, 2008).

The results of this study showed that most parameters were maintained without significant deterioration for three days at BHT concentrations of 0.5 in BX and 1.0 mM in TEY and CEY extenders, as opposed to other treatment groups and controls, where most parameters deteriorated daily. In this research, better results were achieved at a lower BHT concentration in BX than in TEY and CEY. This could be because while TEY and CEY extenders contain egg yolk as a cold shock protectant (Pace & Graham, 1974), Bioxcell® contains soybean lecithin instead, substituting egg yolk. Soybean-lecithin-based semen extenders such as AndroMed® and Biociphos Plus® have been reported to be superior in bull semen storage when compared with egg-yolk-based extenders (Akhter et al., 2010). This is not unconnected with the fact that soybean lecithin has a high content of low density lipoprotein, which makes it a better shock protectant and may require little synergism from an antioxidant. Moreover, density of the lipoproteins in the egg yolk used may affect sperm quality: low density lipoproteins protect spermatozoa from damage due to cold shock while high density lipoproteins in egg yolk result in reduction of respiration and sperm motility (Amirat et al., 2005). Since the quality of the egg yolk is not constant, the deleterious effect due to high density lipoproteins might have affected the spermatozoa in the TEY and CEY extenders. Furthermore, Khalifa et al. (2008) suggested that it is possible that BHT has higher solubility to egg lipids rather than the plasma membrane of the spermatozoa. Therefore, when BHT is added, much of its potency would affect the egg yolk, and very little would affect the spermatozoa membrane. This phenomenon may explain why higher concentrations of BHT were required for optimum effect in the egg-yolk based extender. However, if the BHT concentration had risen much higher than required such as 2.0 and 3.0 mM/mL in the current study, perhaps the BHT would have caused high fluidity within the cytoplasm of the spermatozoa beyond a critical limit, causing it to rupture.

The present study indicated less sperm damage during preservation in the TEY

than in the CEY semen extender. Even though the reason is not well understood, this is in line with previous studies carried out in other species such as buffalo bulls and rams, where significantly better sperm quality in TEY compared to CEY in chilled spermatozoa was seen (Akhter et al., 2010; Rakha et al., 2013).

CONCLUSION

In conclusion, addition of BHT at 0.5 concentration in Bioxcell and 1.0 mM/mL to Tris egg yolk and citrate egg yolk extenders improved chilled bull semen quality parameters, signalling that addition of BHT at this concentration has potential for protecting sperm progressive motility.

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Pertanika J. Trop. Agric. Sci. 40 (1): 143 - 160 (2017)

PERTANIKA JOURNALS

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Physicochemical Characteristics of Oil Palm Frond (OPF) Composting with Fungal Inoculants

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ABSTRACT

This investigation highlights the chemical and physical properties of oil palm frond (OPF) observed during 14 weeks of composting. Composting is a controlled biological decomposition process that converts organic waste into humus-like material. Two white rot fungi species, *Trametes versicolor* and *Schizophyllum commune*, were introduced as inoculants for the composting process. Oil palm fronds (OPF) were composted for 14 weeks, with four treatments, i) control (untreated OPF), ii) OPF treated with *T. versicolor*, iii) OPF treated with *S. commune*, iv) OPF treated with both *T. versicolor* and *S. commune*, with four replications. The resulting compost was brown in colour with a homogenous appearance, and no unpleasant odour was detected. In this study, C/N ratio and percentage volume reduction became the most important parameters to be monitored. Inoculation with *S. commune* achieved the acceptable C/N ratio of 63.2 at the end of the composting period. Compared to other treatments, single inoculation of *S. commune* indicated a higher percentage of volume reduction with a value of 62.8%. Single inoculation of *S. commune*, therefore, provides a suitable medium for composting of OPF.

Keywords: composting, oil palm frond, white-rot fungi, inoculants, compost

INTRODUCTION

In Malaysia, cultivated oil palm covers 4.49 million hectares with a production of 17.73 MnT of palm oil and 2.13 tonnes of palm kernel oil (MPOC, 2011). The oil palm industry is a major provider of employment in Malaysia. The addition of cultivated areas from 1.02 million ha in 1980 to 4.24 million ha in 2007 expanded the workforce from 92,352 workers in 1980 to about 405,000 workers in 2007 (Yusof, 2008). According to Salathong (2007), one hectare of oil

ARTICLE INFO

Article history:
Received: 18 April 2016
Accepted: 10 November 2016

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ISSN: 1511-3701

palm plantation can produce about 50 to 70 tonnes of biomass residue, while its oil consists only 10% of the biomass produced in the plantation. Hassan and Yacob (2005) reported that the main contributor of biomass in Malaysia is the palm oil industry, and this biomass consists of empty fruit bunch (EFB), palm oil mill effluent (POME), mesocarpfiber, palm kernel shells and residue of palm kernel cake. Oil palm fronds (OPF) are normally left to rot in between oil palm trees in plantation sites or as mulching component. Fronds can be recycled as mulch, paper pulp and animal feed (Chan, 1999). Oil palm fronds cut during pruning and harvesting can be processed into green feed or silage, and the fibrous characteristic is similar to rice straw (Abu Hassan & Yeong, 1999). The possible uses of OPF in Malaysia still lack attention; however, several studies have been carried out regarding fronds as ruminant feed (Abu Hassan et al., 1996), herbivore feedstock (Dahlan, 2000), pulp production (Wanrosli et al., 2007), fuel pellet (Trangkaprasith & Chavalparit, 2010) and pressed juice (Zahari et al., 2012).

Composting of organic waste is now seen as an option for restoring soil health, building soil environment complex and achieving sustainability in agricultural production. Composting is an alternative method of dealing with agricultural and industrial waste produced worldwide. Transformation of agricultural waste into compost is one of the validated recycling methods; it is also a good way to produce bio-fertilisers and soil conditioners. The stable composted product helps in replenishment of plant nutrients, maintenance of soil organic matter and improving the physical and microbial properties of soil. In nature, animal and plant residue will undergo complex microbial degradation and transformation by various microbiological processes. Panda and Hota (2007) concluded that the biodegradation of biomass is carried out by different groups of heterotrophic microorganisms, bacteria, fungi, actinomycetes and protozoa.

The objectives of this research were to investigate the usefulness of oil palm fronds (OPF) as composting material with a microbial system and to assess the suitability of filamentous fungi as decomposing agents of frond waste. In this study, the inoculants of *Trametes versicolor* and *Schizophyllum commune* were introduced in composting of OPF as decomposing agents. The physical and chemical changes that occurred during 14 weeks of composting were observed.

MATERIALS AND METHODS

Preparation of Composting Media

Pruned oil palm fronds (the petiole and leaflets) were obtained from a plantation in Batu 10, Jalan Sungai Batang, Sandakan. These collected fronds were already about four weeks into decomposition, providing a suitable inoculation medium than freshly pruned fronds. The frond samples were manually shredded to reduce their size to about 3 to 5 cm for efficient microbial action during the decomposition process. Shredded OPF were dried in a drying oven (Memmert, Germany) at 60°C for 24 h and ground prior to analytical tests. Fungal strains of

Trametes versicolor and *Schizophyllum commune* were selected as inoculants for the composting purposes. *T. versicolor* and *S. commune* were used as inoculants due to their ability as white rot fungi to degrade components of lignin, hemicellulose and cellulose in woody materials. They were maintained on potato dextrose agar (PDA, Merck), prepared and sterilised according to the manufacturer's instructions for strain characterisation.

Decomposition of OPF Waste

Composting of shredded frond waste (500 g on dry basis) was carried out in a white polystyrene box measuring 30 cm (length), 30 cm (width) and 15 cm (height) using Complete Randomised Design (CRD). The compost mixture was left for 14 weeks (98 days). Turning was done once every 10 days to mix the outer and inner part of the compost. A volume of 100 mL of water was sprayed on each compost mixture to maintain their moisture level. The experiments were conducted in an open area with ambient temperature (27 to 30°C), and consisted of a control (un-inoculated fronds), inoculated fronds with *T. versicolor*, inoculated fronds with *S. commune* and co-inoculated fronds with *T. versicolor* and *S. commune*, with four replicates each. Their weight was recorded as initial mass before being decomposed further with the selected fungi. The inoculations were carried out by direct transfer of agar block (1 cmx1 cm) and the microbial suspension method (Haddadin et al., 2009; Zeng et al, 2009; Wang et al., 2011).

Chemical Analysis

A total of 20 g of composted samples was collected from each box every three days in the first week and then at intervals of 12 to 15 days until the end of the experiment to measure the physical and chemical changes in the material. All analyses were done in triplicate and data presented as mean values with standard deviation.

Macronutrient, Micronutrient and Heavy Metal Content. Analysis of selected micronutrients and heavy metals (Pb, Cd, Cu, Ni, Zn and Fe) and macronutrient (Mg) were carried out using inductively coupled plasma-optical emission spectroscopy, ICP-OES (Perkin Elmer, USA) based on the aqua-regia method. The digestion was made using hydrochloric acid (HCl) and 65% nitric acid (HNO3) solution in a volume ratio of 3:1.

pH Determination. The pH was determined in a suspension of 1:10 (w/v) compost and distilled water using a pH meter (Mettler Toledo FE20).

Temperature Measurement. Temperature was measured daily during composting at specific times (day 1, 5, 14, 25, 37, 48, 57, 70, 84, 97). Measurement was made at four different points in the composting heaps, at the corner and centre of the heaps using a thermometer. The thermometer was placed inside the compost heaps to about approximately 3 to 4 inches, for 5 min before the data were recorded.

Moisture Content Determination. Compost moisture content was determined by gravimetric method whereby 10 g of compost was air-dried at ambient temperature overnight before samples were placed in the oven at 105°C for 24 h. The samples were then removed from the oven and placed at room temperature to cool off. The weight of the oven-dried compost was recorded.

C/N Ratio Determination. Organic C was determined according to the combustion method whereas total N was determined using the Kjeldahl method. In the combustion method, 3 g of the oven-dried (60°C) sample was thoroughly ground and sieved through a 0.2-mm sieve and then transferred to a crucible and weighed accurately. The contents of the crucible were ignited in a furnace (Thermo Scientific, USA) at 550°C for 4 h. The crucible was cooled in a desiccator and the loss in weight during ignition was calculated. As for total N determination, the Kjeldahl method was divided into three steps: i) sample digestion in digestion block (Gerhardt, Germany) at 180°C for 1 h and 320°C for 4 to 5 h until the content became colourless, ii) distillation of 10 mL of sample and 10 mL of 40% (w/v) NaOH in a distillation apparatus (Büchi, Switzerland) and the distillate was collected in 10 mL of 2% boric acid-indicator solution, iii) the collected distillate was titrated against 0.01 M H2SO4 until the colour changed from green to purple.

Physical Characteristics

The physical characteristics of composted OPF were observed throughout the composting period. The criteria monitored were compost texture and colour displayed. Percentage volume reduction of composted OPF was evaluated at the end of the composting process, by taking the difference between the volume of the composting material at the beginning and volume of the compost after 14 weeks of the composting period. Percentage of volume reduction was calculated using the procedure suggested by Kala et al. (2009). The calculations took into account all the composted samples (in gram) that were removed throughout the composting period.

Compost Maturity Indices

In order to evaluate the maturity of the composted OPF, the germination of mustard (*Brassica chinensis*) and mungbean (*Vigna radiata*) seeds in extracts of the compost were analysed by the method proposed by Tam and Tiquia (1994) and Zeng et al. (2009). Glass petri dishes were used for the experiment. A fresh compost sample was extracted with distilled water at a compost to water ratio of 1:10 (w/v) and shaken for 1 h and then filtered. The compost was allowed to settle for approximately 20 min. The top solution was skimmed off and filtered leaving the filtrate (extract). For the germination experiments, 7 mL aqueous extract was dispensed into glass petri dishes lined with Whatman filter paper.

Experiments were conducted in triplicate and distilled water was used as the control. Ten seeds were placed in each dish and then incubated at 27°C in the dark. The dishes were labelled. Seed germination and root length were measured after three days. The germinated seeds were counted and of these, the length of the radicle, the part that looks like a root, was measured properly. The end point of the germination process was considered when the seeds started to germinate with a primary root ≥5mm.The relative seed germination (SG), relative root elongation (RE) and germination index (GI) were calculated as suggested by Zeng et al. (2009):

SG (%) = (number of seeds germinated in extract / number of seeds germinated in control) x 100

RE (%) = (mean root length in extract / mean root length in control) x 100

GI = [SG (%) x RE (%)] / 100

Statistical Analysis

Statistical analysis was carried out using the SPSS 21 for Windows. All experimental data were analysed statistically using analysis of variance (ANOVA). Duncan's Multiple Range Test was used for comparison of treatment means when F values were significant at $p<0.05$.

RESULTS AND DISCUSSION

In this study, the whole branch of OPF consisting of petiole and leaflets was selected as composting material. In composting, the particle size of the substrate influences the decomposition time and the extent to which fungi inoculants penetrate the substrate. Thus, a smaller size of OPF was required to maximise the surface area of the substrate for efficient decomposition by introduced and indigenous microorganisms. Morphologically, OPF fibre contains a huge amount of hemicellulose but lacks lignin. It has a comparatively high ash content, with polysaccharide monomers made up of glucose and xylose and a low concentration of arabinose, mannose and galactose (Wan Rosli et al., 2007). Zahari et al. (2012) found that the content of cellulose, hemicellulose and lignin in fresh OPF was 41.7%, 16.4% and 15.5%, respectively. Table 1 shows some of the chemical characteristics of oil palm frond waste used in composting.

Table 1
Some Chemical Characteristics of Oil Palm Frond Waste Used in Composting

Parameter	Composting substrate
	Oil palm frond (OPF)
pH	6.35±0.15
Moist. cont. (%)	53.93±2.25
TOC (%)	48.07±0.08
N (%)	0.59±0.04
C/N	82.2±6.02
Mg ($g.kg^{-1}$)	0.06±0.01
Cu ($mg.kg^{-1}$)	4.89±3.12
Zn ($mg.kg^{-1}$)	1.10±0.09
Fe ($mg.kg^{-1}$)	7.24±0.35
Ni ($mg.kg^{-1}$)	0.02±0.01
Cd ($mg.kg^{-1}$)	0.04±0.01
Pb ($mg.kg^{-1}$)	2.18±0.37

*Moist. cont. - moisture content
Data include mean±SD of four replicates

Turning was done once every 10 days and watering was done every two days to prevent dryness. Turning of compost helps to break up clumps of compost and provides new surfaces for microbial attack; however, it must be carried out carefully and not too often to avoid interference with growth of some microorganisms such as filamentous fungi. The moisture content of 50-60% will encourage dispersal of inoculated fungi, and it was observed that when moisture content was low (below 30%), fungi dispersal was retarded. Diaz et al. (2002) proposed that turning not only allows aeration in the windrows, but also facilitates in producing homogenous compost mass. The chemical and physical characteristics of OPF after 14 weeks of composting were evaluated and the results are summarised in Table 2.

Macronutrient, Micronutrient and Heavy Metal Content

The presence of Ni, Cd and Pb at high concentration enhances toxicity levels as they are absorbed by the plants and then introduced into the food chain system. High concentration of these elements in the plant samples might have been contributed by improper waste or land management and application of excessive fertilisers. Kala et al. (2009) found that addition of sludge to oil palm waste significantly increased heavy metal content due to higher heavy metal content in sewage sludge. The analysis of

Table 2
Chemical Characteristics of Composts Obtained After 14 Weeks of Composting

Parameter	Treatment			
	T1	T2	T3	T4
pH	5.80±0.46 a	5.97±0.07 a	6.78±0.10 b	6.53±0.05 b
Vol. red.	50.4±3.37 a	57.5±5.27 bc	62.8±6.03 c	54.3±1.24 ab
Moist. cont.	58.26±0.27 ab	58.19±0.47ab	58.55±0.22 b	57.85±0.37 a
TOC%	48.66±1.74 a	50.33±1.42 ab	51.30±0.48 b	51.88±0.33 b
N%	0.64±0.01 b	0.70±0.02 c	0.81±0.01 d	0.59±0.02 a
C/N	75.7±2.62 a	72.4±5.42 a	63.2±1.39 a	88.6±6.15 b
Mg ($g.kg^{-1}$)	0.07±0.00 c	0.06±0.00 a	0.07±0.01 d	0.07±0.00 b
Cu ($mg.kg^{-1}$)	0.95±0.85 c	0.38±0.11 a	0.34±0.19 a	0.67±0.34 b
Zn ($mg.kg^{-1}$)	1.55±0.17 b	4.49±0.85 c	0.90±0.12 a	0.94±0.13 a
Fe ($mg.kg^{-1}$)	8.87±0.02 d	7.46±0.08 c	6.26±0.07 b	6.16±0.02 a
Ni ($mg.kg^{-1}$)	0.04±0.01 c	0.06±0.01 d	0.03±0.01 b	0.01±0.01 a
Cd ($mg.kg^{-1}$)	0.03±0.00 a	0.04±0.02 a	0.03±0.01 a	0.04±0.01 a
Pb ($mg.kg^{-1}$)	6.45±0.15 d	0.46±0.11 a	4.91±0.05 b	5.52±0.08 c

T1, T2, T3 and T4 represent the control (without inoculation), inoculation of *T. versicolor*, inoculation of *S. commune* and co-inoculation of *T. versicolor* and *S. commune*, respectively
*Vol. red.-volume reduction; moist. cont. - moisture content
**Data include mean±SD of four replicates. Means with a different letter within the row indicate significant differences (p<00.5) using Duncan's multiple range test

heavy metal was important in this study as good compost should contain little or almost no heavy metal that can induce phytotoxic effects on plant growth in the field. Micronutrient and heavy metal content observed in the OPF compost was within the safety limit for general agricultural use as outlined by HKORC (2005) and TAS (2005).

pH

At the end of 14 weeks of composting, pH for the control (un-inoculated) was reduced to 5.80, inducing higher acidity compared to the initial pH of 6.37. This decrease in pH probably occurred due to organic acids being released when fungi and bacteria digested the organic materials, as described by Kala et al. (2009). The OPF inoculated with *T. versicolor* recorded a pH of 5.97 at the end of composting. There were slight increases in pH observed for OPF treated with *S. commune* and co-inoculated with both species (*T. versicolor* and *S. commune*), which gave pH values of 6.78 and 6.53, respectively. The control (un-inoculated) treatment had no significant difference when compared with inoculation of *T. versicolor* at $p<0.05$; however, both were significantly different in comparison to inoculation of *S. commune* and co-inoculation of both species ($p<0.05$) based on Duncan's multiple range test. Haddadin et al. (2009), who studied olive pomace composting, stated that the increase of pH value from 4.0 to 6.7 in treatment bioreactors could be influenced by degradation of organic acids and production of ammonium. Liu et al. (2011) added that decreasing pH was probably due to organic acid production and incomplete oxidation of organic matter. The decrease in pH might have occurred due to mineralisation of nitrogen and organic acids and easily degradable carbon sources, such as monosaccharide and lipids (Nair & Okamitsu, 2010).

Temperature Fluctuation

Figure 1 illustrates the changes in temperature of the composting substrate during 14 weeks of composting. The highest temperature for all composting substrates could be observed on day 70; un-inoculated fronds recorded 30°C, whereas the inoculated fronds with *S. commune* and *T. versicolor* were 34°C and 35°C, respectively. The temperature for co-inoculated frond (*S. commune* and *T. versicolor*) also showed 35°C, which was the highest point reached during the whole composting process.

Tuomela et al. (2000) pointed out that in the composting mass that achieved

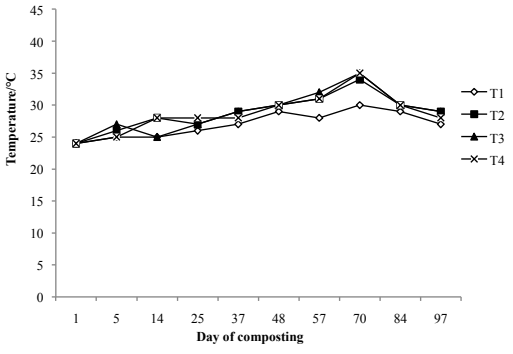

Figure 1. Changes in temperature of the composting substrate during 14 weeks of composting. T1, T2, T3 and T4 represent the control (without inoculation), inoculation of *T. versicolor*, inoculation of *S. commune* and co-inoculation of *T. versicolor* & *S. commune*, respectively

50°C elevation, the microbial population started with thermophilic fungi and was continued by bacteria and actinomycetes, which developed on the fungal mycelium. In this study, the temperature did not reach the thermophilic phase (>45°C) in all the treatments, probably because of heat dissipation due to the small volume of substrate used (Kala et al., 2009). Nair and Okamitsu (2010) stated that the compost needed to be kept at 55°C for 15 days for efficient maturity and pathogen reduction; however, their compost thermophilic phases only lasted a week at the maximum temperature of 55°C for two days only. This was commonly observed in a system that used small volumes since they were prone to temperature fluctuation and heat loss contributed by high surface-to-volume ratio (Nair et al., 2006; Nair & Okamitsu, 2010). Taiwo and Oso (2004) concluded that large heaps generally generate high temperature, while small heaps generate lower temperature, and suggested the use of plastic pots as they conserve the heat produced by microorganisms.

Moisture Content

At Week 1, the initial moisture content of the control (un-inoculated OPF), inoculation with *T. versicolor*, the inoculation with *S. commune* and the co-inoculated fronds recorded moisture content of 53.90%, 54.80%, 54.05% and 52.97%, respectively. The moisture content ranging from 50% to 55% was recommended for composting (Riahi & Fakhari, 2004). The high moisture content at initial composting times would encourage fermentative metabolism, which produces incomplete decomposed product such as organic acid, therefore reducing the compost pH (Liu et al., 2011). Apparently, after 14 weeks of composting, moisture content for the control (un-inoculated OPF) was 58.26%, while for the inoculation with *T. versicolor* and *S. commune*, the moisture content recorded was 58.19% and 58.55%, respectively. Co-inoculated fronds with both species (*T. versicolor* and *S. commune*) gave moisture content values of 57.85%, which was the lowest compared to other treatments. This slightly lower value might be due to vaporisation in compost treatment (Tsai et al., 2007). In spite of that, it was observed that the moisture content of all treatments increased gradually compared to their initial moisture content at Week 1. This could be due to the presence of water vapour inside the composting materials that resulted from the gradual watering process. Aini (2005) elaborated the squeeze ball test, in which moisture can be analysed by squeezing a ball of compost taken from the centre of the heap. If the ball crumbles at a slight tap of the finger, then the moisture is adequate. If water oozes through the clenched fingers, this indicates too much moisture, and if the compost does not form a ball at all, then it is too dry. Haddadin et al. (2009) mentioned that enough moisture in a compost pile will promote biological activity without obstructing the amount of accessible oxygen in the pile. Nevertheless, moisture content exceeding 70% would be unfavourable to fungal growth as fungi rarely survive in an environment with too

high a moisture content (Thambirajah, 1994).

C/N Ratio

Figure 2 summarises the mean percentage of TOC in the OPF compost during 14 weeks of composting.

In all treatments, except T2 (inoculation with *T. versicolor*) showed a similar pattern

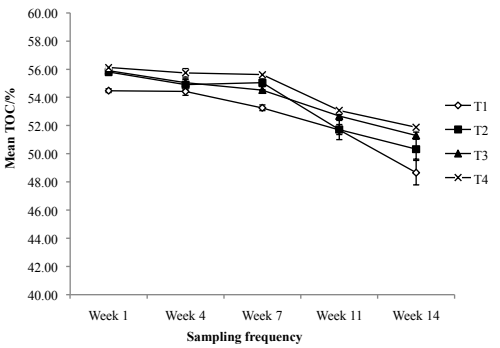

Figure 2. Mean percentage of TOC in composted OPF during 14 weeks of composting. T1, T2, T3 and T4 represent the control (without inoculation), inoculation of *T. versicolor*, inoculation of *S. commune* and co-inoculation of *T. versicolor* & *S. commune*, respectively

of decreasing TOC as the composting continued until the end. This was correlated with findings by Lopez et al. (2006), Goyal et al. (2005) and Fang et al. (1999), who reported that organic carbon content decreased as decomposition proceeded due to conversion of TOC to carbon dioxide (CO_2). During decomposition of compost substrate, the mineralisation of organic matter causes a reduction in TOC values (Liu et al., 2011). Bernal et al. (1998) stated that a decrease in organic matter and organic carbon in waste mixtures reflected the degradation of organic materials during the composting process. The highest value of TOC reflected the presence of recalcitrant carbon compounds, such as lignin, which is slowly decomposed (Goyal et al., 2005). At the end of the sampling, the highest TOC recorded in this study was in the OPF compost co-inoculated with *T. versicolor* and *S. commune* with a value of 51.88%. Interestingly, the TOC value for un-inoculated OPF was the lowest at 48.66%, indicating that the rate of substrate decomposition was the highest in that treatment when compared to inoculated OPF. This could have been due to the environmental and nutrient conditions that favoured the growth of indigenous micro-organisms inside the heap even though no inoculants were added.

Figure 3 illustrates the percentage of nitrogen (N) in the OPF compost during 14 weeks of composting. Before the composting started, the N content of the OPF compost was recorded as 0.59%. During the first week of composting, the N content varied from 0.18% to 0.62% in all the treatments. The high N content of 0.62% in T1 (un-inoculated OPF) could be observed at Week 1 possibly due to indigenous microbial activity that resulted in N accumulation. The N content was recorded in a range of 0.18% to 0.22% at the end of Week 4 for all un-inoculated and inoculated treatments. Goyal et al. (2005) discussed that although total N content decreased at early stages of organic waste composting due to nitrogen losses, the main factors were material used

and its initial C/N ratio. Ammonia is highly soluble in water; however, in low moisture content, the ammonium and ammonia present in composting substrate will induce higher vapour pressure, which is most likely to cause nitrogen loss (Hubbe et al., 2010). At Week 7, the treatment of control, inoculation with *T. versicolor*, inoculation with *S. commune*, and co-inoculation with both species recorded 0.24%, 0.15%, 0.20% and 0.14% of N content, respectively.

During Week 11 and Week 14, there were significant changes in N content, as the values increased rapidly towards the end of composting. In co-composting of sewage sludge with coal fly ash, Fang et al. (1999) claimed that total N content increased for all treatments due to a net loss of dry mass. Bernal et al. (1998) also suggested that total N increased due to concentration effect caused by strong degradation of labile organic carbon compound, which also reduced the weight of composting materials. Piškur et al. (2011) observed that N content in wood chips increased (0.14% in fresh wood chips to 0.36% in aged chips) in a 17-month period due to an increase in microbial biomass and N accumulation. In vermicomposting, Pramanik et al. (2007) explained that a smaller value of C/N ratio would accelerate the organic waste decomposition rate, causing the increment of total Kjeldahl nitrogen (TKN) content in vermicompost. At the end of composting, the N values for control, inoculation of *T. versicolor*, inoculation of *S. commune* and co-inoculation of *T. versicolor & S. commune* were 0.64%, 0.70%, 0.81% and 0.59%, respectively. After 14 weeks of composting, it was observed that all treatments of un-inoculated and inoculated OPF were significantly different ($p<0.05$) in N content according to Duncan's multiple range test.

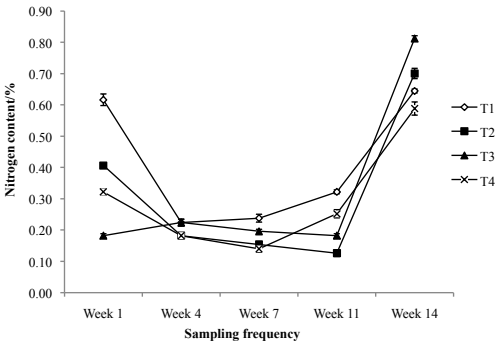

Figure 3. Mean percentage of nitrogen in composted OPF during 14 weeks of composting. T1, T2, T3 and T4 represent the control (without inoculation), inoculation of *T. versicolor*, inoculation of *S. commune* and co-inoculation of *T. versicolor* and *S. commune*, respectively

The C/N ratio of raw OPF was 82.2 at the beginning of the study, which was higher than expected. Based on previous research, it was quantified that OPF C/N ratio was 61.0 (Aini, 2005) and 69.7 (Kala et al., 2009). According to Rao (2007) and Haddadin et al. (2009), C/N ratio of the composting substrate influenced the length of the composting period and degree of compost maturity. In the case of substrate with initial C/N ratio of 25, the composting process could be carried out in a relatively short period. Figure 4 summarises the C/N ratio of OPF compost at each sampling time throughout the 14 weeks of composting. At the first week of composting, C/N ranged

from 88.8 up to 307.3 and continued to increase until Week 4 with a C/N of 249.0 to 310.3. The similar trend could be observed in Week 7 of composting, in which the C/N of the control, inoculation of *T. versicolor*, inoculation of *S. commune* and co-inoculation of *T. versicolor* and *S. commune* were 221.3, 362.8, 280.5 and 405.3, respectively. Goyal et al. (2005) found that C/N ratio of poultry manure increased as a result of N losses mostly through ammonia volatilisation. In Week 11, treatments of the control, inoculation of *T. versicolor*, inoculation of *S. commune* and co-inoculation of *T. versicolor* and *S. commune* recorded C/N ratios of 160.4, 422.0, 289.7 and 211.8, respectively.

After 14 weeks of OPF composting, the C/N ratio of the end product varied from 63.2 to 88.6, and the highest value was recorded in treatment T4 (co-inoculated with *T. versicolor* and *S. commune*, while the lowest of 63.2 was recorded in treatment T3 (inoculated with *S. commune*). This observation is in agreement with those reported by Kostov et al. (1991) that after six months of decomposition, the C/N ratio of bark reduced from 251 to 61, while for sawdust the decrease was from 473 to 41. This reduction in C/N ratio showed significant microbial activity present in the substrate used (Kostov et al., 1991). Nair and Okamitsu (2010) detected that in the first seven days of kitchen waste composting, the initial C/N ratio of 79.1 was decreased to <35.0, probably due to carbon loss, which directly correlates to volume reduction in the composting substrate. Rao (2007) stated that in composting, irrespective of substrate used, the C/N ratio will drastically decrease in the final product compared to the initial C/N value. Goyal et al. (2005) mentioned that as decomposition proceeded with time, the carbon content in compost materials decreased and nitrogen per unit material increased, resulting in a decrease of C/N value. Liu et al. (2011) also agreed that C/N ratio decreased during composting due to carbon loss and nitrogen content increment per unit material. In the case of wood degradation, mineralisation of carbon components and nitrogen accumulation led to a decrease in the C/N ratio for beech wood chips from 360 (starting material) to 230 after six months and 160 after 17 months (Piškur et al., 2011). It was observed that at the end of composting, all treatments were significantly different in their C/N ratio except for treatment of T1 (un-inoculated OPF) and T2 (inoculation of *T. versicolor*)

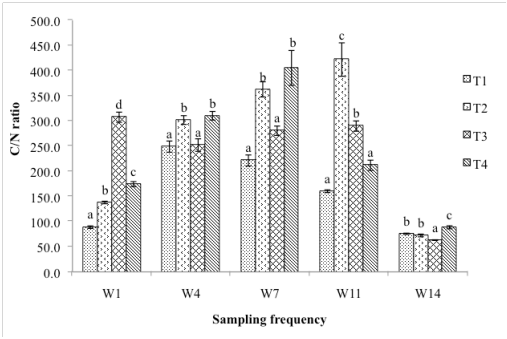

Figure 4. C/N ratio of composted OPF during 14 weeks of composting. T1, T2, T3 and T4 represent the control (without inoculation), inoculation of *T. versicolor*, inoculation of *S. commune* and co-inoculation of *T. versicolor* & *S. commune*, respectively. Columns displaying different letters are significantly different ($p<0.05$) according to Duncan's multiple range test

at p<0.05, based on Duncan's multiple range test. In this experiment, the C/N ratio of OPF inoculated with *S. commune* was the lowest (63.2), which, remarkably, corresponded with the highest volume reduction in the same treatment of T3 with 62.8% (Figure 5). Based on these results, it was found that single inoculation of *S. commune* gave the best decomposition rate in OPF composting.

Volume Reduction

Figure 5 illustrates the mean volume reduction of compost recorded during the 14 weeks of the composting process.

The maximum volume reduction of 62.8% was recorded in OPF treated with *S. commune,* whereas the least volume reduction of 50.4% was in the control (un-inoculated OPF). Treatments of T2 (inoculation of *T. versicolor*) and T4 (co-inoculation of *T. versicolor* and *S. commune*) recorded a volume reduction of 57.5% and 54.3%, respectively. All treatments had no significant difference in their percentage volume reduction, except for T1 (control) and T3 (inoculation of *S. commune*), which were significantly different at p<0.05 based on Duncan's multiple range test. In this study, it could be summarised that *S. commune* inoculant degraded OPF efficiently when compared to the other treatments. According to Kala et al. (2009), the percentage of volume reduction depends on starting materials used and initial moisture content in the compost pile. In municipal solid waste compost, it was recorded that the weight reduction of waste was more significant in the first week of composting, and this was attributed to maximum microbial activity during this period (Gautam et al., 2010). In this study, although no inoculant was introduced in the control treatment, the presence of an adequate population of microorganisms helped to decompose the frond substrate progressively. Biologically, it was found that inoculants helped in wood decomposition by penetrating inside the wood components and with their enzymatic reaction, the composting proceeded rapidly.

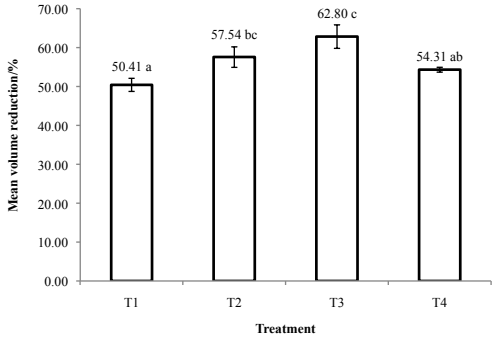

Figure 5. Mean volume reduction of the composting substrate during 14 weeks of composting. T1, T2, T3 and T4 represent the control (without inoculation), inoculation of *T. versicolor*, inoculation of *S. commune* and co-inoculation of *T. versicolor* & *S. commune*, respectively. Columns displaying different letters are significantly different (p<0.05) according to Duncan's multiple range test

Germination Index Analysis

Table 3 illustrates percentage seed germination (SG), root elongation (RE) and germination index (GI) for mungbean and mustard seeds, respectively, at the end of the composting period.

Compost obtained through biodegradation of the OPF promoted seed germination.

Table 3
Percentage Seed Germination (SG), Root Elongation (RE) and Germination Index (GI)

Treatment	Mungbean			Mustard		
	SG (%)	RE (%)	GI	SG (%)	RE (%)	GI
T1	96.7±5.77	109.6±18.36	106.0±11.81	108.0±0.01	94.1±11.91	101.6±12.87
T2	100.0	95.4±5.17	95.4±5.16	100.0±18.34	123.5±27.65	123.5±33.61
T3	100.0	92.3±5.19	92.3±5.18	96.0	78.9±2.91	75.7±2.80
T4	100.0	114.1±17.32	114.1±17.32	92.0±6.93	105.3±13.45	96.9±18.76

for Mungbean and Mustard Seed
T1, T2, T3 and T4 represent the control (without inoculation),inoculation of *T. versicolor*, inoculation of *S. commune*, and co-inoculation of *T. Versicolor* and *S. commune*, respectively.
*Test results were expressed as % control

These results were in contrast with those of Mitelut and Popa (2011), in whose study biodegradable compost from synthetic solid waste inhibited radish seed germination. It was found that all their tested compost was toxic compared to the control, which had a germination rate of 95% and 100%. In this study, the GI of un-inoculated compost for mungbean and mustard was 106% and 102% of the control, respectively. Surprisingly, some of the GI values in these tests were recorded above 100% of the control. All the GI above 100% reflected that the compost had a higher number of total germinated seeds and mean root length when compared to the control (distilled water). Kutsanedzie et al. (2012) observed that in finished compost from agricultural waste and poultry manure, GI exceeded 150%, showing the absence of phytotoxicity in the final compost. In evaluating olive pomace compost, Haddadin et al. (2009) observed that wheat seed germinated up to 100% in a treated sample; however, no germination was found in the control. Wang et al. (2011) reported that by the end of straw-cattle manure composting, Run 1 (un-inoculated) exhibited 100% of GI, while Run 2 and 3 (inoculated with *Penicillium expansum*) achieved 150% of GI. The high GI of inoculated samples implied that phytotoxic substances such as NH_3 and organic acid were removed faster in that respective sample (Wang et al., 2011).

van Heerdeen et al. (2002) found that cress-seed germination index increased gradually with maturation of the compost from 0% at days 1, 10 and 25 to 77% at day 90, showing a decrease in phytotoxicity with time. Mitelut and Popa (2011) performed radish seed germination using different concentrations of the compost extract (25%, 50%, 75% and 100%), and concluded that the increase in compost concentration affected radish seed germination. A study by Riffaldi et al. (1986) showed that GI increased faster during the first 20 days, and after 30 days of composting, phytotoxicity almost completely disappeared in waste water sludge compost. Diaz et al. (2002)

observed that a decrease in phytotoxicity during composting resulted perhaps from a breakdown of toxic substances in compost.

It was observed that the resulted compost was brown in colour with a homogenous appearance and no unpleasant odour was detected. This was in agreement with studies by Pacheco et al. (2010), in which the organic waste compost had a homogenous appearance, was brown in colour and emitted a fungal odour. As summarised by Aini (2005), at the end of composting, the temperature slowly recedes to ambient temperature, the compost turns brownish black and the original constituents are no longer recognisable. Based on the high germination index of mungbean and mustard seeds in composted OPF, it is obvious that the OPF compost has potential for use in soil amendment as it leads to decrease in phytotoxicity levels over time.

CONCLUSION

Oil palm frond (OPF) is an eco-friendly compost that contains appropriate amounts of micronutrients and heavy metals; therefore, it is safe for general agricultural use. In this study, although all parameters correlated with each other, C/N ratio and percentage volume reduction became the most important parameters to be monitored. Inoculation with *S. commune* promoted the acceptable C/N ratio of 63.2 at the end of the composting period. Compared to other treatments, single inoculation of *S. commune* indicated a higher percentage of volume reduction, with a value of 62.8%. Therefore, single inoculation of *S. commune* is suggested as a medium in OPF composting. Nevertheless, further studies are required to discover a suitable species or strain of microorganism specific to OPF as a substrate and to enhance the composting process for generating compost of high chemical and physical quality.

ACKNOWLEDGEMENT

The first author gratefully acknowledges the award of the UMS Graduate Teaching Assistance Scheme Scholarship from Universiti Malaysia Sabah during the course of this study.

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Pertanika J. Trop. Agric. Sci. 40 (1): 161 - 172 (2017)

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Journal homepage: http://www.pertanika.upm.edu.my/

Morphological and Molecular Identification of Sea Cucumber species *Holothuria scabra, Stichopus horrens* and *Stichopus ocellatus* from Kudat, Sabah, Malaysia

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ABSTRACT

The abundance of sea cucumbers (Phylum Echinodermata: Class Holothuroidea) in Malaysian waters has been gradually declining in past decades. Due to the lack of recent studies on the status of sea cucumber populations in Kudat, Sabah, Malaysia, this study was conducted. This study aimed to identify the species of a *timun laut* morphospecies i.e. *Holothuria (Metriatyla) scabra* and two *gamat* morphospecies i.e. *Stichopus horrens* and *Stichopus ocellatus* that were collected from Limau Limawan based on ossicle shapes and non-protein-coding 12S mitochondrial rRNA gene sequences. A number of five main ossicle shapes were microscopically identified without microscopic size measurement i.e. rod, plate, rosette, button and table. However, a number of ossicle shapes for *S. horrens* and *S. ocellatus* recorded in the previous studies were not observed in this study and this could be due to the body deformation of the specimens. Interestingly, five specimens of *H. scabra* exhibited additional ossicle shapes other than the smooth button and the table. Despite the absence of common ossicles and the presence of additional ossicle shapes, 12S mitochondrial rRNA gene sequences analysed using the Basic Local Alignment Search Tool programme for Nucleotide (blastn) resulted in the species identification of the specimens of morphospecies *H. scabra* and *S. horrens* as *H. scabra* and *S. horrens*; however, the specimen of morphospecies *S. ocellatus* was identified only up to the genus level i.e. genus *Stichopus*, showing the lack of 12S mitochondrial rRNA gene sequences of *S. ocellatus* in the GenBank until 15 September, 2016. In total, 31 partial sequences of 12S mitochondrial rRNA gene were registered with the GenBank (Accession No.: KX913672-KX913702). The findings also suggested that species identification based on 12S mitochondrial rRNA gene sequence

ARTICLE INFO

Article history:
Received: 28 April 2016
Accepted: 10 November 2016

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ISSN: 1511-3701

showed better inference than the ossicle shape identification. In summary, the three morphospecies were morphologically and genetically verified as *H. scabra, S. horrens* and *S. ocellatus*. Despite the fact that more specimens and more molecular techniques are required to generate better conclusive outcomes, the current findings give better insight into the importance of morphological and molecular approaches and the present status of the *timun laut* species and *gamat* species in Kudat.

Keywords: 12S mitochondrial rRNA gene, *gamat, Holothuria scabra*, Kudat, ossicle shape, *Stichopus horrens, Stichopus ocellatus, timun laut*

INTRODUCTION

In Malaysia, sea cucumber (Phylum Echinodermata: Class Holothuroidea) species can be categorised into two groups i.e. *gamat* species and *timun laut* species (Kamarudin et al., 2015). *Gamat* species refers to all species of sea cucumber from the family Stichopodidae including the genus *Stichopus* and the genus *Thelenota*. The term *gamat* also refers to sea cucumber species that are believed traditionally or proven scientifically to contain medicinal properties e.g. *Stichopus horrens* (Selenka, 1867) or the dragonfish. The *timun laut* or non-*gamat* species refers to all species of sea cucumber from other than the family Stichopodidae. *Timun laut* species are usually exploited as food in the *beche-de-mer* or *trepang* industry in Sabah, Malaysia. Choo (2008) reported that 19 sea cucumber species are commercialised by Malaysia, making the country the fourth top producer of sea cucumber products in the world after Indonesia (35 commercial species), China (27 commercial species) and the Philippines (26 commercial species).

Holothuria (Metriatyla) scabra (Jaeger, 1833) is a *timun laut* species. However, in Malaysia, some also regard it as a *gamat* species. It is locally known in Malaysia as *bat putih* (Kamarudin et al., 2015) or *balat harimau putih* (Abdullah, 2016). Its English name is sandfish. It has a brackish grey dorsal part, dark wrinkle lines (upper side) and a light grey ventral part (underside). The sandfish is one of Malaysia's commercial species of sea cucumber that are exploited as food (Choo, 2008). However, the species is regarded as "endangered, or at a high risk of extinction" based on the International Union for Conservation of Nature (IUCN) Red List for aspidochirotid holothuroids (Conand et al., 2014). In order to restore the sea cucumber species in Langkawi, sea ranching of *H. scabra* is being carried out by the Langkawi Development Authority (LADA) in Teluk Yu, Temoyong, Langkawi and Tuba Island, Langkawi (Sharif & Osman, 2016). Langkawi and Pangkor Islands are well known in the sea cucumber-based traditional medicine industry in Peninsular Malaysia for the production of body fluid extracts (*air gamat*) and lipid extracts (*minyak gamat*).

S. horrens and *Stichopus ocellatus* (Massin, et al., 2002) are among the *gamat* species in Malaysia. The total number of Malaysia's *gamat* species to date is 10, including eight *Stichopus* species and two *Thelenota* species (Kamarudin et al., 2015).

S. horrens or dragonfish is locally known as *gamat emas* or golden sea cucumber in Malaysia due to its grey-brown colour and its use as the main ingredient in the production of *air gamat* and *minyak gamat*. Its old scientific name was *Stichopus variegatus* because its body is often variegated with dark patches; however; the scientific name is no longer accepted. Meanwhile, a living specimen of *S. ocellatus* is yellow-orange mottled with a green-grey colour and has eye-like, large papillae in its dorsal part (Massin et al., 2002). The dragonfish is also one of Malaysia's commercial species of sea cucumber but *S. ocellatus* was not listed by Choo (2008). Moreover, *S. horrens* and *S. ocellatus* are not included in the IUCN Red List for aspidochirotid holothuroids as endangered or at risk of extinction and by Conand et al. (2014) as vulnerable or at risk of extinction; however *Thelenota ananas* and *Stichopus herrmanni* from the gamat species group are in their lists.

There are a lot of sea cucumber studies focussing on Sabah, Malaysia as the main study site (Kamarudin et al., 2015). The report by Ridzwan and Che Bashah (1985) on the distribution of sea cucumbers in Sabah and their use as a food resource is believed to be the first documentation of the sea cucumber. According to Kamarudin et al. (2009), at least three factors have contributed to the unique level of richness of the sea cucumber species in Sabah i.e. the extensive distribution of coral reefs, low level marine pollution and the possibility of biogeographical factors within and out of the Sunda Platform area. Furthermore, Sabah is the most significant sea cucumber fishery in Malaysia and the Sabah Fisheries Department reported that about 139 tonnes of sea cucumber was landed in Sabah between 2000 and 2005 (DOF, 2000-2005). Kudat, a town in the state of Sabah, is geographically located near the northernmost point of Borneo Island in East Malaysia. Due to the lack of recent studies on sea cucumber in Kudat, this town was chosen as the study site for this study.

In general, the aims of this study were to identify the species of a *timun laut* morphospecies i.e. *Holothuria (Metriatyla) scabra* and two *gamat* morphospecies i.e. *Stichopus horrens* and *Stichopus ocellatus* that were collected from Limau Limawan based on ossicle shapes and non-protein-coding 12S mitochondrial rRNA gene sequences. Ossicles are small parts of calcified materials from sea cucumber. Their shapes, in fact, have continued to be an important characteristic for morphological identification of sea cucumber (Kamarudin & Mohamed Rehan, 2015). Furthermore, mitochondrial DNA containing the 12S mitochondrial rRNA gene has been the main subject of interest in zoological genetic studies due to its considerably effective maternal inheritance, continuous replication, non-recombination, haploid genome and greater rate of substitution as compared to 'single-copy' nuclear cells (Nabholz et al., 2008). Therefore, ossicle and non-protein-coding 12S mitochondrial rRNA genes were used in this study for

morphological and molecular sea cucumber species identification and verification.

MATERIALS AND METHODS

Study Site and Sampling

A number of 30 specimens of *H. scabra* (PKS 1-PKS 30), one specimen of *S. horrens* (PKSH1) and one specimen of *S. ocellatus* (PKSO1) were collected from Limau Limawan, Kudat, Sabah, Malaysia (Figure 1) in February 2015. The specimens of *H. scabra* were fresh and in good form while the bodies of specimen of *S. horrens* and *S. ocellatus* became deformed before the transportation by flight from Kudat, Sabah to Science Research Lab 3.2 (SRL 3.2), Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM), Nilai, Negeri Sembilan (Figure 2). The morphospecies identification was done based on the outward body appearance or external morphology and the information given by the collectors. Prior to the transportation, each specimen was packed and sealed in a plastic bag, left in the freezer for a few days and wrapped in old newspaper before being transferred to an ice box. In the SRL 3.2, the specimens were stored in a -20oC chest freezer for long-term storage with proper cataloguing.

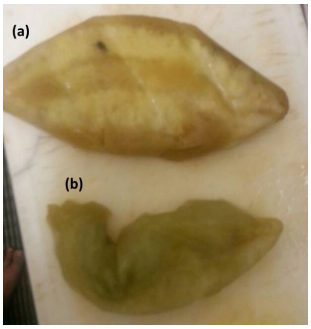

Figure 2. Deformed specimens of *Stichopus horrens* (a) and *Stichopus ocellatus* (b) from Limau Limawan, Kudat, Sabah, Malaysia

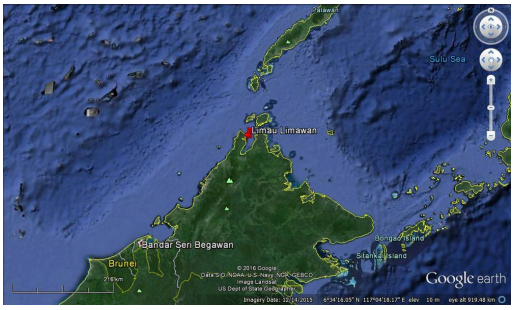

Figure 1. The collection site of *Holothuria (Metriatyla) scabra, Stichopus horrens* and *Stichopus ocellatus* in Kudat, Sabah, Malaysia highlighted in red. [Adapted from Google earth Version 7.1.5.1557 (December 14, 2015)].

Ossicle Extraction and Shape Observation

A little modification was done to the related methods by Kamarudin and Mohamed Rehan (2015). A small piece of tissue from the ventral cuticle of each of the specimens was cut with a sterile blade. The tissue portion was placed on a glass microscope slide. Three tissue portions of different specimens were allocated for each glass microscope slide. Several drops of liquid household bleach were used to dissolve the soft tissue while the ossicles that were usually in the form of white pellets remained in the liquid solution. The prepared slides were observed under the Olympus culture microscope model CKX41 with 400x magnification. The captured images of ossicle shapes were saved for morphological identification. The definite

microscopic size of each ossicle type was not entirely counted due to the specification of the Olympus culture microscope model CKX41. The main focus of this study was to record and identify the shapes of ossicles and then to compare their varieties between *H. scabra,* the *timun laut* morphospecies and *S. horrens* and *S. ocellatus*, the two *gamat* morphospecies.

Amplification of 12S Mitochondrial rRNA Gene

Total genomic DNA extraction was done using the DNeasy mericon Food Kit by QIAGEN with a little modification to the protocol. Standard PCR procedures were then used to amplify the non-protein-coding 12S mitochondrial rRNA gene using the 2x TopTaq Master Mix Kit by QIAGEN [~360 bp of fragment length based on Palumbi et al. (1991)].

AB12SA-Lf (forward) 5'- AAA CTG GGA TTA GAT ACC CCA CTA T -3' (25 bases)

AB12SB-Hr (reverse) 5'- GAG GGT GAC GGG CGG TGT GT -3' (20 bases)

Cycle parameters for the PCR run were 2 min at 95°C for initial denaturation, 30 s at 95°C for denaturation, 30 s at 50.3oC for annealing and 45 s at 72°C for extension. Repetition of step 2-4 was done for another 34 cycles and final extension was for 5 min at 72°C. The purified PCR products were sent for DNA sequencing at the First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor, Malaysia. QIAquick PCR Purification Kit by QIAGEN (for direct purification of single PCR fragment) and QIAquick Gel Extraction Kit by QIAGEN (for purification of desired PCR fragment from agarose gel) were used for the PCR product purification.

Basic Local Alignment and GenBank Submission

The online Basic Local Alignment Search Tool programme for Nucleotide (blastn) was used to align and match each gene sequence (i.e. the query sequence) from this study with a particular sea cucumber species or genus. The Sequin Version 15.10 programme was then used to prepare sequence data for the GenBank submission in order to obtain the accession numbers from the GenBank, National Centre for Biotechnology Information (NCBI), U. S. National Library of Medicine.

RESULTS AND DISCUSSION

All specimens of *H. scabra* from Kudat, Sabah, Malaysia shared two main shapes of ossicles i.e. smooth button and table (Figure 3). In terms of quantity, more smooth tables were observed compared to the tables.

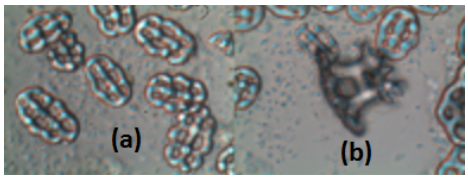

Figure 3. Two common ossicle shapes in the ventral cuticles of *H. scabra* specimens from Kudat, Sabah, Malaysia. (a) smooth button, and (b) table

Regardless of the microscopic size of each shape, the results showed good congruence with the study of Massin et al. (2000), who found that from the specimen length of 30 mm and above i.e. for the adult specimens, the ossicles of *H. scabra* contained more buttons and fewer tables than did the juveniles. In this study, the average length of the specimens of *H. scabra* adults was 70 mm. Therefore, the results supported the species identification based on the outward body appearance or external morphology of *H. scabra* from Kudat, Sabah.

Interestingly, five out of 30 specimens of *H. scabra* from Kudat, Sabah (approximately 17%) also showed additional ossicle shapes in their ventral cuticles. Figure 4 indicates that at least six different types of ossicle shape were present in specimens PKS 14, PKS 19, PKS 20, PKS 21 and PKS 27 (Figure 5). In terms of outward body appearance, all 30 specimens of *H. scabra* had the same body colour, shape and length; thus, the presence of the additional ossicles may represent some uniqueness of the specimens. Dabbagh et al. (2012) reported the presence of large perforated I-shaped rod in the tube feet of *H. scabra* from the Persian Gulf (see Figures 4b-4c) and branched rod from the ventral body wall and tube feet (see Figure 4e). The presence of a large perforated I-shaped rod in specimens PKS 14 and PKS 19 from Kudat, Sabah (Figures 4b-4c) and a branched rod in specimen PKS 21 (Figure 4e) could have originated from the tube feet of *H. scabra* from Kudat, Sabah. The presence of a Y-shaped rosette in the ventral cuticle of specimen PKS 14 (Figure 4a), a J-shaped rod in specimen PKS 20 (Figure 4d), an H-shaped rod in specimen PKS 21 (Figure 4f) and an I-shaped rod in specimen PKS 27 (Figure 4g) were in very small quantity. It is speculated that the Y-shaped rosette in specimen PKS 14 (Figure 4a) and the H-shaped rod in specimen PKS 21 (Figure 4f) could be the broken parts of a button-shaped ossicle.

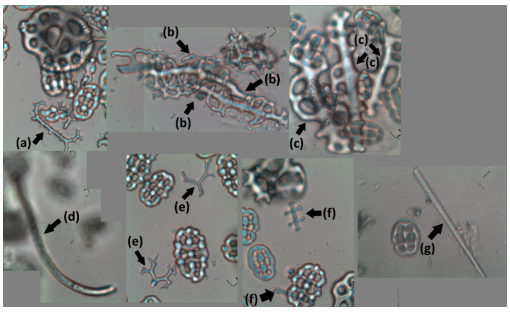

Figure 4. Six additional ossicle shapes in the ventral cuticles of five *H. scabra* specimens from Kudat, Sabah apart from the three common shapes (Figure 3). (a) Y-shaped rosette from specimen PKS 14, (b) large perforated I-shaped rod from specimen PKS 14, (c) large perforated I-shaped rod from specimen PKS 19, (d) J-shaped rod from specimen PKS 20, (e) branched rod from specimen PKS 21, (f) H-shaped rod from specimen PKS 21, and (g) I-shaped rod from specimen PKS 27

Figure 5. Five specimens of *H. scabra* from Kudat, Sabah, Malaysia with additional ossicle shapes extracted from their ventral cuticles (Figure 4).

Nevertheless, all the specimens remained to be verified as *H. scabra* despite the presence of the additional ossicles.

There were at least four different shapes of ossicle extracted from the ventral cuticle of *S. horrens* specimen from Kudat, Sabah (Figure 6). The morphospecies name of the specimen was determined based on the information given by the collectors. Even though its body became deformed prior to the ossicle extraction (Figure 2a), the ossicles were still observable. Figure 6 shows the presence of a large boomerang-shaped rod (Figure 6a), a large perforated plate (Figure 6b), a table (Figure 6c) and an X-shaped rosette (Figure 6d). These findings were supported by Kamarudin and Mohamed Rehan (2015), who also recorded the same observation for three *S. horrens* specimens from Pangkor Island, Perak, Malaysia. However, Massin et al. (2002) listed a C-shaped rod as one of the ossicle shapes in the ventral cuticle of *S. horrens* from Pulau Aur, Johor; this was not observed in this study. The body deformation of *S. horrens* in this study could have led to the absence. Notwithstanding the absence of the C-shaped rod, Kamarudin and Mohamed Rehan (2015) successfully confirmed the species status of the specimens from Pangkor Island, Perak as *S. horrens* using the cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene-sequencing technique.

Regarding the specimen of *S. ocellatus* from Kudat, Sabah, at least five ossicle shapes were recorded i.e. C-shaped rod, X-shaped rod, rosette, X-shaped rosette and table (Figure 7). Nonetheless, the large rod and large plate shapes were not observed in this study although the two shapes were recorded by Massin et al. (2002). In the specimen of *S. horrens* from Kudat, Sabah, the body deformation of *S. ocellatus* (Figure 2b) in this study could have led to the absence. According to Toral-Granda (2005), the ossicles of *Isostichopus fuscus* samples in the forms of fresh, salted and dried specimens showed no difference in

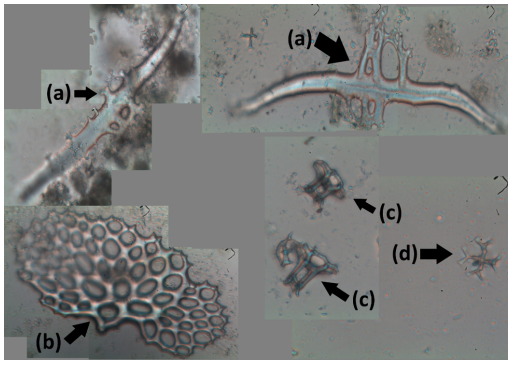

Figure 6. Ossicle shapes of *Stichopus horrens* specimen from Kudat, Sabah, Malaysia. (a) large boomerang-shaped rod, (b) large perforated plate, (c) table, and (d) X-shaped rosette

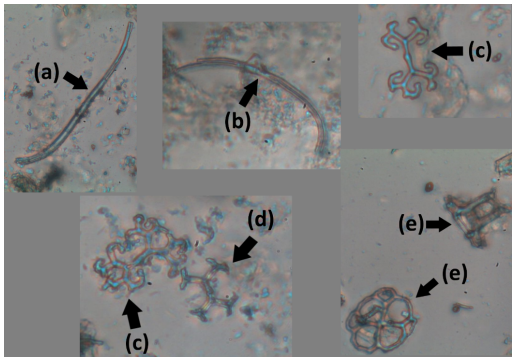

Figure 7. Ossicle shapes of *Stichopus ocellatus* specimen from Kudat, Sabah, Malaysia. (a) C-shaped rod, (b) X-shaped rod, (c) rosette, (d) X-shaped rosette, and (e) table

terms of the proportion, size and shape. The results suggested that ossicle shape as a feature is still useful and informative for morphological identification of sea cucumber in any form. The observable ossicles from *S. horren*s and *S. ocellatus* specimens from Kudat, Sabah in this study further support this suggestion.

However, in the absence of common ossicles, the DNA sequencing technique using mtDNA genes, for instance, is required to confirm the species status of a sea cucumber specimen. For this reason, non-protein-coding 12S mitochondrial rRNA gene sequencing was incorporated in this study. Approximately 360 bp non-protein-coding 12S mitochondrial rRNA gene fragments were successfully amplified. In terms of DNA sequencing results, a range of 327-372 nucleotide bases of the 12S mitochondrial rRNA gene was successfully obtained except for specimen PKS 30. The DNA sequencing failure for specimen PKS 30 was due to the presence of bad sequence parts. Moreover, the blastn results showed that the specimens of morphospecies *H. scabra* were specifically identified as *H. scabra* with identity scores (Ident) ranging from 98% to 100% when aligned against the corresponding sequence (GenBank accession number: KP257577), while the specimen of morphospecies S. horrens was specifically identified as *S. horrens* with an Ident of 99% when aligned against the corresponding sequence (GenBank accession number: HQ000092). Interestingly, the morphospecies specimen, *S. ocellatus*, was identified only up to the genus level i.e. genus *Stichopus*, with an Ident of 96% when aligned against the corresponding sequence (GenBank accession number: HM853683.2, an unknown *Stichopus* species). However, the morphospecies identification based on outward body appearance and the information given by the collectors suggested that the specimen was *S. ocellatus*. The score of Query cover for the blastn of the morphospecies *S. ocellatus* was 99% and the Expect value (E value) was $2e^{-160}$, showing the most significant score and alignment with the corresponding sequence. The other scores were maximum score and total score; each was 575. Therefore, the findings suggested that 12S mitochondrial rRNA gene sequence for *S. ocellatus* was lacking in the GenBank until 15 September, 2016. The genetic information using the non-protein-coding 12S mitochondrial rRNA gene supported the species status of the specimens as *H. scabra* (the *timun laut* species), *S. horrens* and *S. ocellatus* (the *gamat* species) as suggested earlier through the morphological information. All the 31 partial sequences of the 12S mitochondrial rRNA gene were registered with the GenBank, NCBI, U. S. National Library of Medicine (Accession No.: KX913672-KX913702). The results from ossicle shape observation as well as the non-protein-coding 12S mitochondrial rRNA gene sequencing complemented each other for a more concrete conclusion.

Despite the fact that more specimens, especially for *S. horrens* and *S. ocellatus* (the *gamat* species), and more molecular techniques are required to generate better

conclusive outcomes, the current findings gave a better insight into the importance of morphological and molecular approaches and the present status of H. scabra (the *timun laut* species) and the two *gamat* species in Kudat, Sabah, Malaysia. The non-protein-coding 16S mitochondrial rRNA gene is among the most common genes used in genetic studies. It was suggested as being able to correlate the relationship between the morphology and genetics of sea cucumber (Clouse et al., 2005; Kerr et al., 2005). Moreover, protein-coding genes such as COI and *cytochrome b* mtDNA genes are also common in genetic studies (Kamarudin & Mohamed Rehan, 2015; Kamarudin & Esa, 2009) and useful for confirming results from non-protein-coding mitochondrial rRNA gene sequencing.

CONCLUSION

Ossicles were successfully extracted from the specimens of *H. scabra, S. horrens* and *S. ocellatus*, even though the specimens of *S. horrens* and *S. ocellatus* became deformed prior to the analyses. Rod, plate, rosette, button and table were the five main ossicle shapes extracted from the specimens. However, a number of ossicle shapes for *S. horrens* and *S. ocellatus* recorded in the previous studies were not observed in this study; this could have been due to the body deformation of the specimens. In terms of ossicle varieties, five specimens of *H. scabra* showed additional ossicle shapes other than the common shapes i.e. smooth button and table. Despite the presence of additional ossicle shapes and the absence of common ossicle shapes, all the specimens from Kudat, Sabah, Malaysia remained to be morphologically identified and verified as *H. scabra* (the *timun laut* species), *S. horrens* and *S. ocellatus* (the *gamat* species). Generally, species identification and species status verification using ossicles have continued to be useful. In addition, 12S mitochondrial rRNA gene sequences analysed using the blastn resulted in species identification of the specimens of morphospecies *H. scabra* and *S. horrens* as *H. scabra* and *S. horrens*; however, the specimen of morphospecies *S. ocellatus* was only identified as being from genus *Stichopus*, showing that 12S mitochondrial rRNA gene sequences of *S. ocellatus* was lacking in the GenBank until 15 September, 2016. A number of 31 partial sequences of 12S mitochondrial rRNA gene were registered with the GenBank (Accession No.: KX913672-KX913702). Despite the absence of common ossicles and the presence of additional ossicle shapes, the three morphospecies were morphologically and genetically verified as *H. scabra, S. horrens* and *S. ocellatus*. Moreover, the current findings gave a better insight into the importance of morphological and molecular approaches and the present status of the *timun laut* species and *gamat* species in Kudat. More specimens of different species and molecular techniques are required in order to generate better conclusive outcomes in the future. Future studies may focus on this.

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Pertanika J. Trop. Agric. Sci. 40 (1): 173 - 184 (2017)

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Effect of Combined Application of Poultry Manure and Inorganic Fertiliser on Yield and Yield Components of Maize Intercropped with Soybean

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ABSTRACT

A field experiment was conducted to evaluate the effect of the combined application of poultry manure and inorganic fertiliser on yield and the yield component of maize (*Zea mays L*) and soybean (*Glycine max L. Merril*) intercrops. Treatments comprised combinations of three intercropping systems (sole maize, sole soybean and maize+soybean) and four nutrient management practices [control, 100% NPK, 100% poultry manure (PM) and 50 % NPK+50% PM]. The experiment was laid out in a randomised complete block design (RCBD) with three replications. The results revealed that intercropping of maize with soybean significantly reduced soybean yield and yield components, but maize yield and yield traits were not significantly affected by intercropping. Land Equivalent Ratio (LER) was >1, indicating a beneficial effect of intercropping soybean with maize. For both maize and soybean, there were no significant differences in yield between application of 100% NPK and the 50% NPK+50% PM fertiliser. A combined application of 50% NPK and 50% PM gave the highest monetary advantage in intercropping system. It can be concluded that a 50% substitution of inorganic fertiliser with poultry manure is recommended to reduce use of chemical fertilisers without sacrificing crop yield..

Keywords: fertiliser, intercropping maize/soybean, NPK, poultry manure, yield

ARTICLE INFO

Article history:
Received: 30 May 2016
Accepted: 10 November 2016

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INTRODUCTION

Maize (*Zea mays L*) and soybean (*Glycine max* L. Merril) are important cereal and legume crops, respectively. The maize-soybean intercropping system is one of the profitable cropping systems. Nevertheless, the productivity of the system in most developing countries is low and has been

ISSN: 1511-3701

ascribed to several constraints. Low soil fertility due to use of unbalanced and inadequate fertilisers and biomass removal accompanied by restricted use of organic manures are some of the major causes for declining productivity of the system (Negassa et al., 2007). Due to the high cost of chemical fertilisers, marketing problems and poor infrastructure, only a limited number of farmers in developing countries apply inorganic fertiliser and, in most cases, at lower rates than recommended (Morris et al., 2007). Long-term use of only chemical fertilisers in a continuous cropping system leads to nutrient imbalance in the soil, which adversely affects soil and crop productivity (Mahajan et al., 2008).

On the other hand, continuous use of organic fertiliser improves soil organic matter and soil productivity. However, nutrients in organic fertilisers are released much more slowly and cannot increase crop yield within a short time as compared to inorganic fertilisers (Negassa et al., 2007). Therefore, use of organic fertiliser alone does not result in remarkable increase in crop yields due to their low nutrient status. This has led to a quest for the use of integrated nutrient management as a sustainable solution and better utilisation of resources to produce crops with less expenditure. Integrated use of different fertiliser sources with an appropriate cropping system can result in improved soil fertility, crop productivity and a better environment for future generations. This study was aimed at determining the effect of combined application of poultry manure and inorganic fertiliser on yield and yield component of maize and soybean intercrops.

MATERIALS AND METHODS

A field experiment was carried out in 2014 at the University Research Park of Universiti Putra Malaysia. The initial physicochemical properties of the experimental field soil were as follows: pH 5.6, organic carbon 1.14%, N 0.08 %, S 0.007%, P 10 ppm, K 0.167 cmol/kg and sandy loam in texture.

The experiment was conducted using 3x4 factorial combinations of intercropping systems (sole maize, sole soybean and maize+soybean) and nutrient management practices [control, 100% NPK, 50% NPK+50% poultry manure (PM) and 100% PM] and laid out in Randomised Complete Block Design (RCBD) with three replications. The plot size of 3.6 m x 3 m was used for all treatments. The plant materials used for this study were the maize variety Sweet Corn 926 and the soybean variety Willis.

The amount of PM was based on N equivalence and applied on dry weight basis two weeks before planting. The amount of PM in sole maize, sole soybean and intercropping plot were 3 t ha^{-1}, 0.4 t ha^{-1} and 3 t ha^{-1}, respectively. The chemical composition of PM is presented in Table 1.

The amount of $N:P_2O_5:K_2O$ for 100% NPK treatment was 120:60:40 and 20:60:40 kg ha^{-1} for maize and soybean monocrop, respectively. The intercropped plots received the recommended fertiliser rate for maize ($N:P_2O_5:K_2O$ at 120:60:40 kg ha^{-1}). The full dose of P and K and one third of N fertiliser

were applied at planting time. The remaining two thirds of N fertiliser was applied at the 8-leaf stage of maize, while for soybean plots the entire dose of NPK was applied at planting. All other agronomic practices were kept uniform for all treatments.

Table 1
Chemical Composition of Poultry Manure

Nutrient Element	Values (%)
N	4.50
P2O5	2.50
K2O	2.00
CaO	2.00
MgO	1.00
S	0.50
Fe	0.04
Mn	0.09
Zn	0.09
Other characteristics	0.50

Maize and soybean were planted manually by placing two seeds per hill on 10 April, 2014. The plants were thinned to one plant per hill two weeks after emergence to maintain the recommended intra-row population for both crops. The sole maize and soybean samples were seeded in six rows spaced 60 cm between rows in the monoculture plots. Maize and soybean were intercropped in 1:1 alternate rows with 60cm space between maize and soybean rows. The spacing between plants for maize was 25 cm and for soybean, 15 cm. Maize and soybean were harvested manually at physiological maturity and samples were taken from a sample quadrat of 2 m x 2 m for each plot.

Data recorded for maize include plant height, cob length, green cob weight, 1000 kernel weight, number of kernels per cob, green cob yield (kg/ha) and total dry biomass (kg/ha). For soybean, data taken included plant height, number of branches per plant, number of pods per plant, seeds per pod, seed yield per plant, hundred seed weight, seed yield (kg/ha) and total dry biomass (kg/ha). All the measured data were subjected to analysis of variance (ANOVA) appropriate to a factorial experiment in RCBD. Analysis of variance was computed using the SAS (Version 9.4) statistical software programme. The Least Significant Difference (LSD) was used for mean separation at 0.05% probability levels. The model for the two-way ANOVA with interaction is:

$$Y_{ijk} = \mu + \tau A_i + \tau B_j + (\tau A \tau B)_{ij} + \beta_k + \varepsilon_{ijk} \quad (1)$$

where Y_{ijk} is the random variable representing the response for treatment i

j observed at block K, μ is the overall mean of all experimental units

τA_i is the effect of factor A at level i

τB_j is the effect of factor B at level j

$(\tau A \tau B)_{ij}$ is the interaction between factor A and B at levels i and j

β_k is the effect of block k, and

ε_{ijk} are random errors for the i, j and ij in the k block.

The advantage of intercropping was measured through land equivalent ratio (LER) based on equation 2:

$$LER = (Yij/Yii) + (Yji/Yjj) \quad (2)$$

where Yii and Yjj are yields of sole crop i and j, respectively

Yij is the intercrop yield of i, and

Yji is the intercrop yield of j.

The profitability of intercropping over monocropping was measured by Gross Monetary Value (GMV) and Monetary Advantage (MA) (Mead & Willey, 1979). GMV was calculated based on market price [Malaysian Ringgit (RM)] of a green cob of maize and seed of soybean. Therefore, GMV and MA were calculated based on equation 3 and 4, respectively (Willey, 1979):

$$GMV = (\text{yield of component crops}) \times (\text{market price}) \quad (3)$$

$$MA = (\text{value of combined intercrops yield}) \times (LER-1) / LER \quad (4)$$

The market price of a green cob of maize (RM 1.15 per cob) and seed of soybean (RM 9 per kg) was estimated based on their average price in 2015 at three main markets in the study area.

The net return (NR) was calculated from the difference of gross monetary value and variable cost (CIMMYT, 1988). Total variable cost (TVC) was the sum of the cost of fertiliser and cost of time expended to spread the fertilisers. Fertilisers were valued at an average price of poultry manure (PM) (RM 0. 977 per kg), Urea (RM 2.3 per kg), Triple Super Phosphate (TSP) (RM 3.38 per kg) and Muriate of potash (MOP) (RM 2.88 per kg). The daily wage rate was based on the wage rate in the study area (RM 70 per day).

RESULTS AND DISCUSSION

Maize

The results showed that nutrient management had a significant effect on plant height (Table 2). However, there was no significant effect of intercropping on plant height of maize. Among nutrient management treatments, 100% NPK and 50% NPK+ 50% PM gave the tallest maize followed by 100% PM, while the shortest plant was obtained in control plots. The plant growth was inhibited due to limited supply of nutrients in the control plots. Greater plant height was attributed to the gradual release of essential nutrients as required by the plant in a combined application of NPK and PM. These results are in agreement with those of Ayoola and Makinde (2009).

Yield traits such as cob length, cob weight, 1000 grain weight and number of kernels per cob were significantly affected by nutrient management, but not by the intercropping system (Table 2). This result is consistent with that of Aziz et al. (2012), who reported that cob length and weight of seeds were not affected by the intercropping system.

Table 2
Effect of Intercropping System and Nutrient Management on Yield and Yield Traits of Maize

Treatment*	PH (cm)	CL (cm)	GCW (g)	NK/C	TGW (g)	GCY (kg/ha)	TDB (kg/ha)
Cropping system (C)							
Sole Maize	162	31	321	530	377	21,591a	7,634
Maize +soybean	150	30	317	503	370	21,394a	7,401
LSD ($p<0.05$)	ns	ns	ns	ns	ns	ns	ns
Nutrient Management (N)							
Control	107c	28b	229c	391c	320c	9,808c	4,466c
100% NPK	203a	32a	387a	591a	414a	28,340a	9,186a
100% PM	145b	31a	306b	492b	370b	19,557b	6,752b
50%NPK+ 50% PM	185a	32a	355ab	593a	390ab	28,264a	9,666 a
LSD ($p<0.05$)	18.2	3.4	51	46	34	3443	1248
C*N	ns	ns	ns	ns	ns	ns	ns
CV (%)	8.98	9.10	13.03	7.35	7.54	13.5	13.6

Means followed by the same letter within columns are not significantly different
* PH=plant height, CL = cob length, CW = green cob weight, NK/C = number of kernel per cob, TGW = thousand grain weight, GCY = green cob yield, TDB = total dry biomass

Longer cobs, bold and heavy grain and more number of grains per cob were observed from 100% NPK and the 50% NPK+50% PM followed by 100% PM. The highest yield components in the application of 100% NPK and 50% NPK+50% PM might be due to greater availability and uptake of nutrients throughout the growing season. The lowest value for all yield components was recorded in control plots due to inadequate supply of nutrients. These results are in line with Akintoye and Olaniyan (2012) and Rehman et al. (2008), who reported higher yield components of maize in combination of organic and inorganic fertiliser.

The green ear yield and total dry biomass of maize were significantly affected by nutrient management, but not by the intercropping system (Table 2). This shows that the presence of soybean did not adversely affect the growth of maize. The present result is supported by that of Mudita et al. (2008) and Waktola et al. (2014), who reported non-significant difference between monocropped maize and intercropped maize with soybean on yield and yield components. The highest green ear yield as well as total dry biomass of maize was obtained from 100% NPK and the 50% NPK+50% PM treatment, while the lowest were recorded from the control. The use of 100% PM gave a higher yield than the control, but it was lower than the yield of the other two fertiliser treatments. This is probably due to the fact that nutrients in the inorganic fertiliser were being rapidly

released and the maize was able to utilise it for its growth and yield. This result is supported by that of Ayoola and Makinde (2009), who reported the highest yield in maize that was fertilised with inorganic fertiliser. Farhad et al. (2009) also reported that yield and yield traits of maize were significantly increased by application of different levels of poultry manure compared to the control.

Soybean

The results revealed that plant height of soybean varied significantly in response to different intercropping systems and nutrient management (Table 3). The soybean plant grown as a monocrop was shorter than that intercropped with maize. This result might have been due to the shading effect of maize, which reduced the interception of light by the soybean. The result is supported by Ijoyah et al. (2013), who reported taller soybean plants in the intercropped sample compared to that of the monocropped soybean due to the competition for light from the greater population of plants in intercropping. Among nutrient management treatments, the tallest plant was observed in the 50% NPK+50% PM and the 100% NPK treatment. The shortest soybean was observed in the control plots, followed by the poultry manure sample. The results of increasing plant height in the integrated application of organic and inorganic fertiliser may be due to the steady release of essential nutrients as required by the soybean plant. The interaction effect of the intercropping system and nutrient management was not significant on plant height of the soybean.

Number of branches per plant was significantly influenced by the intercropping system and nutrient management (Table 3). A higher number of branches per plant was obtained in the sole soybean sample compared with the soybean intercropped with maize sample. Among nutrient management, the maximum number of branches per plant was observed in the application of 50% NPK+50% PM and 100% NPK followed by 100% PM. In contrast, the number of branches per plant was noted in the control. This is in agreement with Yagoub et al. (2015), who reported the highest number of branches per plant in the combination of organic and inorganic fertilisers.

Both intercropping and nutrient management had a significant effect on the number of pods per plant and seed yield per plant (Table 3). Intercropping maize with soybean reduced the number of pods and seed yield per plant of soybean. This might be due to the inhibition of initiation of pods by soybean plants attributed to strong competition with maize. This result is supported by Zerihun et al. (2013), who reported the reduction of seed yield and number of pods per plant of soybean in intercropping. Application of 50% NPK+100% PM and the 100% NPK treatments produced the highest number of pods per plant and seed yield per plant. The lowest number of pods per plant and seed yield per plant was found in the control treatment and the 100% PM treatment.

Table 3
Effect of Intercropping System and Nutrient Management on Yield and Yield Components of Soybea

Treatment*	PH (cm)	NB/P	NP/P	NS/P	HSW (g)	SY/P (g)	SY (kg/ha)	BY (kg/ha)
Cropping system (C)								
Sole soybean	80.9b	6.97a	136a	2.75a	11.3a	55.0a	3,236a	7,071a
Maize +soybean	95.1a	6.28b	116a	2.73a	10.8a	43.4b	2,560b	5,487b
LSD ($p<0.05$)	7.7	0.58	14	ns	ns	4.80	208.61	397
Nutrient Management (N)								
Control	66.7c	4.11c	83c	2.68a	10.7a	30.4c	1,618c	3,642c
100% NPK	98.4a	8.16a	160a	2.78a	11.2a	59.7a	3,475a	7,350a
100% PM	83.0b	5.88b	103.2b	2.75a	11.2a	45.9b	2,862b	6,641b
50 % NPK+ 50% PM	103.9a	8.35a	163a	2.77a	11.2a	60.9a	3,637a	7,483a
LSD ($p<0.05$)	10.8	0.822	20	ns	ns	6.8	295	561
C*N	ns	ns	ns	ns	ns	ns	ns	ns
CV (%)	10.04	10.14	13.20	2.58	11.5	8.47	8.20	7.2

Means followed by the same letter within columns are not significantly different
* PH = plant height, NB/P = number of branches per plant, NP/P = number of pods per plant (NP/P), NS/P = number of seeds per pod, HSW = hundred seed weight, SY/P = seed yield per plant, SY= seed yield and TDB = total dry biomass

The highest number of pods and seed yield per plant in the combined application of organic and inorganic fertilisers was probably due to a balanced supply of nutrients to the crops throughout the crop growth period as PM undergoes decomposition, during which a series of nutrient transformation phases take place that makes the nutrients available to the crops. This result is in agreement with Khaim et al. (2013), who reported that the highest seed yield per plant and number of pods per plant of soybean was in the application of 100% NPK and NPK+PM.

The main and interaction effect of intercropping systems and nutrient management was not significant on the number of seeds per pod and 100 seed weight (Table 3). This result is supported by Ali et al. (2015), who reported that the cropping system had no significant effect on the weight of the 100 seeds and the number of seeds per pod of soybean.

Seed yield and total biomass of soybean were significantly affected by the intercropping system and nutrient management (Table 3). The seed yield of soybean was reduced by 26% and total biomass of soybean was reduced by 28% when intercropped with maize. This might have been due to competition for light between maize and soybean in the mixtures. The result is consistent with that of Dolijanović et al. (2013), who reported that the yields of soybean in maize-soybean intercrops were significantly lower than

those obtained in the sole crop. Among nutrient management treatments, the highest seed yield and total biomass was obtained from 50% NPK+50% PM and the 100% NPK treatments. The increase in seed yield was due to the increase in the number of branches and pods per plant. Application of 100% PM also gave higher yield than the control treatment. These results are in agreement with Khaim et al. (2013) and Yamika and Ikawati (2012), who reported higher yield due to the application of PM compared to control.

Intercropping Advantage

Land equivalent ratio (LER) reflected the advantage of intercropping over the sole cropping system. The partial LER of maize was greater than that of soybean in all nutrient management treatments (Table 4). Higher partial LER of maize indicated the dominance of maize over soybean in intercropping. The LER of intercropping was more than one in all nutrient management samples, indicating the yield advantage of maize-soybean intercropping over monocropping (Ofori & Stern, 1987). The better utilisation of growth resources by component crops in intercropping systems led to the higher LER. This result is supported by Shaker-Koohi and et al. (2014), who explained that the greater LER could be attributed to the morphological differences of the two crops and the optimal utilisation of resources.

Table 4
Intercropping Productivity Index as Influenced by Nutrient Management

*Treatment	PLER		TLER	GMV RM/ha	MA RM/ha	TVC RM/ha	NR RM/ha
	Maize	Soybean					
Control	0.86b	0.69b	1.55c	30,693c	10,381c	0c	30,693d
100% NPK	0.97ab	0.83a	1.75b	68,061a	28,512ab	6,920a	61,141b
100% PM	0.90a	0.76ab	1.70b	51,748b	25,089b	7,157a	44,591c
50% NPK +50% PM	0.97a	0.77a	1.82a	72,405a	30,691a	6,753b	65,652a
LSD (P< 0.05)	0.10	0.14	0.15	6503	5022	1281	4411

Means followed by the same letter within columns are not significantly different
*PLER = partial land equivalent ratio, TLER = total land equivalent ratio, GMV = gross monetary value MA= monetary advantage, TVC= total variable cost, NR = net return

Among nutrient management treatments, a higher total LER was observed in the 50% NPK+50% PM treatment followed by the 100% NPK and 100% PM treatment. In contrast, the lowest LER was recorded in the control; this confirms that the productivity of intercropping systems was reduced by low soil fertility (Table 3). This result was in agreement with that of Zerihun et al. (2013), who found that the highest LER of the maize-soybean intercropping system was from the integrated use of 55/23 N/P_2O_5 kg ha^{-1} and 8 t ha^{-1} FYM.

Gross monetary value (GMV) of maize/soybean intercropping was significantly affected by nutrient management (Table 4).

The highest GMV was observed in the 50% NPK+50% PM and 100% NPK treatment followed by the 100% PM treatment, while the lowest GMV value was recorded in the control. This might have been due to higher green cob yield and seed yield values from the 50% NPK+50% PM plots, which contributed more to gross monetary value than the other treatments.

Monetary Advantage (MA) in maize/soybean intercropping system was positive, indicating productivity and profitability of the system compared to monocropping. The MA was mainly dependent on the market price of the produce and the harvested economic yield. Similarly, the MA was significantly different among nutrient managements (Table 4). The highest MA was obtained from the combined application of 50% NPK+50% PM, while the lowest was from the control in the intercropping system. The result was in agreement with that of Muyayabantu et al. (2013), who reported higher yield and monetary advantage index (MAI) for maize/soybean intercropping under a combined application of organic and inorganic fertiliser.

The highest net return was observed in the combined application of 50% NPK+50% PM followed by the application of 100% NPK. The application of 100% PM gave a higher net return compared to that of the control treatments. The increased yield from the combined application of 50% NPK+50% PM contributed to increasing net benefits and net return. The combined application of organic and inorganic fertiliser increased the synchrony between the nutrients released and plant uptake and hence, enhanced crop yields. This suggests that integration of organic and inorganic fertilisers is a more economically profitable approach among smallholder farmers in tropical developing countries. This result is in agreement with Mutegi et al. (2012) and Moghadamla and Mirshekari (2014), who found the highest gross and net benefit in the combined application of organic and inorganic fertiliser compared to that of the sole application of either fertiliser.

CONCLUSION

The results of this study showed that intercropping reduced soybean yield by 21% but had no significant effect on maize yield. Maize-soybean intercropping was shown to have greater productivity per unit area and profitability than monocropping of either maize or soybean alone based on Land Equivalent Ratio (LER) and Monetary Advantage (MA). Among nutrient management treatments, 50% NPK+50% PM and 100% NPK were found to be the best nutrient management options for a high green cob yield of maize and seed yield of soybean. Therefore, 50% substitution of inorganic fertiliser with poultry manure is recommended for reduction in use of chemical fertilisers without sacrificing crop yield for both monocropping and intercropping of maize and soybean.

ACKNOWLEDGEMENTS

The author is grateful to the Organisation for Women in Science for the Developing World (OWSD), the Swedish International

Development Cooperation Agency (SIDA) and University of Putra Malaysia (UPM) for sponsoring this study.

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Pertanika J. Trop. Agric. Sci. 40 (1): 185 - 202 (2017)

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TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Analysis of Gamma Irradiated-Third Generation Mutants of Rodent Tuber (*Typhonium flagelliforme* Lodd.) Based on Morphology, RAPD, and GC-MS Markers

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ABSTRACT

Rodent tuber is an anticancer plant. The natural genetic diversity of rodent tuber is low due to vegetative propagation. It is important to increase the plant's genetic diversity in order to obtain plants with a high amount of anticancer compounds. *In vitro* calli were irradiated with gamma rays to increase its genetic diversity. Seventeen clones of the first generation of vegetative mutants in a green house (MV1) were propagated until MV3. This research aimed to analyse the stability of mutation in MV3 based on morphology, RAPD and GC-MS markers. Clone 6-1-2 had the highest increase of shoots and leaf number than the control and the other MV3 clones while clone 6-1-3-4 had the highest fresh and dry weight. RAPD analysis using 15 primers produced 67 polymorphic DNA bands and showed four main clusters at the similarity coefficient cut-off of 0.87. The GC-MS showed that MV3 contained at least eight types of anticancer compound in the leaves and six types in the tubers; these were higher than in the control. MV3 leaves and tubers contained at least eight new anticancer compounds that were not found in the control. This research proved that rodent tuber MV3 clones were solid mutants and had a high potential for being developed into anticancer drugs.

Keywords: anticancer compounds, gamma irradiation, GC-MS, morphology, RAPD, *Typhonium flagelliforme* Lodd

ARTICLE INFO

Article history:
Received: 26 May 2016
Accepted: 10 November 2016

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ISSN: 1511-3701

INTRODUCTION

Rodent tuber (*Typhonium flagelliforme* Lodd.) is a herbal plant from the Araceae family (Essai, 1986) that contains detoxification and anticancer compounds. Rodent tuber is a plant native to Indonesia and has been used as traditional medicine for years. Bioactive compounds of rodent tuber are alkaloids, saponins, steroids and glycosides (Syahid, 2007). Anticancer compounds can be found in all parts of the rodent tuber plant, including the root, tuber, stem and leaf (Choo et al., 2001).

Rodent tuber has been reported to be cytotoxic against cancer of the lung, breast (Lai et al., 2010), liver (Lai et al., 2008), blood (leukemia) (Mohan et al., 2010), colon, prostate gland and cervix (Hoesen, 2007). Rodent tuber extract has also been reported to be able to prevent breast and cervical cancers (Syahid & Kristina, 2007). Rodent tuber hexane extract was toxic against Artemia salina (Sianipar et al., 2013a). Other biological activities of rodent tuber have been found to be antibacterial and antioxidant (Mohan et al., 2008) and able to induce apoptosis of cancer cells (Lai et al., 2008).

The development of Indonesian rodent tuber into anticancer drugs was inhibited by its low genetic diversity, which is caused by the conventional clonal vegetative propagation method. Low genetic diversity is followed by low bioactive compound diversity in rodent tuber (Syahid, 2008). Mutation induction of *in vitro* somatic cell population (calli) or shoot culture could increase its genetic diversity, which in turn might increase the probability of generating plant clones that contain a higher amount of anticancer compounds. Mutation can be induced by irradiation with physical mutagens such as gamma ray.

Genetic diversity can be analysed using molecular markers such as RAPD, RFLP, AFLP and SSR (Powell et al., 1996). The Randomly Amplified Polymorphic DNA (RAPD) marker is able to detect genetic diversity of a plant whose genome has not been sequenced yet (McClelland et al., 1994), such as rodent tuber. Genetic characterisation of a plant's germplasm is essential for harnessing the maximum potency of the plant's genetic diversity (Rout, 2006).

Embryogenic calli of rodent tuber plant have been induced, proliferated and regenerated using the single node culture method (Sianipar et al., 2011). Rodent tuber mutant clones have also been successfully generated by combining the effects of gamma irradiation with somaclonal *in vitro* culture variation. A somatic cell population (calli) of rodent tuber was irradiated with 6-Gy gamma rays. The irradiated *in vitro* plantlets exhibited various growth responses (Sianipar et al., 2013b). Those irradiated in vitro plantlets (mutant) and control plants were found to have genetic differences based on analysis with RAPD molecular markers (Sianipar et al., 2015a).

Thirty-seven clones of first generation vegetative mutant of rodent tuber induced in a green house (MV1) were analysed based on morphological and RAPD markers. MV1 clones had various morphological

characteristics (Sianipar et al., 2013c). Out of those 37 MV1 clones, there were 17 that had a diversified genetic profile and showed genetic differences from the control plants based on RAPD molecular analysis (Sianipar et al., 2015b).

Genetic mutation might also affect the relative abundance of bioactive compounds. Gas Chromatography-Mass Spectrometry (GC-MS) is a method for analysing the metabolomic profile of an organism. GC utilises gas as the mobile phase to separate chemical compounds. GC is able to separate a lot of compounds in one run and can be combined with MS to identify the compounds based on the database (Kayser & Quax, 2007). Rodent tuber MV1 clones were propagated and regenerated into MV3 clones. This research aimed to analyse the third generation of vegetative mutant clones of rodent tuber (MV3) based on morphological analysis, molecular marker with RAPD profiling and GC-MS to identify the relative abundance of bioactive compounds.

MATERIALS AND METHODS

Plant Material

This research analysed the third generation of vegetative mutant clones of rodent tuber (MV3) (in the patenting process). Rodent tuber mother plants were obtained from Bogor (West Java, Indonesia).

Morphological Characterisation

The parameters of observation were the number of shoots, number of leaves, plant height and fresh and dry weight of the rodent tuber control and mutant plants. Morphological characteristics were analysed using the NTSYS DIST coefficient and UPGMA.

Molecular Analysis with RAPD Marker

DNA isolation was done based on Doyle and Doyle (1987). In this method, 2.5 g of leaf sample was homogenised with PVP 0.1% and liquified nitrogen. A volume of 2 mL of CTAB buffer (CTAB 10% b/v, EDTA 0.5 M pH 8.0, Tris-HCl 1M pH 8.0, NaCl 5M) and 10 µL of 1-merkaptoetanol 1% (b/v) was added to the sample powder. The sample was homogenised with vortex, incubated at 60°C for 20 min and cooled at room temperature. About 750 µl of chloroform:isoamyl alcohol (24:1) solution was added to the sample and then vortexed. The sample was centrifuged at 11.000 rpm for 10 min. A supernatant was added to 1 mL of chloroform:isoamyl alcohol (24:1) and centrifuged at 11.000 rpm for 10 min. About 750 µL of cold isopropanol, homogenised and stored at -20°C for one night was added to the supernatant. The sample was centrifuged at 11.000 rpm for 10 min. A DNA precipitate was dried in a vacuum for one h. The dried DNA sample was solubilised in 200 µL of buffer TE (Tris-HCl 1M pH 8.0; EDTA 0.5M pH 8.0). About 200 µL of DNA solution was added to 20 µL of RNase (10 mg/mL) and incubated at 37°C for one h. DNA was incubated at 4°C for one night. DNA solution was stored at -20°C. The DNA sample was amplified with 15 decamer primers using

the Thermal Cycler Gene PCR (ABI 9700). The composition of 1x PCR reaction was 5 µL of the DNA template (5 ng/µL), 0.2 µL of dNTP 0.2 mM, 2.5 µL of PCR buffer + $MgCl_2$ (1x), 1 µL of 10 pmol primer, 0.2 µL of 1U Taq polymerase and 16.1 µL of ddH_2O with a total volume of 25 µL. The PCR reaction thermal cycle was repeated 45 times in stages as follows: 94°C for one min, 36°C for one min, 72°C for 2 min and 72°C for 4 min. Extension time was conditioned at 72°C for 4 min. PCR product was fractionated by electrophoresis method in 1.4% agarose gel (w/v) submerged in 40 mL of 1x TAE. Electrophoresis was run at 75 volt for 1.5 h. Agarose gel was submerged in ethidium bromide solution for 10 min and washed with distilled water. Electrophorised gel was visualised under UV light and documented with Kodak gel logic. PCR-RAPD for each sample was replicated at least three times. The size of DNA bands was determined by comparison with 1 kb DNA ladder. Quantitative data were standardised based on Steel and Torrie's (1981) findings. The molecular data of the DNA bands were converted to binary numbers (0 and 1) and formulated in matrix. The relationship between mutant clones was analysed using SHAN clustering UPGMA. Genetic distance and clone grouping were determined using NTSYS ver. 1.70. The similarity index used in this research was Jaccard's (1901) Version 2.4. Chemical Compounds Analysis with GC-MS.

The leaves (including the stem) and tubers of rodent tuber were macerated in 96% ethanol. Extraction was done twice with a sonificator and incubated at room temperature for 48 h. The extracts were filtered with Whatman filter paper and analysed with a GC-MS detector. A volume of 5 µL of extract was injected into the column at a split ratio of 5:1 at 250°C. Helium was used as a carrier gas with a flow rate of 0.8 µL/minute. The initial column oven temperature was 70°C. It was then increased at a rate of 5°C/min until the temperature reached 200°C, after which it was stabilised for 1 min and then increased at the rate of 20°C/min until it reached 280°C for 28 min. The mass spectrometer was adjusted at electron impact ionisation mode at a voltage of 70 eV. Chemical compounds were identified by comparing the MS fragmentation pattern profile with the NIST database.

RESULTS AND DISCUSSION

Morphological Characterisation

Morphological characteristics, specifically the number of shoots, number of leaves, plant height and the fresh and dry weight of 17 MV3 clones were different from those of the control plants (Figure 1 and Table 1). After eight weeks, clone 6-1-2 was found to have the highest increase in shoot (4.5) and leaf number (15) compared to the control and the other MV3 clones. Clone 6-3-2-5

Figure 1. MV3 clones after eight weeks of growing in a green house. a: 6-3-2-5; b: 6-1-1-2; c: 6-9-1

Table 1
Morphological Characteristics of MV3 Clones and Control Plants

Num	Clone	Increase from 1st to 8th week			Total plant weight (g)*	
		Shoot	Leaf	Plant height (cm)	Fresh weight	Dry weight
1	Control	0.0	1.0	3.5	24.33	4.13
2	6-3-3-6	1.0	6.0	4.0	7.25	1.59
3	6-9-3	2.5	3.5	4.0	12.36	1.54
4	6-9-4	0.4	4.0	12.5	14.43	1.9
5	6-2-5-3	0.5	7.0	12.0	7.32	1.77
6	6-3-2-5	1.5	8.0	13.5	27.96	4.10
7	6-1-1-2	3.5	2.0	6.0	24.59	3.67
8	6-9-1	2.5	11.0	4.5	15.82	3.36
9	6-2-4-1	0.0	2.0	3.0	12.6	2.7
10	6-6-3-7	0.5	6.0	7.5	9.03	2.05
11	6-6-3-6	1.0	6.0	12.5	16.03	5.99
12	6-2-7	0.0	5.5	12.0	4.13	0.82
13	6-2-6-3	0.0	5.0	5.5	10.98	2.24
14	6-1-2	4.5	15.0	8.3	22.17	3.96
15	6-1-1-6	1.0	2.0	5.0	16.17	3.62
16	6-2-8-2	2.5	11.5	6.5	21.17	4.67
17	6-9-5	0.0	12.5	10.3	5.32	1.29
18	6-3-3-10	0.0	1.5	7.5	5.54	0.99

* Fresh and dry weight were cumulative of root, leaf, stem, tuber and flower weight

had the highest increase in plant height (13.5 cm) compared to the control and the other MV3 clones. Clone 6-3-2-5 had the highest fresh weight (27.96 gr), while clone 6-6-3-6 had the highest dry weight (5.99 gr) compared to the control and the other MV3 clones. Thus, based on morphological characterisation, MV3 clones were able to produce the higher biomass (number of shoots, number of leaves and plant height) compared to the control (Table 2).

The morphological characteristics of the rodent tuber control and MV3 clones were analysed using the NTSYS programme to generate relative similarity between the clusters. The dendrogram showed six clusters at the similarity coefficient cut-off of 0.73 (Figure 2). Clones in one cluster had a short genetic distance between one another based on morphological observation.

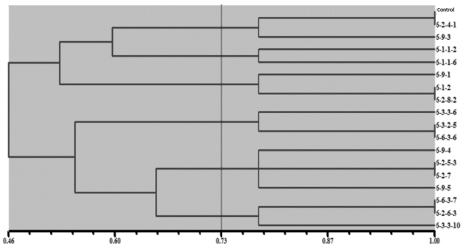

Figure 2. A dendrogram of MV3 clones based on morphological characteristics. The dendrogram was made from binary scores of the number of shoots, number of leaves and plant height data using the NTSYS software at the similarity coefficient cut-off of 0.73

Table 2
Grouping of MV3 Clones Based on Morphological Characteristics

Morphology	Interval	Clone
Increase in the number of shoots from 1st to 8th week	<1	Control, 6-9-4, 6-2-5-3, 6-2-4-1, 6-6-3-7, 6-2-7,6-2-6-3, 6-9-5, 6-3-3-10
	1-2	6-3-3-6, 6-3-2-5, 6-6-3-6, 6-1-1-6
	>2	6-9-3, 6-1-1-2, 6-9-1, 6-1-2, 6-2-8-2
Increase of the number of leaves from 1st to 8th week	<5	Control, 6-9-3, 6-9-4, 6-1-1-2, 6-2-4-1, 6-1-1-6, 6-3-3-10
	5-10	6-3-3-6, 6-2-5-3, 6-3-2-5, 6-2-6-3, 6-6-3-7, 6-6-3-6, 6-2-7
	>10	6-1-2, 6-2-8-2, 6-9-5, 6-9-1
Increase of plant height from 1st to 8th week	<5	Control, 6-3-3-6, 6-9-3, 6-9-1, 6-2-4-1
	5-10	6-1-1-2, 6-6-3-7, 6-2-6-3, 6-1-2, 6-1-1-6, 6-2-8-2, 6-3-3-10
	>10	6-9-4, 6-2-5-3, 6-3-2-5, 6-6-3-6, 6-2-7, 6-9-5

Table 3 shows the similarity coefficient matrix, which represents the genetic distance between the mutant clones based on morphological characteristics. A similarity coefficient of 1.00 (100%) indicates that there is no genetic difference, while one lower than 0.95 (95%) indicates that there is genetic difference between two mutant clones. Based on the morphological analysis, MV3 clones 6-3-2-5, 6-6-3-6, 6-1-2 and 6-2-8-2 were shown to have the lowest genetic similarity (33%) to the control, while MV3 clone 6-2-4-1 showed 100% similarity to the control. The other MV3 clones had 56% (nine clones) and 78% (three clones) genetic similarities to the control. According to this research, gamma irradiation at a dose of 6 Gy was able to increase the number of shoots, number of leaves and plant height of rodent tuber MV3 clones compared to the control plants (Table 1). The observed increase in the MV3 plant's biomass compared to that of the control is a crucial beneficial factor for commercial production of medicinal plants.

Gamma irradiation could induce the release of free radicals in plant cells; this induces somatic genetic cross-over, DNA sequence modification and chromosomal aberration (changes in the number and structure of chromosome). These genetic modifications alter the structure and metabolism of a plant as well as its morphological characteristics (Kovacs & Keresztes, 2002). A low dose of gamma irradiation has also been able to induce morphological alteration of potato plants (Afrasiab & Iqbal, 2010). The diversity of morphological characteristics observed between MV3 clones (Figure 2 and Table 3) was due to random mutation induced by gamma irradiation, which could change a gene's structure and function in a different way in different plants (Surya & Soeranto, 2006; Pillay & Tenkouano, 2011).

Table 3
Genetic Similarity Matrix of MV3 Clones Based on Morphological Characterictics

	Kontrol	6-3-3-6	6-9-3	6-9-4	6-2-5-3	6-3-2-5	6-1-1-2	6-9-1	6-2-4-1	6-6-3-7	6-6-3-6	6-2-7	6-2-6-3	6-1-2	6-1-1-6	6-2-8-2	6-9-5	6-3-3-10
Kontrol	1.00																	
6-3-3-6	0.56	1.00																
6-9-3	0.78	0.56	1.00															
6-9-4	0.78	0.33	0.56	1.00														
6-2-5-3	0.56	0.56	0.33	0.78	1.00													
6-3-2-5	0.33	0.78	0.33	0.56	0.78	1.00												
6-1-1-2	0.56	0.33	0.78	0.56	0.33	1.00												
6-9-1	0.56	0.56	0.78	0.33	0.33	0.33	0.56	1.00										
6-2-4-1	1.00	0.56	0.78	0.78	0.56	0.33	0.56	0.56	1.00									
6-6-3-7	0.56	0.56	0.33	0.56	0.78	0.56	0.56	0.33	0.56	1.00								
6-6-3-6	0.33	0.78	0.33	0.56	0.78	1.00	0.33	0.33	0.33	0.56	1.00							
6-2-7	0.56	0.56	0.33	0.78	1.00	0.78	0,33	0.33	0.56	0.78	0.78	1.00						
6-2-6-3	0.56	0.56	0.33	0.56	0.78	0.56	0.56	0.33	0.56	1.00	0.56	0.78	1.00					
6-1-2	0.33	0.33	0.56	0.33	0.33	0.33	0.78	0.78	0.33	0.56	0.33	0.33	0.56	1.00				
6-1-1-6	0.56	0.56	0.56	0.56	0.33	0.56	0.78	0.33	0.56	0.56	0.56	0.33	0.56	0.56	1.00			
6-2-8-2	0.33	0.33	0.56	0.33	0.33	0.33	0.78	0.78	0.33	0.56	0,33	0.33	0.56	1.00	0.56	1.00		
6-9-5	0.56	0.33	0.33	0.78	0.78	0.56	0.33	0.56	0.56	0.56	0.56	0.78	0.56	0.56	0.33	0.56	1.00	
6-3-3-10	0.78	0.33	0.56	0.78	0.56	0.33	0.78	0.33	0.78	0.78	0.33	0.56	0.78	0.56	0.78	0.56	0.56	1.00

According to Van Harten (1998), the normal irradiation doses that are commonly applied to plant cells were at the range of 5-100 Gy. Morphological diversity, which is induced by gamma irradiation, has also been observed in mutant plants of yardlong bean [*Vigna unguiculata* (L.) Walp.], whose number of shoots, number of leaves and plant height were higher compared to those of the control plants (Gnanamurthy et al., 2012). Potato var. Silana (Hamideldin & Hussin, 2013) and soybean (Mudibu et al., 2012), which had been induced by gamma irradiation also had higher plant height compared to controls. However, gamma irradiation at doses of 5-24 Gy on ginger vegetative plants (*Zingiber officinale*) (Rashid et al., 2013) and at doses of 10 and 20 Gy on *Curcuma alismatifolia* (Taheri et al., 2014) resulted in a lower number of leaves and shorter plants compared to the controls. Like MV1 clones (Sianipar et al., 2013c), MV3 clones also had morphological variations due to gamma irradiation (Table 3). Besides genetic mutation, environmental factors also influence morphological characteristics. Therefore, morphological characterisation should be accompanied with molecular marker analysis in order to obtain a more accurate estimation of genetic diversity.

RAPD Molecular Marker Analysis

The concentration of DNA extracts was between 641.70 and 4246.8 ng/µL. The OD_{260}/OD_{280} ratio was between 1.8 and 2.0, which indicated that the extracts were relatively pure (without RNA or protein) (Sinden, 1994). PCR-RAPD of rodent tuber's DNA extracts was done using 15 primers (Table 4), which had also been used for research on the other plants from *Typhonium* genus (Acharya et al., 2005). Primers used in this research were reproducible according to previous RAPD profile analyses of rodent tuber MV1 clones (Sianipar et al., 2015b). There were 67 polymorphic bands (190-3000 bp) out of the total 132 bands produced by 15 primers. The OPB-18 primer produced the highest number of polymophic bands as well as total bands compared to the other primers. Specifically, the OPB-18 primer produced 14 polymorphic bands out of the total 15 DNA bands. RAPD amplification of MV3 with OPB-18 primer produced polymorphic bands at 390 bp, 750 bp, 1350 bp and 3000 bp (Figure 3). The RAPD profile of OPB-18 primer revealed that MV3 clones had new DNA bands but also underwent the loss of DNA bands compared to the RAPD profile of control plants.

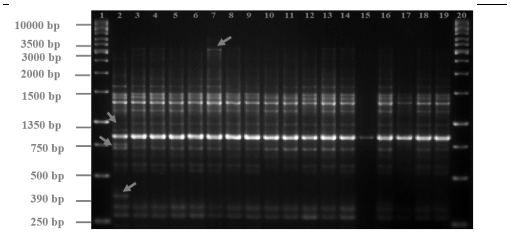

Figure 3. RAPD profile of MV3 clones amplified with OPB-18 primer. 1: Marker 1 kb; 2: Control; 3: 6-3-3-6; 4: 6-9-3; 5: 6-9-4; 6: 6-2-5-3; 7: 6-3-2-5; 8: 6-1-1-2; 9: 6-9-1; 10: 6-2-4-1; 11: 6-6-3-7; 12: 6-6-3-6; 13: 6-2-7; 14: 6-2-6-3; 15: 6-1-2; 16: 6-1-1-6; 17: 6-2-8-2; 18: 6-9-5; 19: 6-3-3-10; 20: Marker 1 kb

Table 4
The Number of DNA Bands Produced by RAPD Amplification of MV3 DNA with 15 Primers

Num	Primer	Sequence	Total bands	Polymorphic bands	Size (bp)
1	OPA-02	5'-TGCCGAGCTG-3'	5	1	450-1250
2	OPA-03	5'-AGTCAGCCAC-3'	10	6	530-2830
3	OPA-09	5'-GGGTAACGCC-3'	10	3	425-1900
4	OPA-14	5'-TCTGTGCTGG-3'	10	8	500-2500
5	OPB-18	5'-CCACAGCAGT-3'	15	14	310-3000
6	OPC-05	5'-GATGACCGCC-3'	13	9	250-1900
7	OPC-08	5'-TGGACCGGTG-3'	10	4	540-2170
8	OPC-11	5'-AAAGCTGCGG-3'	4	3	650-2200
9	OPC-14	5'-TGCGTGCTTG-3'	8	3	600-3000
10	OPD-08	5'-GTGTGCCCCA-3'	6	1	570-2830
11	OPD-10	5'-GGTCTACACC-3'	11	6	190-2000
12	OPD-18	5'-GAGAGCCAAC-3'	7	3	400-1350
13	OPD-20	5'-ACCCGGTCAC-3'	10	5	575-2400
14	OPE-03	5'-CCAGATGCAC-3'	5	0	350-1400
15	OPE-07	5'-AGATGCAGCC-3'	8	1	350-1500
Total			132	67	

RAPD profiles were scored using the NTSYS software to produce a dendrogram for determining relative genetic similarity between clones. According to Rout (2006), a dendrogram represents the genetic relationship between different plant varieties of the same species. A dendrogram of MV3 clones based on the RAPD analysis with a similarity coefficient cut-off of 0.87 showed four main clusters (Figure 4). The genetic similarity matrix showed the genetic distance between MV3 clones (Table 5). The matrix showed that there was no 100% genetic similarity between 17 MV3 clones and the control. Mutant clones 6-9-3, 6-2-5-3, 6-1-1-2, 6-2-6-3, 6-1-2 and 6-3-3-10 each had 71% genetic similarity to the control, which was the lowest among all the clones. Clone 6-6-3-6 had 80% genetic similarity to the control, which was the highest among all the clones. Table 5 showed that there were three clones with 73%, one with 74%, one with 80%, one with 75%, four with 76%, two with 77% and one with 78% genetic similarity to the control.

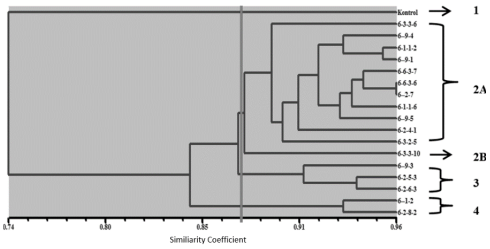

Figure 4. Dendrogram of MV3 clones based on RAPD profile. The dendrogram was generated by scoring of DNA bands in RAPD profiles of 15 primers using NTSYS UPGMA and SHAN clustering at the similarity coefficient cut-off of 0.87

The RAPD molecular marker is able to detect DNA polymorphism without taking environmental factors into account (Guimaraes et al., 2007). The RAPD molecular marker, which utilises random primers, was able to show genetic diversity between the control and the MV3 clones. Single random primers have been known to be able to amplify DNA and show the polymorphism of mutant DNA (Williams et al., 1990). DNA mutation could change primer annealing sites in a plant's genome, which is followed by change in the RAPD profile of DNA bands (Tindall et al., 1988). The number of DNA bands produced by a primer is determined by the number of complementary sequences in the genome (Singh & Singh, 1995).

DNA polymorphism of MV3 clones is due to gamma irradiation on somatic cell population. Gamma irradiation is able to induce the production of reactive free radicals, which can change the DNA sequence and break DNA double bonds. Gamma irradiation also causes chromosomal aberration such as deletion, invertion, translocation and duplication (Gorbunova & Levy, 1997). The loss of DNA bands observed in the RAPD profile of MV3 clones (Figure 3) was probably caused by destruction of DNA, rearrangement of chromosomes and deletion or insertion of DNA nucleotides. The existence of new DNA bands observed in the RAPD profile of MV3 but not in the control was probably caused by deletion or invertion of DNA nucleotides (Yunus et al., 2013).

RAPD analysis of *Rhododendron* also showed genetic differences between the control and MV3 clones, which were irradiated with gamma ray at doses of 5 and 10 Gy (Atak et al., 2011). Yunus et al. (2013) also proved that RAPD analysis was effective in deleting DNA polymorphism of ginger plants (*Etlingera elatior*) irradiated with 10 Gy gamma ray. Polymorphism analysis of gamma-irradiated DNA has also been done on potato plants (Yaycili & Alikamanoglu, 2012), Hibiscus *Sabdariffa* L. (Sherif et al., 2011) and anthurium plants (Puchooa, 2005). A genetic similarity matrix (Table 5) of the results shows the diversity of genetic modification between the MV3 clones. The observed genetic similarity variation between the MV3 clones was due to random mutation caused by gamma irradiation (Pillay & Tenkouano, 2011). According to Pillay and Tenkouano (2011), the DNA repair mechanism of potato plants was different from one cell to another; therefore, they showed different genetic profiles. Based on morphological markers, MV3 clone 6-2-4-1 had 100% similarity to the control, while, based on the molecular marker, clone 6-2-4-1 had 73% genetic similarity to the control. The difference in genetic similarity between morphological and molecular markers was due to the influence of the environment on morphological analysis.

The genetic difference between different rodent tuber mutant clones (MV0 and MV1) has also been assessed by applying the PCR-RAPD technique (Sianipar et al., 2015a; Sianipar et al., 2015b).

Table 5
Genetic Similarity Matrix of MV3 Clones Based on RAPD Molecular Marker

	Kontrol	6-3-3-6	6-9-3	6-9-4	6-2-5-3	6-3-2-5	6-1-1-2	6-9-1	6-2-4-1	6-6-3-7	6-6-3-6	6-2-7	6-2-6-3	6-1-2	6-1-1-6	6-2-8-2	6-9-5	6-3-3-10
Kontrol	1.00																	
6-3-3-6	0.73	1.00																
6-9-3	0.71	0.90	1.00															
6-9-4	0.74	0.90	0.88	1.00														
6-2-5-3	0.71	0.86	0.91	0.91	1.00													
6-3-2-5	0.75	0.89	0.90	0.90	0.89	1.00												
6-1-1-2	0.71	0.92	0.86	0.94	0.91	0.92	1.00											
6-9-1	0.76	0.89	0.83	0.92	0.89	0.87	0.95	1.00										
6-2-4-1	0.73	0.83	0.83	0.92	0.85	0.86	0.89	0.89	1.00									
6-6-3-7	0.78	0.92	0.86	0.93	0.87	0.89	0.93	0.93	0.90	1.00								
6-6-3-6	0.80	0.88	0.86	0.92	0.87	0.92	0.90	0.92	0.90	0.94	1.00							
6-2-7	0.77	0.90	0.89	0.92	0.88	0.92	0.91	0.92	0.92	0.95	0.96	1.00						
6-2-6-3	0.71	0.87	0.91	0.86	0.94	0.87	0.88	0.88	0.83	0.89	0.86	0.88	1.00					
6-1-2	0.71	0.80	0.79	0.85	0.82	0.83	0.85	0.85	0.86	0.86	0.87	0.86	0.80	1.00				
6-1-1-6	0.77	0.89	0.90	0.90	0.87	0.89	0.90	0.90	0.90	0.92	0.94	0.95	0.89	0.87	1.00			
6-2-8-2	0.73	0.82	0.80	0.87	0.80	0.85	0.84	0.86	0.87	0.88	0.91	0.89	0.80	0.93	0.89	1.00		
6-9-5	0.76	0.89	0.86	0.92	0.86	0.90	0.92	0.92	0.91	0.93	0.93	0.92	0.88	0.85	0.93	0.89	1.00	
6-3-3-10	0.71	0.87	0.85	0.85	0.85	0.86	0.89	0.88	0.85	0.87	0.87	0.88	0.86	0.82	0.90	0.81	0.91	1.00

Analysis of Chemical Compounds with GC-MS

GC-MS analysis revealed that the leaves of MV3 clones contained at least eight anticancer compounds i.e. more than in the control plants (Table 6). The quantity of anticancer compound stigmasta-5,22-dien-3-ol (3-beta) in leaves of clone 6-1-1-6 was 10.96% higher than in the controls and was also the highest compared to the other MV3 clones. The leaves of MV3 clones also contained new anticancer compounds that were not found in the control plants, such as coumaran, gamma tocopherol, 1-phenanthrenecarboxylic acid and oleic acid (Table 6).

Table 6
The Number of DNA Bands Produced by RAPD Amplification of MV3 DNA with 15 Primers

Compounds	% Relative abundance in control	% Relative abundance in mutant clones							
		6-9-1	6-3-3-6	6-1-1-6	6-3-2-5	6-6-3-6	6-1-1-2	6-1-2	6-2-8-2
Hexadecanoic acid	11,64	10,5	13,09	2,19	16,85	22,48	7,51	19,21	14,92
Alpha-tocopherol	3,36	0	0	0	2,84	0	3,5	4,11	0
Coumaran	0	0,7	0	0	0	0	0	0	0
Gamma-tocopherol	0	1,45	0	0	1,38	0	0	0	0
Ergost-5-en-3-ol (3 beta)	6	7,42	0	13,58	3,3	0	2,69	0	0
9-Octadecenamide	1,11	1,75	2,63	4,2	1,06	1,72	2,83	1,83	1,71
Squalene	3,8	2,65	0	0	3,46	0	4,6	0	0
Stigmasta-5,22-dien-3-ol (3 beta)	12,84	13,68	0	23,8	13,41	0	13,37	9,89	9,02
Stigmast-5-en-3-ol (3.beta.,24s)	2,42	4,02	0	7,28	0	0	0,52	0	0
1-Phenanthrenecarboxylic acid	0	0	0	0	0	0	0,38	0	0
Oleic acid	0	0	0	0	0	0	0,62	0	0
4-vinyl-2-methoxy-phenol	0,28	0	0	0	0	0	0	0,43	0

The highlights indicate the higher quantities of anticancer bioactive compounds in MV3 clones compared with the control

The GC-MS analysis of tubers of MV3 clones revealed that they contained six anticancer compounds, more than in the control plants (Table 7). The quantity of hexadecanoic acid methyl ester of tubers of clone 6-6-3-6 was 15.37% higher than in the control and was also the highest compared to the other MV3 clones. Tubers of MV3 clones also contained new anticancer compounds that were not found in the control plants i.e. alpha-tocopherol, stigmasta-5,22-dien-3-ol (3-beta), beta elemene and dodecanoic acid.

Table 7
The Number of DNA Bands Produced by RAPD Amplification of MV3 DNA with 15 Primers

Compounds	% Relative abundance in control	% Relative abundance in mutant							
		6-9-1	6-3-3-6	6-1-1-6	6-3-2-5	6-6-3-6	6-1-1-2	6-1-2	6-2-8-2
Hexadecanoic acid	8,91	11,81	5,2	10,91	13,57	13,2	0,06	0,1	14,29
Alpha-tocopherol	0	0,3	0,16	0	0	0	0	0	0
9-octadecenamide	0,76	0,35	1,13	0	0,5	0,64	0	0	0
Stigmasta-5,22-dien-3-ol (3 beta)	0	0	0	0	0	4,78	3,8	0	0
Stigmast-5-en-3-ol (3.beta.,24s)	2,44	2	0	0	2,21	2,83	0	0	0
Tanshinone II-b	0,17	0,59	0	0	0	0	1,64	0	0
Hexadecanoic acid ethyl ester	20,98	13,61	20,58	15,08	18,07	0	24,26	35,9	7,57
Hexadecanoic acid methyl ester	0,20	0,30	0,28	0	0,25	15,57	0	0	0
Beta elemene	0	0	0	0	0	0	0,12	0	0
Dodecanoic acid	0	0	0	0	0	0	0	0,7	0
Oleic acid	0	0	0	0	0	0	0,62	0	0
4-vinyl-2-methoxy-phenol	0,28	0	0	0	0	0	0	0,43	0

The highlights indicate the higher quantities of anticancer bioactive compounds in MV3 clones compared with the control

The GC-MS analysis showed that the leaves of MV3 clones contained at least eight anticancer compounds, more than in the control plants. The quantity of anticancer compound stigmasta-5,22-dien-3-ol (3-beta) of clone 6-1-1-6 was the highest compared to the control and the other MV3 clones (Table 6). Stigmasta-5,22-dien-3-ol (3 beta) (stigmasterol) is a phytosterol that could reduce the number of *Ehrich Ascites Carcinoma* (EAC). Stigmasterol is able to activate the protein phosphatase 2A by ceramide to promote the apoptosis of cancer cells. Stigmasterol also exerts an antioxidant effect because it is able to reduce lipid peroxidation and increase gluthathionin, superoxide dismutase and catalase activities in EAC mice liver (Ghosh et al., 2011).

The leaves of the MV3 clones also contained new anticancer compounds that were not found in the control plants, such as coumaran, gamma-tocopherol, 1-phenanthrenecarboxylic acid and oleic acid. Coumaran (2,3-dihydrobenzofuran) and its derivatives are able to inhibit the polymerisation of tubulin, thus inhibiting mitosis (Pieters et al., 1999). Vitamin E (α dan γ-tocopherol) has been proven to be able to reduce the risk of developing several kinds of cancer (Jiang et al., 2001). The compound, 1-phenanthrenecarboxylic acid, shows anticancer activity against KB nasopharyngeal cancer, Hop62 lung cancer, ME180 cervical cancer and K562 leukemia (Tatiya et al., 2014). Oleic acid is a monounsaturated fatty acid n-9 with anticancer activity against breast cancer (Escrich et al., 2008) and colon adenocarcinoma (Carrillo et al., 2011).

The GC-MS analysis of tubers of MV3 clones revealed that they had six anticancer compounds; this was more than in the control plants. The quantity of hexadecanoic acid methyl ester of tubers of clone 6-6-3-6 was the highest compared to the control and the other MV3 clones. Hexadecanoic acid methyl ester has been able to induce the apoptosis of human gastric cancer cells (Yu et al., 2005).

The tubers of the MV3 clones also contained the new anticancer compounds that were not found in the control plants i.e. alpha-tocopherol, stigmasta-5,22-dien-3-ol (3-beta), beta elemene and dodecanoic acid. Beta-elemene has anti-proliferative activity against prostate, lung, colon, cervical, breast and brain cancers (Li et al., 2010). Dodecanoic acid (lauric acid) has been proven to be able to induce apoptosis of colon cancer cells (Fauser et al., 2013). This finding was consistent with previous analysis of rodent tuber MV1 phytochemicals, whose shoots and tubers contained at least eight anticancer compounds, which is a higher number of anticancer compounds than in the control. The shoots and tubers of MV1 also contained new anticancer compounds that were not found in the control plants (Sianipar et al., 2015c). Because MV3 clones have a higher plant biomass than the control and contain a higher amount of anticancer compounds than the control, they have a high potential for becoming a new source of anticancer bioactive compounds for the formulation of commercial anticancer drugs.

CONCLUSION

MV3 clones underwent genetic changes not seen in the control according to morphological, RAPD profile and bioactive compound analyses. Based on the RAPD molecular marker, four genetic diversity clusters were shown, indicating that MV3 clones were different from the control. The GC-MS analysis showed that the leaves of the MV3 contained at least eight kinds of anticancer compound, more than in the control. MV3 leaves also contained coumaran, gamma-tocopherol, 1-phenanthrenecarboxylic acid and oleic acid, which were new anticancer compounds not found in the control. The GC-MS analysis also showed that tubers of MV3 contained at least six types of anticancer compound, more than in the control. MV3 tubers also contained new anticancer compounds that were not found in the control i.e. alpha-tocopherol, stigmasta-5,22-dien-3-ol (3-beta), beta elemene and dodecanoic acid. Clone 6-1-1-2 had the lowest genetic similarity to the control, contained a higher amount of anticancer compounds compared to the control and contained new anticancer compounds that were not found in the control. Therefore, rodent tuber MV3 clones are solid mutant clones that have a high potential for being developed into anticancer drugs.

ACKNOWLEDGEMENT

This work was funded by The Directorate General of Higher Education through a competitive grant project. The authors would like to thank The Directorate General of Higher Education, Ministry of National Education, Indonesia. The authors also thank Prof. Ika Mariska for reviewing the final manuscript.

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Pertanika J. Trop. Agric. Sci. 40 (1): 203 - 214 (2017)

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Forming Process Analysis in Environmentally-Friendly Composite Production from Fibres of Oil Palm Empty Fruit Bunches

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ABSTRACT

The empty fruit bunch of oil palm (EFB) is the solid waste that is generated at palm oil mills. In 2012 only, it was available in large amounts by more than 50 million tons worldwide. Its utilisation for technical purposes is unfortunately very limited and so far, it is still less value-added. It would be interesting to discover the appropiate technology so that the biomass has an added-value such utilisation for technical and commercial products. Fibre from EFB can be processed to be environmentally-friendly composites by mixing the fibres with renewable binding agents based on starch such as potato starch. There are some options to forming the between product into a composite such as creating a dough base first that is then laminated and finally mixed (nonwoven). Experiments to produce possible composites can be done where the combination between product and technology matches so that the fibres are spread randomly and homogenously throughout the body of the composite. The variables for this option of producing a composite with a thickness of 2 mm are a temperature of 180°C, press duration of 5-15 min and pressure of about 10-30 bar where the modulus of elasticity is about 7 GPa.

Keywords: biomass, composite, empty fruit bunch, forming process, oil palm

ARTICLE INFO

Article history:
Received: 19 November 2015
Accepted: 04 May 2016

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INTRODUCTION

In 2012, crude palm oil (CPO) was the most produced vegetable oil with a world production of about 53.3 million tons. Indonesia is the largest producer of CPO in the world, producing 26.9 million tons a year. The same amount of empty fruit bunches (EFB) was also produced i.e. about 53.3 million tons of EFB worldwide and

ISSN: 1511-3701

26.9 million tons of EFB just from Indonesia in 2012 (Arya et al., 2015). The production of CPO is increasing from year to year, so that the amount of EFB produced every year is also increasing. EFB is an economical biomass. It can produce about 50-53% fibre, which is a potential industrial raw material (Arya et al., 2015). Scale enlargement of EFB fibre under the microscope and from using Structural Equation Modelling (SEM) it is known that fibre has a honey-comb structure, which is the basic structure for lightweight construction (Arya, 2005; Arya, 2015) as shown in Figure 1 below.

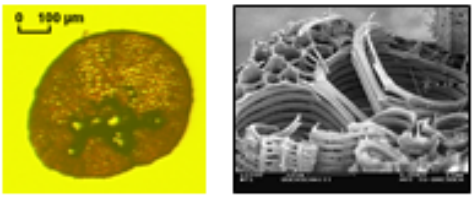


Figure 1. Structure of EFB fibre (Arya, 2005; Arya, 2015)

This research focussed on studying the structure of EFB fibre to consider its potential use as an industrial raw material especially for technical purposes as a composite.

Of the various types of waste (gas, liquid, solid) produced in the palm oil mill, the solid waste of EFB has the potential to be utilised as raw material for industrial use as a natural fibre that can be utilised to produce composites. Composites can be applied in various technical and commercial products. A composite material has a distinctive specification as a lightweight material with high strength. Thus, the basic ingredients of composite products can be further applied as an exterior component of automotive parts or bulletproof (hard plate) items. Hard plate today is produced from synthetic material such as aramid, HPPE, minerals (ceramic) or metals. Synthetic and mineral raw materials¬ are difficult to be composted without the help of sustainable chemical and mechanical processes but EFB fibre is a natural fibre that is renewable and biodegradable. In comparison with non-organic components such as minerals, aramid, which is made from a complex process and also through the addition of metals to achieve the desired strength so that in bulletproof items, five layers of composites are able to withstand the bullets of a certain calibre. The technology to produce the composites requires binding agents based on starch such as potato starch or protein such as casein to be added. This technology produces an environmentally-friendly composite that is biodegradable, possesses strong mechanical characteristics (a high mechanical strength) by more than 7 GPa, has good heat resistance up to 240°C, is resilient to electrical conductivity, has good thermal insulating and has sound-proofing characteristics. Composites made out of EFB are expected to have mechanical strength equal or equivalent to the mechanical strength of conventional materials.

Utilisation of natural fibres in composites for technical products has been carried out in Europe for about 15 to 20 years, especially

for vehicle components. Used natural fibres that are available in Western Europe include fibres of flax and hemp, while natural fibres imported from India include fibres such as kenaf. Efforts to use natural fibres takes into account several factors that support the development of the automotive industry like global markets and the development and production of components that can use non-woven fibres. Generally, composites consist of two main components i.e. fibres as reinforcing material and fibres as fillers in the form of adhesive.

The second major component of a composite that is available now is unfortunately based on non-renewable raw materials e.g. metals, minerals and polymers. Composites may be applied as industrial raw materials in manufacturing, in this case as a filler component, but the materials available are still based on non-biodegradable materials e.g. binding agent of latex, where latex is a derivative of natural rubber and used as an adhesive (Felegi, 1990). On the other hand, a combination of natural fibres and natural adhesives based on starch (potato) can produce composite materials that are renewable and environmentally-friendly.

In the palm oil industry, fresh fruit bunches (FFB) are the main raw material to produce crude palm oil (CPO), where FFB is harvested for palm oil and its derivatives. At the first stage of production, the FFB is sterilised (this is called the evaporation process), where steam at a temperature in the range of 130 to 140°C and by pressure of about 2.8-3.0 kg/cm^2 is channelled to a sterilisation tank. After the sterilisation process, the fruits in the FFB are easily separated in a thresher machine. After the fruits are separated from the bunch, what is left behind is the empty fruit bunch, EFB, or the bunch without fruits. This EFB is the biomass or solid waste produced from CPO production in a palm oil mill.

The oil palm fruit contains palm oil (CPO) in the palm fruit flesh (mesocarp) and its oil is has economic value. The EFBs are a solid waste that is burnt in an incinerator or used as compost instead of fertiliser for oil palm plantations. Both are no-added-value solutions. If the EFB can be utilised, it will be an added-value for the palm oil industry and other industrial sectors. EFB fibres are a potential renewable raw material for producing technical and commercial products. This solution can give added-value for this solid waste for instance if it is used as a component of exterior and interior parts of vehicles or as a bulletproof material. Fiberising EFB for technical and commercial use is done in a hot press at 180°C.

MATERIALS

The Fibres of Oil Palm Empty Fruit Bunch

The first step is the procurement of the main raw material, the EFB. This study traced the route of EFB that was transported from a palm oil mill in PTPN VIII, Kertajaya, located in Lebak, Banten, Malimping, West Java. The EFB is generated at the palm oil mill as biomass. Its availability is centralised;

this is a very good logistic factor. EFBs are generated after the separation process separates the fruits from their bunches in a thresher machine. The EFBs are transported in a truck with a load of about 4-7 tons. The EFB is fiberised manually. Some unused parts such as burnt fibres, thorns, leaves and dust are separated. Only good fibres are used as raw material for composite production.

After the raw material is obtained, the next stage is the process of fiberisation, where the bunches are made into fibre (this requires a duration of one month for 7 tons of EFB). This is followed by washing, drying, election, where brightly coloured fibres are separated from the brown, burnt ones during the sterilisation process in the palm oil mill and finally, packing the fibre. Fibre requires careful packaging as it needs to be properly stored. If possible, it should be supplied to purchasers immediately. Fibre packaging is a new process. It takes about six months for the whole process from washing to packaging to be completed.

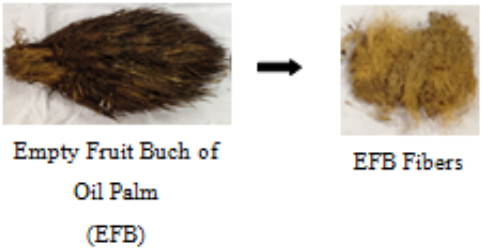


Figure 2. EFB and its fibres

Producing a Binding Agent from Potato Powder

Potato starch is used to make the binding agent. The potatoes are bought at traditional markets. Figure 3 shows how the binding agent from potato starch is produced.

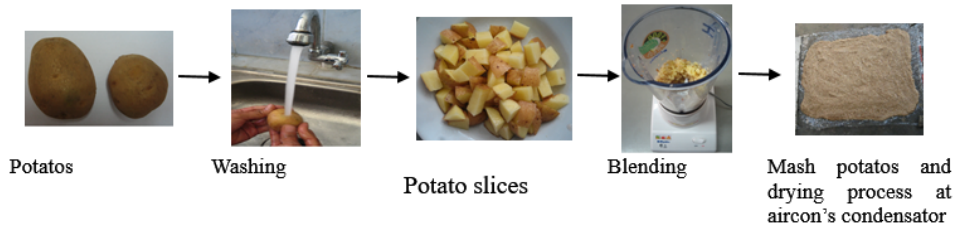


Figure 3. Production of binding agent raw material from potatoes

First, the potatoes are washed. Then they are cut into slices. The slices are blended in a blender and end up as mashed potato. The mash is air dried at about 40-60 0C. The drying process takes about 12 hours. The dried mash is crushed manually or in a blender to form a powder. This powder is the raw material for the binding agent. The powder is mixed in warm water and stirred slowly and consistently by hand. A gel-like substance is produced; this is the binding agent for the composite. The composite must have a viscosity η of 487 mPa s and a surface tension γ of 97 mN/m.

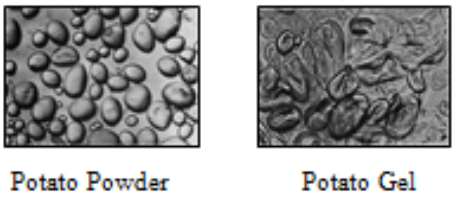


Figure 4. The powder and gelatinised powder of potato starch (Kohler, 1971)

METHODOLOGY

The production process of industrial raw materials (fibres) of the solid waste of palm bunches begins with the process of packaging and storing the industrial raw materials. The solid waste is supplied from palm oil mills. Next, the fibre is carried manually to reduce the possibility of damage to it. The fibre is collected and packed into a box that is then transported and stored in a humidity-controlled room. Figure 5 illustrates the forming process where the raw materials are mixed and formed before being turned into the end product (Arya, 2015).

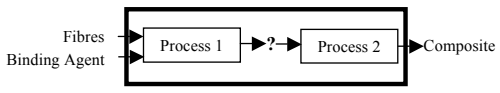


Figure 5. Process stages of end product forming

The question mark (?) in Figure 5 indicates the between product. The composite may take three forms, namely dough, laminate (fibres in row) and mixture (Arya, 2015), as explained below:

Option 1: Dough

According to Tsiapouris (Felegi et al., 1990), enthalpy bilance plays a big role in the process by when the option of dough is selected.

Accumulation of enthalpy = Temperature flows from hot plates – steam is given out, as shown by the formula below.

$$c_p M \frac{dT}{dt} = \alpha_e A[T_w(t) - T(t)] - \dot{M}_v(r_w + c_w T) \qquad (1)$$

Hot-pressing to form a dough as the between-product in producing the composite, unfortunately, did not yield a good result as the fibre was not homogenous throughout the composite. Figure 6 shows that the fibre did not spread out homogenously throughout the composite.

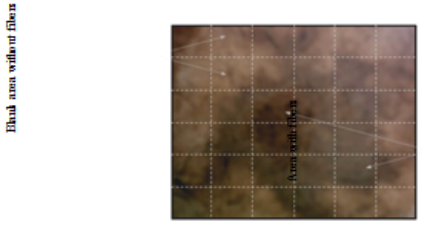


Figure 6. Expasion type of the fibres in composite (Felegi et al., 1990)

Option 2: Laminate

In lamination, the fibre is aligned in rows and coloums. The hole room of the positioned fibre is filled with binding agent so that

the fibre is filled out with binding agent. This is the structure of built composite (see Figure 7).

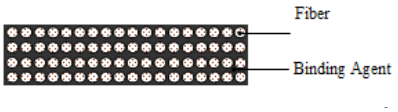


Figure 7. Composite built by fibres and binding agent (Felegi et al., 1990)

According to Föll (2003), all the fibre are straight and in parallel position throughout the matrix so that the composite material possesses elasticity as a mechanical property (fibres and matrix). An ideal composite material of fibre modulus of elasticity is greater than the matrix modulus (EF > EM) of hard fibre in a soft matrix. According to Hooke's law, the relationship between direct stress (σ in N/mm2), direct strain (ε) and modulus of elasticity (E in MPa) is formulated as follows (Köhler, 1971):

$$\sigma = E \cdot \varepsilon \qquad (2)$$

Direct strain ε is the relative change in length (Köhler, 1971):

$$\varepsilon = \Delta l / l_0 \qquad (3)$$

where

Δl: Change of measuring length

lo: initial length

l = lo +Δ l

The load on the fibre position (parallel or perpendicular) to produce direct stress σ on the composite body also depends on the position of the fibre, as shown in Figure 8 and Figure 9.

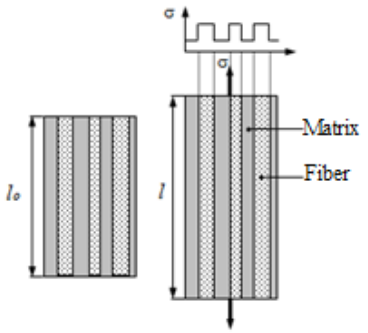


Figure 8. Tensile test parallel to the fibre position

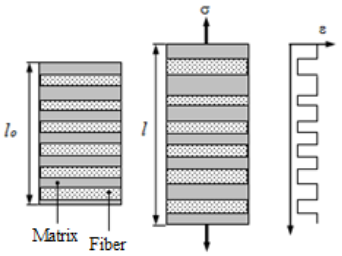


Figure 9. Tensile test perpendicular to fibre position

The modulus of elasticity of the composite by tensile test is found to be parallel to fibre position. In order to stretch the fibre to ε more power must be applied to the cross-sectional area of the fibre than to an equally large area of the matrix (Tsiapouris, 2000).

$$\sigma VB = \varepsilon \, [E_F \cdot V_F + EM \cdot (1 - V_F)] \qquad (4)$$

or

$$Ep = E_F \, . \, V_F + E_M \cdot (1 - V_F) \qquad (5)$$

The modulus of elasticity of the composite by tensile test is found to be perpendicular to fibre position. Here, the fibre is less stretched than the matrix (Föll, 2003).

$$\varepsilon = \sigma\left(\frac{V_F}{E_F} + \frac{1 - V_F}{E_M}\right) \quad (6)$$

or

$$E_s = \frac{1}{\frac{V_F}{E_F} + \frac{1 - V_F}{E_M}} \quad (7)$$

where

Ep: Modulus of elasticity of composite by tensile test parallel to fibre position

Es: Modulus of elasticity of composite by tensile test perpendicular to fibre position

σ_{VB}: Tension on cross-sectional area of the composite material

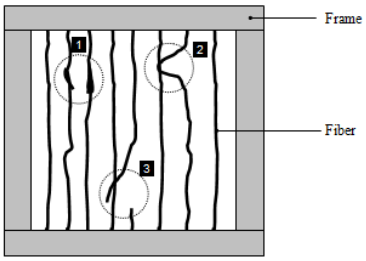
V_F: Volume fraction of the fibre

E_F: Fibre modulus of elasticity

E_M : Binding agent or matrix modulus of elasticity

According to Arya (2005), the production of a laminate of oil palm fibre produced in relation to the manufacture of fibreglass indicates the following (see Figure 10):

- Oil palm fibre is a corrugated fibre (wave type) and has non-uniform fibre thickness that ensures that parallel fibre order cannot be realised
- Fibre breaks indicate that the fibre laminate has not formed well because the fibre strands are no longer parallel to one another.
- Where fibre strands overlap between the upper and lower strands, no fibre bonding through the binding agent takes place and that location is a weak spot in the composite.
- EFB fibre is not an endless fibre; the dimensions of the sample will depend on the maximum fibre length of this natural fibre and the preparation process (shredding/crushing the EFB).
- A problem during gluing occurred where the gelantinised binding agent derived from potato starch dried out under normal weather conditions and shrank by about 80%, rendering the laminate option inappropriate for use in forming a composite.



. .: Fibre thickening and corrugated (wave)

. .: Fibre overlap

. .: Fibre fracture

Figure 10. Experimental equipment for laminate specimen production (Arya, 2005)

The laminate option was also problematic as the binding agent lost volume and would not dry as did the synthetic binding agents. Figure 11 shows how the binding agent of potato will lose volume after some days and weeks.

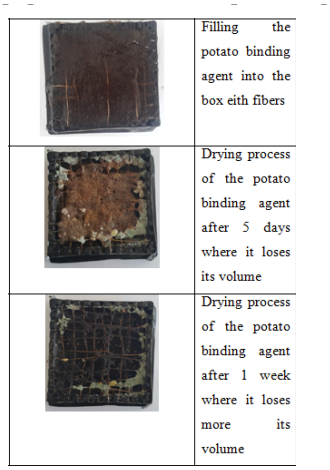


Figure 11. Drying process of potato binding agent

Option 3: Mixture (Nonwoven)

The EFB fibre strands are first mixed with the binding agent and then dosed in unorientated/random fashion in a matrix box. The fibre strands are compressed to a certain thickness and dried, leading to the naming of this option as mixture or non-woven.

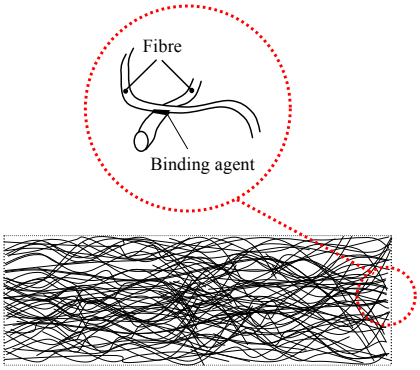


Figure 12. Mixture (non-woven) of EFB fibres (Arya, 2005)

Figure 12 shows that the EFB fibre with length of more than 1 cm (long fibre) is positioned in random direction. The fibre have a wave structure that naturally provides support, giving strength to the composite. If bent, the strands of fibre will be bent but they will not break, as shown in Figure 13 below.

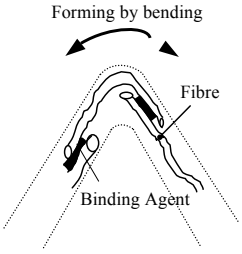


Figure 13. Composite flexibility through fibre flexibility (Arya, 2005)

The long fibres of EFB ensure flexibility of the composite. The binding agent is carried in steam (moisture content) in soft condition and remains deformed after the steam leaves the body of the composite. This option was chosen for the development of a production process for this research. With EFB fibres, unfortunately the modulus of elasticity calculation, according to Föll, cannot be produced. Due to non-orientated fibre positioning, uneven fibre length and thickness of the EFB fibre strands and the extreme reduction in volume of the binding agent after drying, laminating was considered an inappropriate option for forming a composite. Where the structure of the processing goods (fibre and binding agent) and adhesive strength may have influence on product specificcation, a different approach can be taken for the calculation of composite strength such as through the three-point bending test (bending modulus), where the flexural modulus of elasticity is the relationship between the increasing surface stress of a rod with increasing outer fibre strain and unhindered cross-sectional deformation (Blumenauer, 1994).

In the production process using this mixture option the variables and parameter are 120-180°C hot pressing temperature, pressure at 10-30 bar, pressing duration of 15-30 minutes (Arya, 2015) to gain strength. Another matter that needs to be considered is the humidity of the materials (ϕm); the value of moisture affects the hot-press process duration. In the pressing process, heat is delivered from the top plate and the bottom plate, both of which must reach the same temperature so that the body of the composite receives the same amount of heat and is therefore, homogeneous. Figure 12 below shows the effect of input material humidity (in %) on the hot-press process duration where the body of the composite is homogenous in terms of temperature.

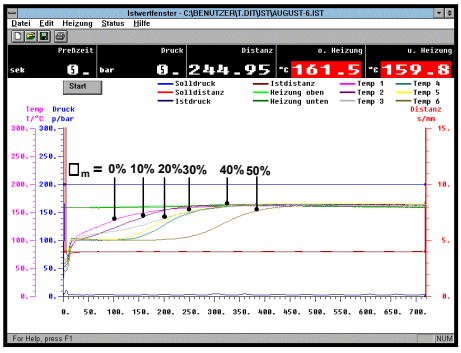


Figure 14. Composite flexibility through fibre flexibility (Arya, 2005)

Figure 14 shows the composite in the hot-press process receiving equitable distribution of heat throughout its body.

Figure 15. Environmentally-friendly composite from EFB fibres and binding agent from potato)

CONCLUSION

The following are the conclusions of this study.

- Paying attention to the uniform distribution of the EFB fibre strands in the composite body established a match for the option of mixture (non-woven) in forming the between product for a composite for this research.

- The option of lamination was discarded because of the reduction in volume (shrinkage) of the binding agent of about 80% that was produced using this option.

- The option of lamination required each strand of fibre to be tied to the frame. This difficulty was another reason for discarding this option.

- The environmentally-friendly composite that was produced can be used in manufacturing automotive (interior or exterior as well) parts and bulletproof material, among other uses.

ACKNOWLEDGEMENTS

The author would like to thank his beloved wife Tiwik Kusmarttanti, his parents (Drs. Arya D. Wisaksana and Mrs. G. V. Kajzel), his brothers and sister (Drs. Yudhi S. Arya, Dhani w. Arya, SE., MBA, Tantri P. McGinnis, DEA) and all his friends. This research paper would not have been possible without them.

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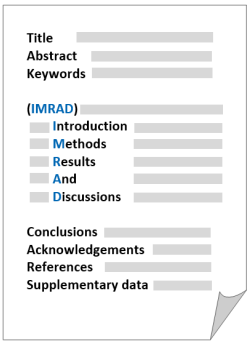

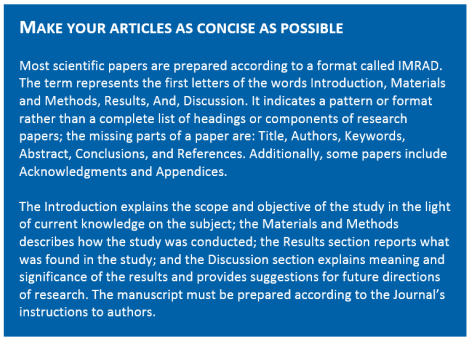

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Pertanika Journal of Tropical Agricultural Science
Vol. 40 (1) Feb. 2017

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ISSN 1511-3701
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