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About the Journal

Overview

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

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

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

The Journal is available world-wide.

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

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

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

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

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

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

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

Manuscript preparation

Refer to Pertanika's **INSTRUCTIONS TO AUTHORS** at the back of this journal.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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ABSTRACTING/INDEXING

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Contents

Foreword

Welcome to the **Second 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 independently owned and managed by the university and run on a non-profit basis for the benefit of the world-wide science community.

This issue contains **10 articles**, out of which **two** are review papers and **eight** are regular papers. The authors of these articles come from different countries, namely, **Malaysia**, **Iraq**, **Indonesia** and **Thailand**.

The first review paper aims to briefly cover the utilisation of oil palm fronds as ruminant feed and its effect on fatty acid metabolism (*Ghani, A. A. A., Rusli, N. D., Shahudin, M. S., Goh Y. M., Zamri-Saad, M., Hafandi, A.* and *Hassim, H. A.*). The second review paper focusses on the formation and utilisation of acid sulfate soils in Southeast Asia for sustainable rice cultivation (*J. Shamshuddin, Q. A. Panhwar, F. J. Alia, M. A. R. S. Shazana, O. Radziah* and *C. I. Fauziah*).

The eight regular papers cover a wide range of topics. In the first research paper, the effects of soaking periods and adhesive concentrations on the properties of phenol-formaldehyde-resin-treated oil palm wood was studies (*Khairunnisha, I. P. N., Bakar, E. S., Rachel, J. L., Halis, R.* and *Choo, A. C. Y.*). The next paper discusses the hypo-osmotic swelling test modification to enhance cell membrane integrity evaluation in cryopreserved bull semen (*Baiee, F. H., Wahid, H., Rosnina, Y., Ariff, O. M., Yimer, N., Salman, H., Tarig, A. A.* and *Khumran, A. M.*). The other papers are studies on: effects of shading and fertiliser on the growth and antioxidant content of olives (*Olea europaea* L.) (*Arlinda Puspita Sari, Triadiati Triadiati* and *Diah Ratnadewi*); enhancing solubility of curcumin in turmeric oleoresin for

improving productive performance of broiler chickens (*Porn-anek, P.* and *Promkot, C.*); translocation and elimination of Cu in *avicennia marina* (*Martuti, N. K. T., Widianarko, B.* and *Yulianto, B.*); utilisation of local crops as alternative media for fungal growth (*Wongjiratthiti, A.* and *Yottakot, S.*); a comparison of ossicle-shape and 12s rRNA gene-sequencing techniques for species identification of *gamat*-based *beche-de-mer* from Langkawi Island, Kedah (*Kamarul Rahim Kamarudin, Maryam Mohamed Rehan, Hanina Mohd Noor, Nur Zazarina Ramly* and *Aisyah Mohamed Rehan*); and finally, the role of heritability as being higher than genetic variability in the estimated selection response of soybean lines on tidal swamp land (*Heru Kuswantoro*).

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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Review Article

Utilisation of Oil Palm Fronds as Ruminant Feed and Its Effect on Fatty Acid Metabolism

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ABSTRACT

Inclusion of oil palm fronds (OPF) pellets (200 g kg^{-1} DM) in a complete animal feed has been found to increase the unsaturated fatty acid content in ruminants. However, given the low-fat content of OPF (21 g kg^{-1} DM), changes in ruminal fatty acid (FA) metabolism will only result in nutritionally relevant differences in animal tissues when OPF enhances conservation of polyunsaturated fatty acid (PUFA) from external sources. Additionally, given the low metabolisable energy value (4.9 to 6.5 MJ (ME) kg^{-1} DM) of OPF, supplementation of OPF with an energy-dense feed compound such as fat is of interest. Thus, this approach could also be used in combination with other dietary fat supplementation strategies to further manipulate fatty acid concentration of ruminant tissues and products for human consumption.

Keywords: Fatty acid, feed, oil palm fronds, ruminant

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INTRODUCTION

Human nutrition is facing sustainability dilemma in ensuring adequate intake of vegetal and animal-based food (Kayouli, 2007). Research is being done to promote the efficient utilisation of non-competitive feed resources from 'marginal areas' not suitable for crop cultivation. In developing

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and emerging countries, feeding systems based on locally available by-products from the agro-industry represent an important feed resource for animals, as forage land is scarce (Nguyen, 1998). In Indonesia, Thailand and Malaysia, large parts of the land are used intensively for oil palm and rice cultivation. For instance, in Malaysia, 4.69 million hectares is currently under oil palm plantation, representing 60% of the total agricultural land (Ng et al., 2011). In Malaysia, 26.9 million tonnes of oil palm fronds (OPF), the major by-product of palm oil production, is utilised as animal feed, which account for 60% of the total OPF production (Ng et al., 2011). Davendra and Thomas (2002) reported that rice straw is fed to more than 90% of the ruminant livestock in Southeast and East Asia (i.e. China and Mongolia), where between 30% and 40% of the total rice straw production was used as ruminant feed.

Indeed, the use of these abundant agriculture by-products in most of the emerging and developing countries could help reduce the cost of feed.. Animal feed is the largest share of expenses incurred by farmers. Therefore, a strategy to develop the livestock industry is by increasing the use of indigenous feed resources (e.g. agriculture by-products) to reduce the cost of importing animal feed. The use of agricultural by-products as animal feed could also help reduce environmental pollution as the normal practice is to burn agriculture by-products (Dahlan et al., 2000).

AGRICULTURE BY-PRODUCTS FROM OIL PALM INDUSTRY

Oil palm (*Elaeis guineensis*) grows well in wet, humid parts of tropical Asia (mainly in Southeast Asia), Africa and Central and South America. It is characterised as a monocotyledonous plant, with long pinnate leaves, without branches and similar to the coconut palm. The OPF, consisting of leaves and petioles, are found at the top of the trees arranged as a crown. Each palm frond has 20 to over 150 pairs of leaves arranged in two rows along each side of the petiole (Figure 1). The oil palm generally has an economic lifespan of 25 years (Ismail et al., 1990).

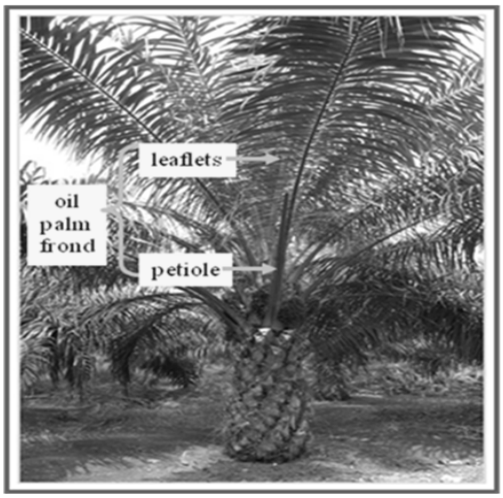

Figure 1. Schematic representation of the oil palm tree

Besides palm oil, massive amounts of oil palm by-products such as oil palm trunks (9%), OPF (30%), empty fruit bunches (22%), palm kernel cake (PKC) (5.5%),

palm press fibre (13.5%) and palm oil mill effluent (POME) (9%) are generated each year world-wide with an estimated production of 1.5 to 25 million tonnes of dry matter (DM) at the mill and 10 to 50 million tonnes DM in the plantations (Figure 2). Most of these oil palm by-products are found in Malaysia and Indonesia. It is estimated that the total oil palm by-products from the palm oil industry in Malaysia in 2009 is 77.24 million tonnes DM per year (Ng et al., 2011). The PKC and POME are regularly incorporated into ruminant feed in Malaysia as cattle and goat fattening finisher.

INCLUSION OF OIL PALM FRONDS AS RUMINANT FEED

The OPF, a major by-product of oil palm plantation, are produced during the life cycle of the palm trees upon pruning and replanting (cycles of 25 years). During its productive life, the OPF are continuously pruned as a part of plantation housekeeping to facilitate harvesting of fruit branches. Pruning and felling of oil palm trees are also carried out upon termination of the plant production cycle for replantation. The fronds are typically considered waste products following the pruning practices. In Malaysia, the total production of OPF is estimated at 44.8 million tonnes DM per year (Ng et al., 2011). The abundance of OPF has resulted in major interest in its potential use as livestock feed in Malaysia. Research has shown that the OPF can be used successfully as a viable ruminant forage feed. In addition, these OPF have been extensively processed as pelleted feed for trade and export. For this, the fresh OPF is first trimmed to a length between 1 and 2 cm, sun-dried for 2 to 3 days and processed

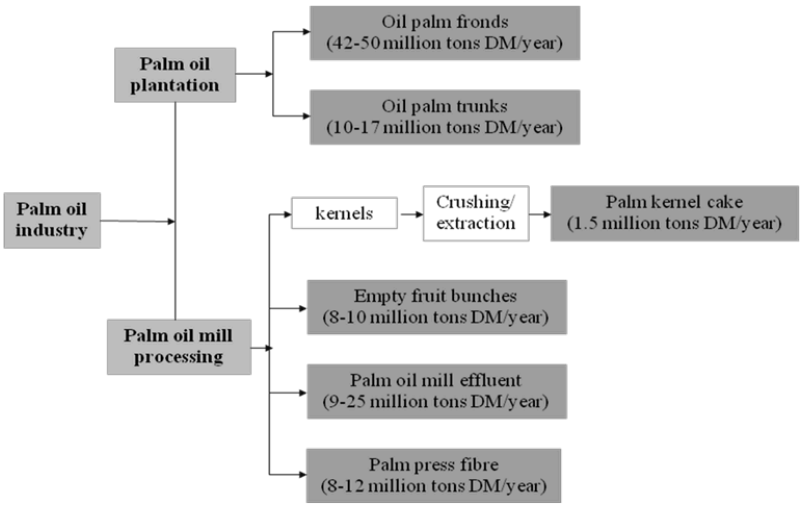

Figure 2. By-products of oil palm production originating during plantation or after processing generated yearly world-wide from palm oil industry (Elbersen et al., 2005)

into pellets or cubes of about 12 mm in size (Figure 3). The process of making OPF pellet and cubes is illustrated in Figure 4.

Figure 3. Picture of oil palm fronds pellet

Toxicity problems have not been reported for OPF (Ishida & Hassan, 1997) and the product seems readily accepted (Dahlan 2000; Rajion et al., 2001) and palatable for cattle, sheep and goat (Asada et al., 1991; Dahlan 2000). Therefore, OPF has been fed to animals as a source of roughage to replace rice straw and other low quality roughages commonly used in ruminant feeding without detrimental effects on livestock production. Besides practical reports illustrating the possibility to include OPF in ruminant diet formulation, some local and technical reports also claimed OPF has increased the unsaturated to saturated fatty acid (SFA) ratio in rumen contents, thereby opening up the possibility of its use to increase the unsaturated fatty acid (UFA) content in ruminant tissues and products.

EFFECT OF FATTY ACIDS IN RUMINANT PRODUCTS ON HUMAN HEALTH

Fatty acids form an essential and integral part of livestock products and influence their

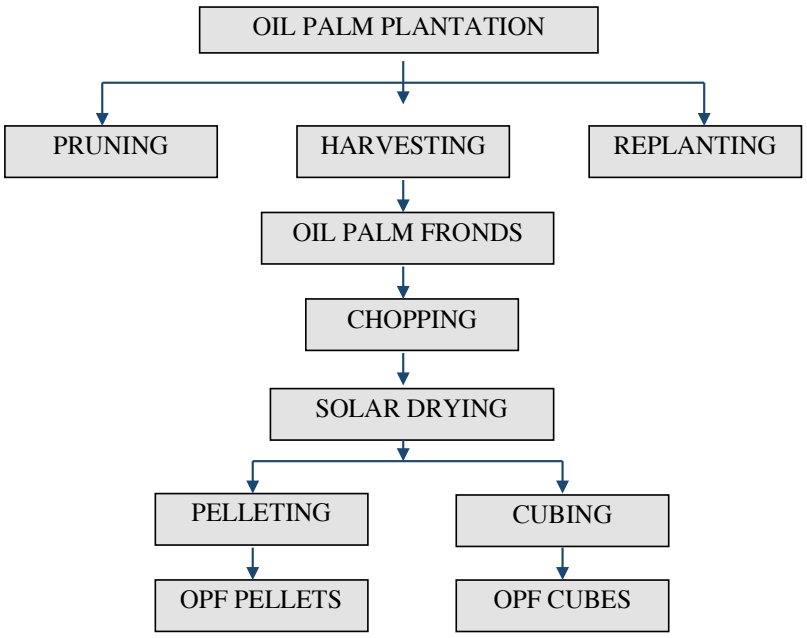

Figure 4. Schematic representation of the process of making OPF pellet and cube (adapted from Mat Daham et al., 2002)

palatability, keeping properties as well as the real and perceived nutritional values (Pariza, 2004). Nutrition and health concerns play a significant role in influencing consumers' food choices. This trend is associated with the perceived relationships between dietary type and amount of fats and fatty acids in animal tissues (Turpeinen et al., 2008). In fact, the human body had not been able to adapt to the recent shifting trends in fat consumption (Newton, 1997). Dietary guidelines published since the early 1980s have proposed reductions in total fat, particularly SFA intake. As recommended by World Health Organization (WHO) total SFA and trans fatty acids should account for between 15% and 30% of one's diet respectively. The SFA should be less than 10% and trans fatty acids less than 1% of dietary energy intake to reduce the prevalence of chronic diseases such as cardiovascular diseases, obesity and diabetes (WHO, 2003). Fatty acids considered beneficial for human health are monounsaturated and polyunsaturated fatty acids (MUFA and PUFA respectively). The most abundant PUFA in ruminant products are linoleic and linolenic acid. Positive health effects attributed to conjugated linoleic acid (CLA) have been shown in many animal studies, e.g. cancer prevention, decreased artheriosclerosis and improved immune response (Whigham et al., 2000; Belury, 2002; Pariza, 2004). In addition, consumption of specific CLA isomers in humans leads to loss of fat and total body weight, reduces plasma concentrations of total and low density lipoprotein (LDL)-cholesterol, and has an anti-inflammatory effect (Gaullier et al., 2007; Close et al., 2007; Turpeinen et al., 2008).

Ruminant products are typically high in SFA, followed by MUFA and PUFA. Red meats, particularly those from ruminant animals, contain more SFA than UFA in terms of ratio compared with meat products from monogastric animals and fishes (Watkins & German, 1998). This is inevitable as the strong reducing conditions in the rumen result in extensive bio-hydrogenation of dietary UFA, leaving only four percent of dietary FA and mostly SFA to be absorbed in the hindgut (Gurr et al., 2002; Jenkins and Thies, 1997). In fact, Dawson and Kemp, (1970) estimated that after normal pasture feeding, the linolenic acid is converted entirely to stearic acid within 10 to 15 hours. Typically, the domesticated ruminant meat has about 45 % total SFA and 55 % UFA, while those from domesticated monogastric animals have only 30% – 35 % fatty acids as SFA (Gurr et al., 2002). These are attributed to the monogastric animals' ability to incorporate dietary fatty acids unchanged (Church & Wood, 1992). Although the rumen seems to be a formidable obstacle for the passage of UFA into the hind gut, it is interesting to note that the muscles of less conventional (buffalo and deer) and wild ruminants (antelope, deer, elk) were found to contain more PUFA than those of range and feedlot animals (Wiklund et al., 2001). In fact, manipulation of the dietary fatty acid composition was shown to modify both plasma and membrane fatty acid profile in human and animal subjects

(Clamp et al., 1997). In the ruminants, modification of the membrane and plasma fatty acid profile occurs via a complex mechanism linked intimately with the rumen functions preceding the absorption and enrichment of fatty acids in both the plasma and membranes (Doreau & Ferlay, 1994). The current study emphasises lipid rumen metabolism in the manipulation of physicochemical events in the rumen for two outcomes. First, the control of the antimicrobial effects of fatty acids which indicate that additional fat can be fed to ruminants without disrupting rumen digestion and fermentation. Second, the regulation of microbial bio-hydrogenation to alter absorption of selected fatty acids that enhance and improve nutritional values of animal food products. The latter had been shown to be possible via both chemical and possible alteration of rumen bio-hydrogenation activity via protozoal activity (Rajion et al., 2001).

It has been demonstrated that it is possible to increase both the milk and tissue fatty acid unsaturation in ruminants by 10-fold if UFA are protected from bio-hydrogenation (Fotouhi & Jenkins, 1992). This is possible since ruminants have higher efficiency to absorb unsaturated fatty acids compared with non-ruminants (Bauchart, 1993). Generally, the intestinal absorption coefficients for individual fatty acids range from 80 % (for SFA) to 92 % for PUFA in conventional diets with low fat content (two to three percent DM) (Bauchart, 1993). These demonstrate that dietary regime, animal husbandry management, dietary habit and preferences have an important role in determining the dynamics of the fatty acid profile within an organism, despite the intrinsic fatty acid metabolism mechanisms in its body (Rajion et al., 2001). Further, these fatty acids form an essential and integral part of fatty acid composition of ruminant products (Pariza, 2004).

Nowadays, consumption of these ruminant products is increasing all over the world and is expected to grow further until 2030, which can lead to excessive fat intake (WHO, 2003). This has led animal scientists to develop strategies to optimise the fatty acid content of ruminant products, particularly by decreasing their SFA to improve the nutritional and health value of ruminant products.

OIL PALM FRONDS AND FATTY ACID CHANGES

Oil palm fronds are characterised by low fat (21 g kg^{-1} DM) and metabolisable energy (4.9 to 6.5 MJ (ME) kg^{-1} DM) content for ruminants, which limit their inclusion in diets of production animals (Dahlan, 2000; Zahari and Alimon, 2005). Therefore, it is important to develop to formulate diets containing OPF which allow optimum growth and productivity for ruminants (Dahlan, 2000). There has been limited scientific research concerning the inclusion of OPF in ruminant diets and only some local and technical reports are available (Dahlan, 2000), promoting relatively high OPF inclusion levels of up to 50% and 30% in beef cattle and dairy cow diets respectively (Ishida & Hassan, 1997). Other

reports claimed appropriate formulation of OPF-based diets could allow live weight gain of beef cattle between 0.6-0.8 kg d^{-1} and, for local crossbred dairy cows, milk yields of about 22 litre d-1 (Zahari et al., 2003). Further, reports suggesting that their incorporation (200 g kg^{-1} DM) could enhance the UFA proportion in rumen contents and sheep plasma (Rajion et al., 2001), increased the interest in this product in the context of functional food development, e.g. meat products with greater amounts of UFA. However, the exact mechanisms of how all these phenomena occur remain to be analysed. It is probable that incorporation of oil palm fronds had altered the rumen environment, increasing the availability of UFA either via regulation of rumen bio-hydrogenation or facilitating the continuous availability of dietary UFA by restricting microbial access to these UFA's. Alteration of rumen environment might be through substances in the OPF which potentially modify rumen FA metabolism and bio-hydrogenation as suggested for some plant secondary metabolites (e.g. Benchaar et al., 2007; Lourenco et al., 2008), or due to differences in dietary macronutrient or micronutrient supply through OPF inclusion. Indeed, other research reported that rumen FA metabolism and apparent bio-hydrogenation of C18:2 n-6 and C18:3n-3 was affected in the presence of plant secondary metabolites, e.g. tannins (Vasta et al., 2009), which have been reported in OPF (Sasidharan et al., 2010).

Furthermore, given the low-fat content of OPF, changes in ruminal FA metabolism will only result in nutritionally relevant differences in animal tissues when OPF enhances conservation of PUFA from external sources. Additionally, given the low metabolisable energy value of OPF, from an animal nutrition perspective, supplementation of OPF with an energy dense feed compound such as fat is of interest. Therefore, the potential of OPF to serve as PUFA supplier is low and the origin of the latter observation requires further investigation. Indeed, Hassim et al. (2010) has shown that the inclusion of OPF in a ruminant diet did not affect fatty acid metabolism and the scope to improve fatty acid composition of ruminant products through OPF supplementation seems limited. This indicates challenges to include this agricultural by-product in ruminant diets. Hence, the utilisation and upgrading of this agricultural by-product are important and has attracted much research and development interest. This is understandable due to a rapid and dynamic increase in consumption of livestock products especially in developing and emerging countries.

CONCLUSION

It is clear that oil palm frond-based diet hold promise for ruminant nutrition, especially in countries with vibrant oil palm industries, such as Malaysia and Indonesia. With the current focus on reducing the SFA content of food stuffs for human consumption, it is believed that dietary approaches utilising oil palm fronds could be further developed to

improve the availability of UFA in ruminant diets, thereby creating base technology for ruminant products (e.g. milk and meat) that have "healthier" fatty acid composition.

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Review Article

Formation and Utilisation of Acid Sulfate Soils in Southeast Asia for Sustainable Rice Cultivation

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ABSTRACT

Large parts of lowland areas in Southeast Asia were submerged in seawater some 4300 years ago due to a rise in sea level. During this period, the coastal plains in the region were pyritised. Agricultural development led to oxidisation of the pyrite (FeS_2) which in turn allowed weathered mineral silicates to be present in the sediments. High levels of Al and/or Fe are thus present in the soils/water that affect plants and aquatic life. Rice grown on the so-called acid sulfate soils suffer from low pH and Al^{3+} and/or Fe^{2+} toxicity, with yields below the national average. The critical pH and Al concentration for rice growth is 6 and 15-30 uM respectively. The soil become infertile due to high concentrations of acid sulfate . Application of ground magnesium limestone (GML) or basalt in combination with bio-fertiliser fortified with phosphate-solubilising bacteria (PSB) can help reduce the acid sulfate. The PSB not only excrete organic acids that inactivate Al and Fe via chelation, but also increase soil pH to the level that precipitates Al as inert Al-hydroxides. Additionally, rice roots are able to excrete organic acids under the presence of high concentration of Al and/or Fe, which further reduces the availability of Al and Fe in the water.

Keywords: Acid sulfate soil, aluminium toxicity, iron toxicity, rice, sustainable production

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INTRODUCTION

Definition and occurrence

Acid sulfate soils contain pyrite (FeS_2), occurring under submerged conditions. Immediately above the pyritic layer is

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usually the sulfuric horizon. According to Soil Survey Staff (2014), sulfuric horizon is defined by the presence of:

1. Mineral jarosite[$(KFe_3(SO_4)_6(OH)_2$]; and
2. More than 0.05% water-soluble sulfate.

The soils, taxonomically classified as Sulfaquents and Sulfaquepts, are sporadically distributed throughout the lowland coastal plains of Southeast Asia. The global occurrence of acid sulfate soils is not related in any way to climatic conditions or the mineralogical properties of the coastal sediments from which they are formed. In Southeast Asia, the soils are found in the Mekong Delta, Vietnam (Husson et al., 2000), Bangkok Plains, Thailand (van Breemen, 1976), Kalimantan, Indonesia (Anda et al., 2009) and Kedah-Perlis Plains, Malaysia (Azmi, 1982; Shamshuddin, 2006). Department of Agriculture estimated about 0.4 million ha of acid sulfate soils in Peninsular Malaysia alone (Figure 1).

Being related to the sea, pyrite-bearing sediments usually occur along the coastal plains throughout Southeast Asia. The areas where these sediments occur are in mangrove swamps, having big and small organisms, living harmoniously side by side. The areas become natural habitat for fish and crabs that co-exist with the forest species. After the areas were opened up to make way for agricultural development, soil acidity and Al and/or Fe toxicity became serious threats to crops and aquatic life

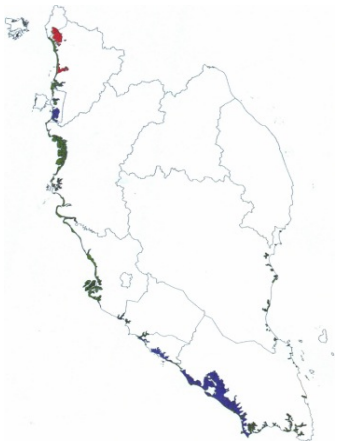


Figure 1. A map of Peninsular Malaysia showing the distribution of acid sulfate soils (Green = Kranji Series; Purple = Sedu-Parit-Botak Associations; Red = Telok- Guar Associations)
Source. Modified from Department of Agriculture, Peninsular Malaysia

(Shamshuddin, 2006). Thus, special soil management practices are necessary to sustain crop production (Shamshuddin, 2006; Shamshuddin et al., 2014).

Acid sulfate soils in Southeast Asia have been extensively studied (van Breemen, 1976; Shamshuddin & Auxtero, 1991; Husson et al., 2000; Shamshuddin, 2006; Anda et al., 2009; Shamshuddin et al., 2014). Major characteristics of the soils are as follows:

1. Pyrite occurs in the sediments under submerged conditions;
2. Jarosite is formed on oxidation of this pyrite;
3. Simultaneous release of soil acidity into the environment, resulting in soil pH < 3.5;

4. Silicates in the soils dissolve to release Al and/or Fe into the soils;
5. The amount of Ca and Mg in the soils are insufficient for crop growth; and
6. P-deficiency is common in acid sulfate soils due to its fixation by Al and/or Fe.

Utilisation for rice cultivation

There is an increased need for land in Southeast Asia to cultivate crops in order to meet the ever-increasing demand for food and fibre. As such, the infertile acid sulfate soils can be put to good use, especially for food production. Studies were carried out to protect the soil for rice (Azura et al., 2014), oil palm (Auxtero and Shamshuddin, 1991) and cocoa cultivation (Chew et al., 1984; Shamshuddin et al., 2004b). Malaysia needs to increase its rice self-sufficiency level (SSL) from the current 70% to > 80% by 2020. However, the country faces difficulties to achieve the set target due to various constraints: 1) Land allocated for rice cultivation is decreasing because paddy fields are being converted to other land uses; and 2) Soil and crop productivity has remained stagnant due to lack of investment on innovative agronomic research.

Agricultural activity is a nutrient mining process; hence, the soil needs to be replenished regularly with plant nutrients. In Peninsular Malaysia, some of the paddy soils adjacent to acid sulfate soils are chemically degraded (Shamshuddin, 2006). The areas in question are in Kedah-Perlis Plains (Azmi, 1982) and Kemasin-Semerak Integrated Agricultural Development Project, Kelantan (Enio et al., 2011). However, they have the potential of becoming good agricultural land if proper agronomic procedures are put in place.

Rice grown on acid sulfate soils produces yield of about 2 t ha^{-1} $season^{-1}$ (Elisa et al., 2012; Elisa et al., 2014), which is far below the national average of 3.8 t ha^{-1} $season^{-1}$. If we can increase the yield to a level above 5 t ha^{-1} $season^{-1}$, farmers growing rice in the affected areas can make a decent living. This is not to mention the increased rice stock in the country, leading to increase in rice SSL.

The root causes of low rice yield are: 1) Acid sulfate soils contain too much acidity; 2) rice planted on the soils are subjected to Al and/or Fe stress; and 3) the availability of P is low due to fixation by Al and/or Fe prevalent in the soils. Infertility is due oxidation of pyrite in the sediments. When the hydromorphic areas are drained, the pyrite is exposed to the atmospheric conditions and subsequently oxidised, resulting in the release of high amount of acidity (Shamshuddin et al., 2004a; Shamshuddin, 2006). A new mineral known as jarosite [$KFe_3(SO_4)_2(OH)_6$] is formed in the end. Under acidic conditions, mineral silicates present in the sediments undergo weathering that result in the release of high amount of Al and/or Fe into the environment which can be toxic. The presence of these acidic metals in the paddy fields can also reduce the availability of P for the uptake by rice.

The pH of water in many of the paddy fields located on acid sulfate soils is 3-4, which is far below the critical pH of 6 needed for the healthy growth of common rice varieties in Malaysia (Azura et al., 2011; Shamshudin et al., 2013; Shamshuddin et al., 2014; Alia et al., 2015). According to Rosilawati et al. (2014) and Azura et al. (2014), soil pH can be increased to the required level for rice cultivation by applying GML or hydrated lime. An alternative method is to apply ground basalt (Panhwar et al., 2014a). Basalt is better than GML in terms of its ameliorative effects as it not only increases soil pH and supplies Ca and Mg, but also supplies K and P (Shazana et al., 2013). Besides, basalt releases Si which improves crop growth, leading to prevention of disease that affect yield, such as rice blast.

Panhwar et al. (2014b) identified three phosphate-solubilising bacteria (PSB) that existed in acid sulfate soils in Malaysia. These PSB have the potential to be effective microorganisms for the formulation of a bio-fertiliser for rice cultivation on acid sulfate soils. These special microbes are not only able to make P more available to rice plants in the fields than otherwise are, they can also excrete organic acids that can reduce Al and/or Fe in the water of the paddy fields via the process of chelation. As such, the rice plants will grow under less stress of Al and/or Fe. Besides, the PSB release a certain chemical that increases water pH to a level above 5 (Panhwar et al., 2014b). The increase in pH would precipitate Al as inert Al-hydroxides, thus further reducing the availability of Al (Shamshuddin et al., 2013; Shamshuddin et al., 2014). Acid sulfate soils will continue to be utilised for rice cultivation in the future given the scarcity of fertile agricultural land in Southeast Asia. The objectives of this paper are: 1) to discuss in detail the formation of acid sulfate soils in Southeast Asia; and 2) to explain how these soils are managed using innovative agronomic practices for sustainable rice cultivation.

PYRITISATION OF THE COASTAL SEDIMENTS IN SOUTHEAST ASIA

Sea level rise in Southeast Asia during the Holocene

Climate change is a global phenomenon, naturally occurring intermittently throughout geological time, with or without human intervention. During the Last Glacial Maximum, some 20,000 years ago, a vast land area was formed in Southeast Asia (Tjia, 2012) that determined the present landscape and geological style of the region (Figure 2). Global ice melts slowly

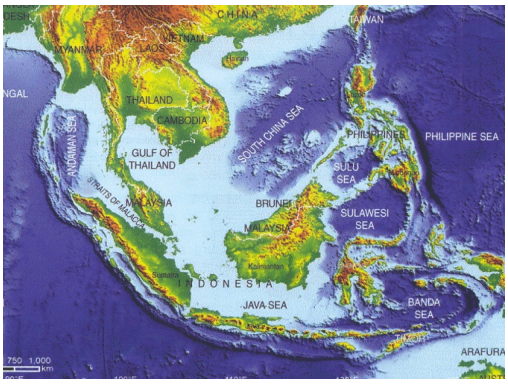


Figure 2. A map of Southeast Asia showing the countries in the region
Source. Geological Society of Malaysia

thereafter, rising the sea level 15 mm year^{-1}. In Southeast Asia, it reached the maximum elevation of 5 m above the present about 4,300 years before present (Tjia et al., 1977; Tjia, 2012).

It was during this period that the low-lying areas in the coastal plains of Southeast Asia were inundated with seawater for a long period of time. Thus, the land area was smaller than it is today. The sediments were pyritised. The phenomenon was proven by a study conducted in the Kelantan Plains, Malaysia (Enio et al., 2011). Using the distribution of pyrite in the sediments, researchers were able to predict the coastline in the plains at the height of the sea level rise during the Holocene (Fig. 3). Similar phenomenon would have occurred in the Bangkok Plains (Thailand), Mekong Delta (Vietnam) and Kalimantan (Indonesia).

The mineralisation of pyrite

Figure 4 shows the mineralisation of pyrite in the coastal sediments in Southeast Asia. The process requires the presence of SO_4^{2-}, supplied by seawater. This sulfate ion goes into the sediments during high tide. Iron required for the pyritisation process is naturally present in the sediments. Under anaerobic condition, both ions undergo reduction process, expedited by the microorganisms living naturally in the sediments:

1. $SO_4^{2-} \rightarrow S^{2-}$
2. $Fe^{3+} \rightarrow Fe^{2+}$

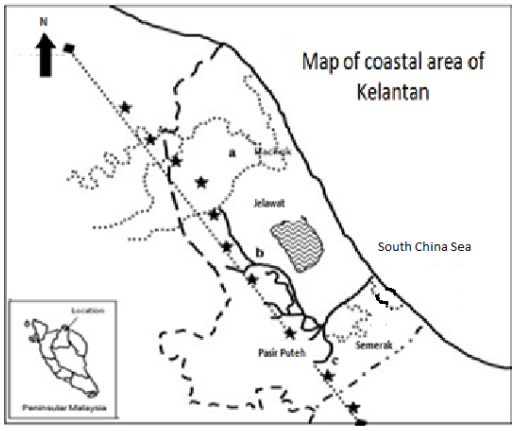


Figure 3. A map showing the predicted coastline in the Kelantan Plains, Malaysia when the sea level was 5 m above the present
Source. Adapted from Enio et al. (2011)

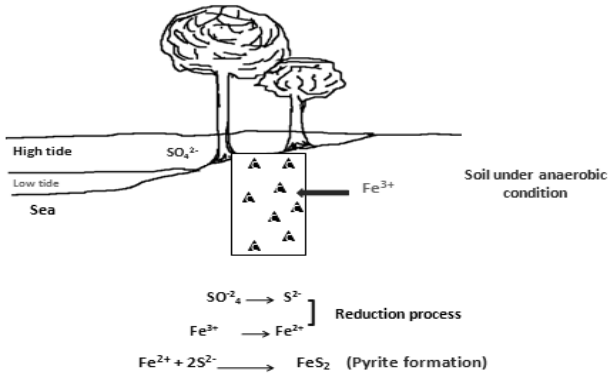


Figure 4. Mineralisation of pyrite in the coastal sediments
Source. Adapted from Shamshuddin (2014)

Eventually, the Fe^{2+} and S^{2-} will react to form FeS_2, the so-called pyrite. The overall reaction as described by Pons et al. (1982) is as follows:

$$Fe_2O_3 + 4SO_4^{2}- + 8CH_2O + ½O_2 \rightarrow 2FeS_2 + 8HCO_3- + 4H_2O$$

This process continues uninterrupted until sulfate is no longer available for the reaction, that is after the seawater recedes. The pyrite so formed remains stable in the sediments provided that it is under submerged condition. Pyrite shown in Figure 3 can be found in the sediments some distance away from the present coastline (Shamshuddin, 2006; Enio et al., 2011). Shamshuddin (2006) reported that some coastal sediments in Peninsular Malaysia contained up to 2-3% pyrite, resulting from thousands of years of pyritisation process.

Pyrite exists in the form of cubic crystal (Figure 5). To get the crystals seen in Figure 5, the soil sample containing pyrite has to be freeze-dried immediately before it is treated for observation under scanning electron microscope. Otherwise, it will undergo immediate oxidation to form yellowish jarosite.

Soil profile containing pyrite occurring under reducing condition looks bluish, indicative of its origin and/or relation with the sea. Figure 6 is a picture of a freshly dug profile of Kranji Series (Sulfaquent) located close to the sea in Peninsular Malaysia (Shamshuddin, 2014). Noordin (1980) studied acid sulfate soils found in Perak and his findings showed the presence of diatoms in the coastal sediments containing pyrite; diatoms are living creatures that thrive in the seabed. This finding has proven beyond doubt that for pyritisation process to occur, seawater is required to supply the required SO_4^{2-} ions.

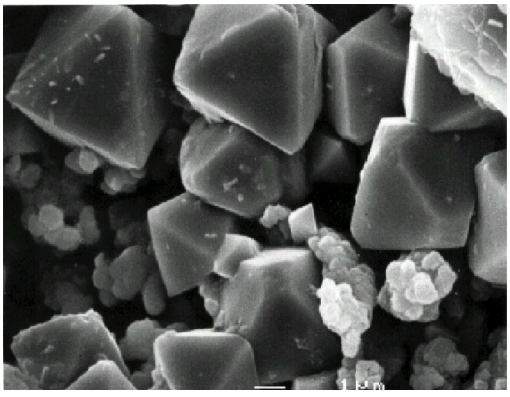


Figure 5. SEM micrograph of pyrite found in the coastal sediments of Malaysia
Source. Shamshuddin et al. (2004a)

Figure 6. A profile of an acid sulfate soil found in Peninsular Malaysia
Source: Shamshuddin (2014)

OXIDATION OF PYRITE UNDER AEROBIC CONDITION

Under flooded environment, pyrite is known to be stable, causing harm to living

creatures in the sediments, either plants or animals. Its crystal structure remains intact until aerobic condition sets in due to drop in water table. When this happens, the pyrite undergoes oxidation and/or disintegration that releases acidity to the soil environment (Shamshuddin, 2006).

Soil containing pyrite in Southeast Asia are usually drained and opened up for the cultivation of rice. In Malaysia, to prevent seawater from entering the land, a specially designed bund is constructed (Figure 7). The excess water is then removed by digging drains at appropriate size and depth. The water table in the soils of the areas drops and subsequently the pyrite is exposed to the atmospheric conditions, resulting in the disintegration/dissolution. This process releases a lot of acidity to the environment, with pH decreasing to the level below 3.5 (Shamshuddin and Auxtero, 1991; Shamshuddin et al., 2004a).

The reaction can be formulated as follows (van Breemen, 1976):

$$2FeS_2(s) + 7O_2(aq) + 2H_2O \rightarrow 2Fe^{2+}(aq) + 4SO_4^{2-} + 4H^+(aq) \quad (1)$$

Further oxidation of Fe^{2+} to Fe^{3+} leads to the generation of more acidity:

$$2Fe^{2+}(aq) + \tfrac{1}{2} O_2(aq, g) + H_2O \rightarrow Fe_2O_3(s) + 4H^+(aq) \quad (2)$$

Reaction 1 shows a lot of acidity is being produced with concomitant formation of the yellowish jarosite (Figure 7). Jarosite is a stable mineral and thus, remains a long time in the soil. However, under extreme weathering condition, the jarosite can further be oxidised to reddish hematite as shown by Reaction 2.

The first sign of pyrite disintegration in the soils is etching of the crystals (Figure 8). Finally, the pyrite crystals are completely disintegrated, with the formation of jarosite soon thereafter (Shamshuddin & Auxtero, 1991; Shamshuddin et al., 2004a).

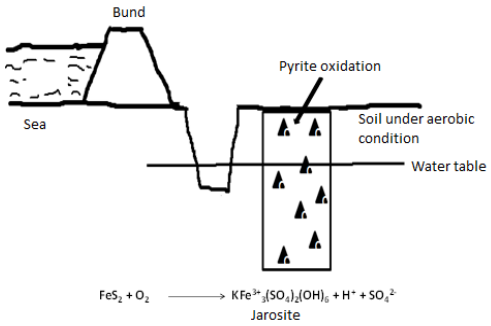


Figure 7. Oxidation of pyrite on exposure to the atmospheric conditions
Source. Adapted from Shamshuddin (2014)

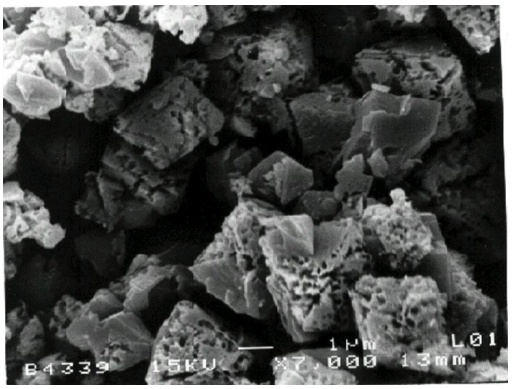


Figure 8. SEM micrograph showing pyrite undergoing oxidation
Source. Shamshuddin et al. (2004a)

Yellowish jarosite mottles can be clearly seen in the sulfuric horizon of acid sulfate soils (Figure 9). In between the yellowish mineral, there are spots of reddish materials so named hematite. Jarosite is partly converted to hematite if extreme weathering condition occurs persistently in the area for a long period of time.

GENERATION OF SOIL ACIDITY

Pyrite, on exposure to the atmospheric conditions, undergoes immediate oxidation, generating a lot of acidity with pH at < 3.5 (van Breemen, 1976; Shamshuddin and Auxtero, 1991; Shamshuddin et al., 2014). The pH of water in the vicinity of acid sulfate soils area is likely to be < 4 (Shamshuddin, 2006; Azura et al., 2011; 2012). The low pH contributes to the enhanced weathering of mineral silicates present in the sediments. In the end, large amounts of Al and/or Fe are released into the soil environment, affecting plant growth and aquatic life in the vicinity of acid sulfate soils.

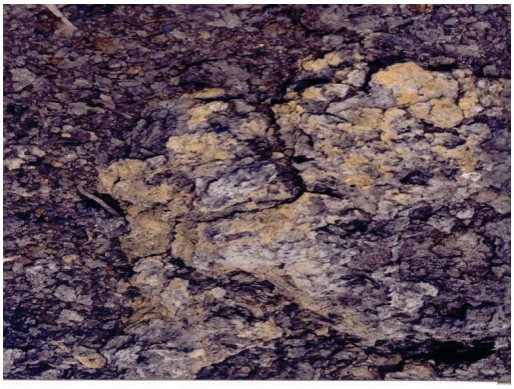


Figure 9. A picture showing yellowish jarosite occurring in the topsoil of the Kelantan Plains
Source. Shamshuddin (2006)

The pKa of Al is 5. When water pH goes down below this level, Al in the water undergoes immediate hydrolysis, releasing more acidity to the environment. This further chemically aggravates the acid sulfate soils. The hydrolysis reaction is described by the following equation:

$$Al^{3+}.6H_2O + H_2O \rightarrow Al^{2+}OH.5H_2O + H_3O^+$$

When water pH goes up above 5, Al begins to precipitate as inert Al-hydroxides [$Al(OH)_3$], which is no longer toxic to rice plants.

Fe, with pKa of 3, undergoes immediate hydrolysis in the same manner as that of Al, but more vigorously, with protons being released as shown below:

$$Fe^{3+}.6H_2O + H_2O \rightarrow Fe^{2+}OH.5H_2O + H_3O^+$$

As such, both elements are regarded as acidic metals, with Fe being more acidic than that of Al. If both metals are present in high amounts in the water of the paddy fields on acid sulfate soils, the pH will be in equilibrium at 3-4 (Azura et al., 2011, Shamshuddin et al., 2013; Shamshuddin et al., 2014). The concentration of Al in the water can reach up to 800 µM (Shamshuddin et al., 2014). Both low pH and high Al concentration will certainly affect the growth of rice plants eventually affecting their yield. Like Al, Fe starts to precipitate as Fe-hydroxides [$Fe(OH)_3$] when pH is above its pKa value. So, to reduce the effects

of Fe toxicity, we only need to increase soil pH to about 4.

EFFECTS OF pH, Al AND Fe TOXICITY ON RICE

Effects of pH

In normal soils with water pH about 6, rice roots grow without limitation. Figure 10(a) shows the morphology of a rice root growing at pH about 6, without H^+ stress. There are many root hairs; hence, the roots are able to absorb sufficient amounts of nutrients required by rice for its growth. On the other hand, at pH below 3.5, rice root grows abnormally. When water pH is about 3, its growth is severely stunted due to absence of root hairs (Figure 10(b)). This could be the scenario when rice is grown on acid sulfate soils without undergoing amelioration.

Under field condition, the pH of water is < 4 (Elisa et al., 2011; Shamshuddin et al., 2014). Worse still, the pH of water in some paddy fields on the chemically degraded acid sulfate soils in the country is 3 or less. Without alleviating the soils using the state-of-the-art agronomic practices, the areas are not suitable for rice cultivation like parts of Kemasin-Semerak Integrated Agricultural Development Project (IADP), Kelantan (Figure 3) where some farmers have abandoned the tradition of growing rice to support their livelihood.

Effects of Al

Rice growing without the presence of Al in the water of their paddy fields is healthy. This is because the cells in its roots are able to function normally. Figure 10(c) shows the structure of the cells in the root of rice

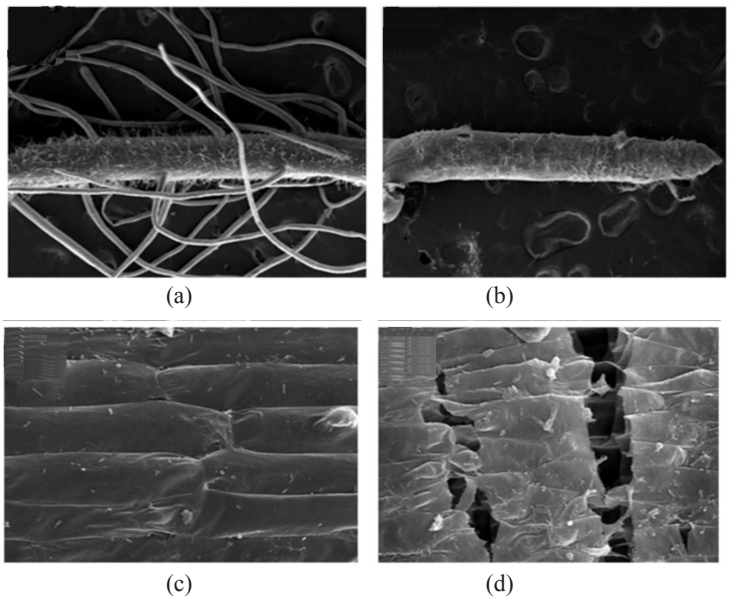


Figure 10. Rice root growing under different pH: (a) & (b) at high pH [6.0]; (c) & (d) at low pH [3.0]
Source. Alia et al. (2015)

plant in the absence of Al. It is seen that the cellular structures of the roots are perfectly normal and healthy. In the presence of high concentrations of Al, rice plants grow under stress, affecting the growth of their root cells; the root cells are damaged severely by Al toxicity as shown in Figure 10(d). The damaged root cells will not be able to absorb sufficient amounts of nutrients; hence, the growth of rice is severely affected.

Under field condition, the concentration of Al is more than 800 µM (Shamshuddin et al., 2013; Shamshuddin et al., 2014). This level is far above the critical Al concentration for the rice growth of 15-30 µM (Azura et al., 2011; Alia et al., 2015). It is clear that the condition is not suitable for rice cultivation unless the soils are treated with appropriate amendments to raise the pH to a sufficient level to precipitate the Al as inert Al-hydroxides. Most of the Al is removed from the water when pH is about 5.2. However, to raise pH of acid sulfate soils to this level is costly (Rosilawati et al., 2013; Azura et al., 2011). We found that it is good enough if the water pH is about 4.5 because rice has a built-in mechanism to protect itself against Al toxicity (Shamshuddin et al., 2013; Shamshuddin et al., 2014; Alia et al., 2015).

Effects of Fe

Rice is also affected by Fe stress. Like Al, the presence of excess amount of Fe in the water reduces rice growth significantly. Iron in the form of Fe^{2+} is toxic to rice plants (Shamshuddin et al., 2013; Shamshuddin et al., 2014; Alia et al., 2015). It was found that excessive amount of Fe was present in the acid sulfate soils of the Kelantan Plains, indicated by the red colour of the water in the paddy fields (Shamshuddin, 2006). When the paddy fields in the area are flooded during the growing season, the water in the paddy fields will look reddish in colour, indicative of the presence of high amount of $Fe(OH)_3$ (Figure 11).

Within two weeks, high amount of Fe^{2+} ions would be produced by reduction of Fe^{3+}. According to Muhrizal et al. (2003), this reduction is expedited by the presence of high quality organic matter. The reaction taking place in the water is described using the following equation (Konsten et al., 1994; Muhrizal et al., 2006):

$$Fe(OH)_3 + ¼CH_2O + 2H^+ \rightarrow Fe^{2+} + ¼CO_2 + 11/4H_2O$$

During the reduction process, protons are consumed, resulting in a slight pH increase. Unfortunately, this mechanism can only increase water pH to about 4, which is

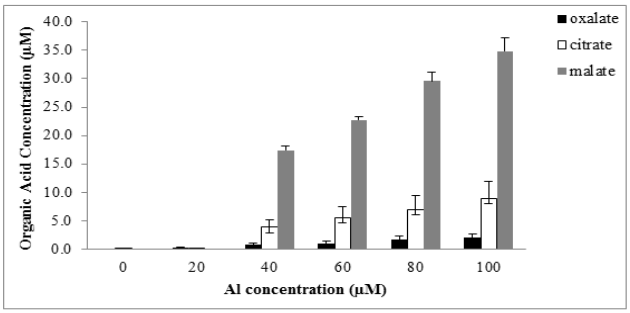
Figure 11. Flooded rice fields in the Kelantan Plains containing Fe-hydroxides
Source. Shamshuddin (2006)

inadequate to precipitate all Al present in the water. We can, however, reduce the effects of toxicity resulting from Fe^{2+} by expediting the reduction process so that the period for the roots exposed to the adverse condition is significantly shortened (Shamshuddin, 2006). This can be achieved by applying organic matter at the appropriate rate (Muhrizal et al., 2003).

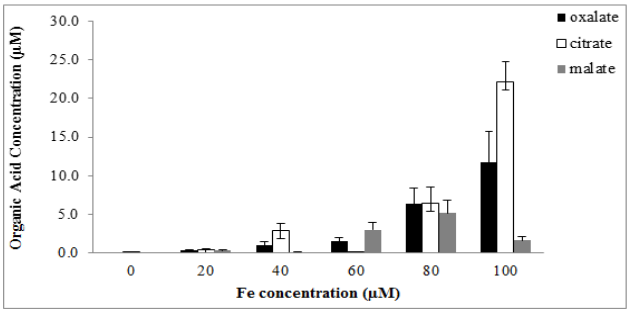
THE MECHANISMS OF RICE TOLERANCE TO Al AND/OR Fe TOXICITY

If the concentration of Al in the water is not too high, rice can withstand stress induced by the former, which is achieved via a special mechanism. An experiment was carried out in the laboratory at Universiti Putra Malaysia, Serdang to determine the mechanism of Al tolerance using rice seedling variety MR 219, the most popular variety grown in Peninsular Malaysia. In this experiment, rice seedlings were subjected to the stress of various Al concentrations. Organic acids secreted by the roots of the seedlings were determined. The results of the experiment are shown in Figure 12.

As the concentration of Al increased, the amount of organic acids secreted also increased. The critical level of the Al concentration is 15-30 µM (Shamshuddin, 2014), which is consistent with the critical Al concentration determined by Alia et al. (2015). Below this level of Al concentration, the amount of organic acids secreted is negligible. It means that if the concentration



(a)



(b)

Figure 12. Excretion of organic acids by rice roots (a) in the presence of Al; (b) in the presence of Fe *Source.* Adapted from Alia et al. (2015

of Al is less than 30 µM, the rice seedlings are not affected significantly by Al stress. In reality, organic acids in this experiment were secreted in response to Al stress. Among the organic acids released by the roots, malic acid was secreted the most while citric acid was the least (Figure 12(a)). Hence, it is believed that malic acid plays an important role in rice tolerance to Al toxicity. Like Al, rice roots respond to Fe stress by secreting organic acids (Figure 12(b)). However, the most important organic acid secreted by the rice roots due to Fe stress was citric acid, followed by citric acid. The least amount of organic acid released was malic acid.

How organic acids help rice defend itself against Al toxicity. The same mechanism can be used to explain its tolerance to Fe toxicity. The surface of rice root is negatively-charged; hence, the positively-charged Al is naturally attracted to it (Shamshuddin et al., 2013; Shamshuddin, 2014; Shamshuddin et al., 2014). As soon as the Al touches the root surface, the root responds by secreting organic acids. The organic acids then start to chelate the Al, making it inactive (Figure 13). The chelated Al and/or Fe, located at the root-water interface, are not toxic to rice plants. In this way, rice plants are able to grow normally, giving good yields.

Structure of a plausible organic acid involved in the chelation of Al and/or Fe is shown in Figure 14. The Al and/or Fe chelated by the organic acid remain stable until they are degraded by the actions of microbes living in the soils.

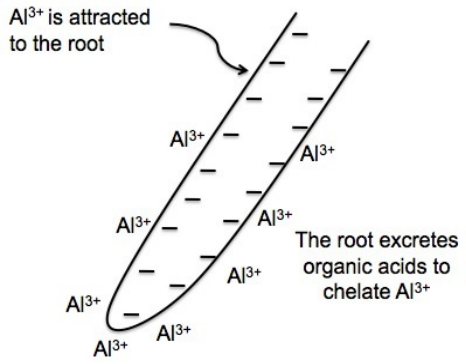


Figure 13. The process of Al chelation by organic acids at the Al-root interface
Source. Adapted from Shamshuddin et al. (2014)

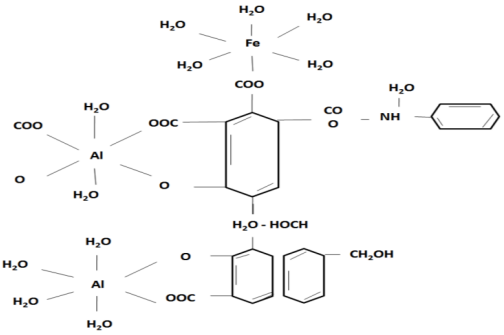


Figure 14. The pictorial representation of Al and/or Fe chelated by an organic acid
Source. Adapted from Shamshuddin (2014)

ALLEVIATION OF SOIL INFERTILITY FOR RICE CULTIVATION

Effects of amendment application on soil pH

A study was conducted in glasshouse in Malaysia to determine the effect of applying various amendments on soil pH (Figure 15). Soil pH of the control treatment did not change significantly, remaining

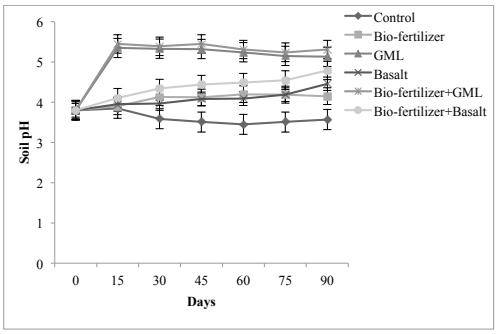


Figure 15. Effect of applying various amendments on soil pH

below 4. However, the pH of the ground basalt treatment increased slightly after 90 days of application. In contrast, GML treatment increased soil pH to a value above 5. The slight pH increase due to basalt treatment can be explained by its low rate of disintegration/dissolution (Shazana et al., 2013). The best treatment in this study was GML in combination with bio-fertiliser.

Effects of applying GML on the growth of rice

Liming acid sulfate soils using GML for rice production is a standard practice in Malaysia (Shamshuddin, 2006). GML reacts with the soil within a short time, increasing soil pH to the level depending on the amount applied. Lime requirement of acid sulfate soils to raise soil pH to a value above 5 is 6 t GML ha^{-1} (Rozilawati et al., 2013), but this rate of application is too costly for farmers in Southeast Asia. The recommended liming rate for acid sulfate soils in Malaysia is 4 t ha^{-1} although soil pH remains below 5 (Shamshuddin, 2006). When GML is applied, the following reactions take place:

$$CaMg(CO_3)_2 + H_2O \rightarrow Ca^{2+} + Mg^{2+} + 2CO_3^{2-}$$

$$CO_3^{2-} + H_2O \rightarrow HCO_3^- + OH^-$$

The hydroxyl ions would then react with the Al in the solution to be precipitated as Al-hydroxides, which over time may be crystallised to form gibbsite [$Al(OH)_3$]:

$$Al^{3+} + 3OH^- \rightarrow Al(OH)_3$$

The GML application not only increases soil pH that remove Al from the solution, but also supplies significant amount of Ca and Mg, which are needed in high amount in the rice fields (Shamshuddin, 2006; Panhwar et al., 2014a). Without GML application, rice plants are affected by low pH and/or Al toxicity and hence their growth are severely stunted (Figure 16(a)). Here it is seen that the rice leaves are brownish in colour, indicative of Al toxicity. The poor rice growth could also be due to insufficient amount of Ca and/or Mg in the soil.

Fortunately, if 4 t GML ha^{-1} is applied onto the soil, rice grows normally (Figure 16(b)). Due to GML treatment, soil pH is increased to the level good enough for the growth of rice. Although soil pH was below 5 with substantial amount of Al present in the water, the plants were able to grow well because they the ability to secrete organic acids that reduce Al toxicity (Shamshuddin et al., 2013; Shamshuddin et al., 2014; Alia et al., 2015).

(a) (b)

Figure 16. Rice grown on acid sulfate soil (a) Control treatment; (b) Soil amended by GML
Source. Azura et al. (2014)

Effects of applying basalt on the growth of rice

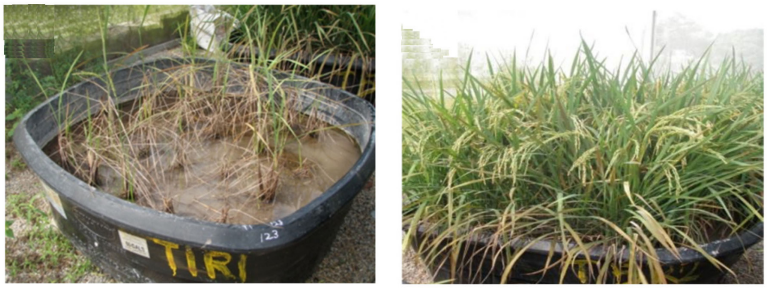
Basalt application is a good long-term solution to treat infertility of acid sulfate soils in Southeast Asia. The reason being it takes time for basalt to disintegrate and dissolve completely in the soil although under low pH condition (Shazana et al., 2013). But once dissolved, it not only increases soil pH significantly and subsequently supplies Ca and Mg, but also improves soil fertility by releasing K and P which are also needed in high amounts by rice plants (Panhwar et al., 2014a). It means that basalt application slightly reduces the cost of rice production because it can cut down the application of K- and P-fertiliser. The only problem is that its ameliorative effects take a long time to be realised. However, basalt dissolution releases Si, an essential element for rice growth. With its uptake, rice plant appears to be healthier and consequently able to prevent the outbreak of rice blast.

The most important mineral in basalt is Mg-olivine. The mineral dissolves in the soils according to the following reaction:

$$4Mg^{2+} + SiO_4^{4-} + 4\ H_2O \rightarrow 4Mg^{2+} + Si(OH)_4 + 4OH^-$$

The reaction shows that Mg-olivine releases huge amounts of hydroxyl ions; in fact, the amount released is more than that of GML reaction. It is seen that silicic acid [$Si(OH)_4$] is simultaneously released by the reaction, the form of Si that can be taken up by rice (Shamshuddin et al., 2014). Rice growth in a pot without the addition of ground basalt is shown in Figure 17(a). It did not grow at all due to the effects of low pH and/or Al toxicity. At harvest, rice plants almost died probably due to the extreme effects of H^+ and/or Al^{3+} stress (Shamshuddin et al., 2014).

Due application of 4 t basalt ha^{-1}, showed that rice in the same experiment grew normally, producing yield more than



(a) (b)

Figure 17. Rice grown on acid sulfate soil (a) growth affected severely by Al toxicity in the control treatment; (b) rice grown normally in the pot treated with basalt
Source. Shamshuddin et al. (2011)

4 t ha^{-1} (Figure 17(b)). This is commendable considering the low rate of basalt dissolution in the soil even though it was undergoing weathering under acidic condition. If rice is grown again in the same pot, the yield is expected to be higher than that of the first crop as more basalt would have been dissolved.

Alleviating Al toxicity and/or soil acidity using microorganisms

Soil contains living microorganisms. Some of these microorganisms can be put to good use, such as to improve the productivity of acidic soils. A case in point is the phosphate-solubilising bacteria living naturally in acid sulfate soils. Panhwar et al. (2014b) had isolated and subsequently identified three potential phosphate-solubilising bacteria in acid sulfate soils in the paddy fields of Semerak, Malaysia. The PSB identified were *Burkholderia thailandensis, Burkholderia seminalis* and *Sphingomona spituitosa*. The PSB were found to be able to make P more available from the otherwise insoluble $FePO_4$ and/or $AlPO_4$ occurring in acid sulfate soils. This phenomenon would partly solve the problem of P-fertiliser being lost via fixation after it is applied in the paddy fields.

The results of this study showed that the PSB inoculated into rice plants released organic acids under the stress of high Al concentration. Like the case of organic acids secreted by rice roots, Al in the soil will be chelated and consequently inactivated. This is another mechanism by which PSB helps improve the environmental condition for rice growth. To prove that inoculation of rice seedling with PSB improves its growth, a short-term experiment was conducted in the laboratory. The results of the experiment are presented in Figure 18. It is clear that rice seedling inoculated with PSB was bigger/taller than those without inoculation.

Results also showed the ameliorative effect of PSB. The microbes existing in the rice seedling produced indole-acetic acid, a plant hormone that helps enhance rice growth significantly. This is yet another finding that excites researchers working with the PSB isolated from acid sulfate soils.

Figure 18. Comparison of rice seedling with and without bacterial inoculation
Source. Panhwar et al. (2014b)

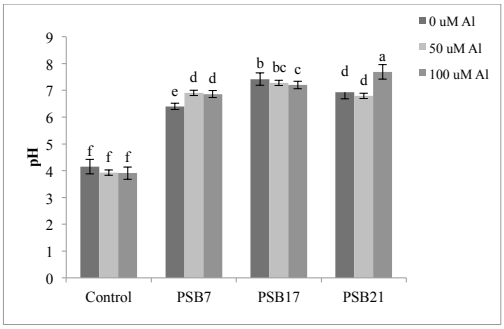


Figure 19. Effects of PSB inoculation on water pH at various concentrations of Al
Source. Adapted from Panhwar et al. (2014b)

Their action in the rice plants had resulted in significant pH increase (Figure 19). For the control treatment, soil pH was about 4, but when the seedling was inoculated with the PSB, soil pH was increased to above 6, regardless of the initial Al concentration. It is believed that during the process expedited by the PSB that takes place in the rice root, exopolysaccharide was released for the increase in soil pH (Panhwar et al., 2014b).

Increasing rice yield in Malaysia

The study on the alleviation of soil acidity as well as Al and/or Fe toxicity that affects rice production is not complete without conducting field trials in the areas subjected to the above-mentioned constraints. Hence, in Malaysia, field work was conducted in the Kelantan Plains (Shamshuddin, 2006) and Merbok, Kedah (Azura et al., 2014). Recently, another field trial was conducted in Semerak. The results on the chemical properties of the Semerak trial are presented in Table 1.

The topsoil pH of the control treatment was about 4. However, deep down the soil profile, the value was < 3.5, indicative of the presence of pyrite and/or jarosite in the soil. The exchangeable Ca and Mg were insufficient for rice growth, but the exchangeable Al and extractable Fe were very high. The exchangeable Al in the subsoil was > 5 $cmol_c$ kg^{-1} soil, above the level known for normal growth of rice plants. On applying the amendments, the fertility of the soil was somewhat improved. This is seen by the increase of exchangeable Ca and Mg with concomitant decrease of exchange Al to a level < 1 cmolc kg^{-1}. Although soil pH was still below 5, the condition was believed to be good enough for the rice plants to grow normally because they have the ability to protect themselves against acidity as well as Al and/or Fe toxicity (Shamshuddin et al., 2013; Shamshuddin et al., 2014; Alia et al., 2015).

The improved soil fertility due to the treatments was reflected by increase in the rice yield (Table 2). T5 in which 6 t GML ha^{-1} was applied in combination with JITU

Table 1
The chemical properties of the topsoil in Semerak, Kelantan, at rice harvest

Treatments	pH	Exchangeable cations ($cmol_c$ kg^{-1})				
		Ca	Mg	K	Al	Fe (mg kg^{-1})
Control	3.7c	0.49d	0.61e	0.26c	2.13a	208.45a
GML (4 t ha^{-1})	3.9b	1.30c	1.23d	0.30b	1.44b	66.15b
GML (4 t) + organic fertilizer (0. 25 t) ha^{-1}	4.3a	1.60b	1.84b	0.35a	0.93c	31.78d
GML (6 t ha-1)	4.1b	1.59b	1.54c	0.31b	1.03c	50.55c
GML (6 t) + organic fertilizer (0.25 t) ha^{-1}	4.4a	1.92a	2.07a	0.39a	0.49d	27.49e

*Organic fertilizer = JITU, GML = ground magnesium limestone
Means within the same column followed by the same letters are not significantly different at P>0.05 (n=5)

Table 2
Effects of different treatments on the rice growth cultivated in acid sulfate soil

Treatments	Grain yield (t ha^{-1})	Panicle number (10^4 ha^{-1})	Spikelet number (panicle^{-1})	Filled spikelet (%)	1000 grain weight (g)
Control	2.12d	553c	77.5c	74.84d	21.78cd
GML (4 t ha^{-1})	3.04c	651b	78.5c	84.71c	22.34c
GML (4 t) + *organic fertilizer (0. 25 t) ha^{-1}	3.99b	707a	83.5b	90.12a	26.41b
GML (6 t ha^{-1})	3.62b	701a	100.5a	88.81b	23/17c
GML (6 t) + *organic fertilizer (0.25 t) ha^{-1}	4.77a	715a	81.25b	91.69a	31.67a

*Organic fertiliser = JITU, GML = ground magnesium limestone
Means within the same column followed by the same letters are not significantly different at P>0.05 (n=5)

(an organic fertiliser fortified with effective microorganisms and growth hormones) gave the best yield of about 5 t ha^{-1} $season^{-1}$. However, it is not economical to apply lime at this rate.

Sustainable rice cultivation on acid sulfate soils

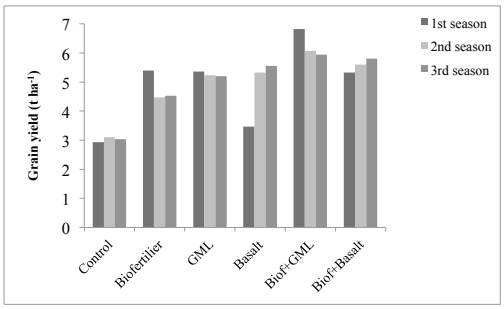
An innovative agronomic practice for adoption by farmers is important for rice production to be sustainable. The best approach is to examine all the important findings of past research on rice and subsequently design a special field trial to test the proposed idea. If the results look promising, the proposed agronomic practice should be fine-tuned before it is tested in a large-scale trial using farmer's land.

We have carried out short-term experiments in a glasshouse to test the suitability of GML, ground basalt, bio-fertiliser as amendments to alleviate the infertility of acid sulfate soils for sustainable rice cultivation. The result clearly showed that bio-fertiliser or bio-fertiliser in

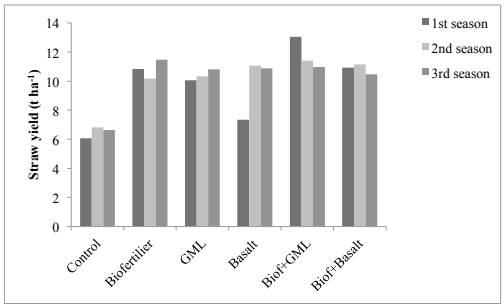
combination with GML gave the best yield. However, it is believed that the ameliorative effects of ground basalt were not been fully realised. Basalt takes longer than 6 months to be completely disintegrated and dissolved (Shazana et al., 2013), and it subsequently increases soil pH and releases Ca, Mg, K, P and Si for the uptake by rice. To be sure, if second crop of rice is grown in the same pots, the results might have been completely different. It is highly probable that basalt treatment would give a much better yield. The best yield would definitely come from basalt treatment in combination with bio-fertiliser.

The results of another experiment using the same amendments are presented in Figure 20. The objective of this experiment was to determine the long-term ameliorative effects of the amendments. It is clear that control treatment gave the lowest grain yield of about 3 t ha^{-1} season^{-1}. The yield increased significantly after the application of bio-fertiliser, However, the yield decreased slightly with time, indicating that bio-fertiliser must be used regularly, perhaps annually.

The ameliorative effects of GML treatment can last for at least three consecutive seasons, with grain yield remaining at about 5 t ha^{-1} season^{-1}. The best yield in this study was obtained by applying GML in combination with bio-fertiliser (Table 3). The authors believe that basalt in combination with bio-fertiliser (having PSB) actually offers the best option for adoption by farmers in Malaysia or even Southeast Asia. It is clear from Figure 20



(a)



(b)

Figure 20. Long-term effects of GML and basalt with or without bio-fertiliser application on rice growth (a) grain yield; (b) straw yield

that grain yield is steadily increasing. As more ground basalt disintegrates, soil fertility improves further resulting in increased yields. The ameliorative effects of basalt application could last for a long time, perhaps 2 to 3 years. This would translate into lower cost of rice production compared with the conventional practice. Hence, we can grow rice sustainably on acid sulfate soils by applying basalt in combination with bio-fertiliser. In this way, the SSL level in Malaysia can be increased significantly.

Research on the application of biochar for agricultural production is being conducted worldwide with conflicting results. Our rice research group at Universiti Putra Malaysia conducted a field trial in Semerak, Kelantan to test the effects of

Table 3
Effects of GML and basalt with or without bio-fertiliser application

Treatments	Number of Panicle plant^{-1}	Size of panicle plant^{-1}	Number of grains pot^{-1} (g)	Weight of grains pot^{-1} (g)	Plant biomass (g pot^{-1})
Control	3d	19.33c	515e	8.10e	14.30e
Bio-fertilizer	6a	23.31a	1106a	17.92a	26.53a
GML	5b	22.77b	854b	14.80b	18.98c
Basalt	4c	22.19b	601d	9.09d	16.07d
Bio-fertilizer+GML	5b	23.12a	879b	14.91b	20.45b
Bio-fertilizer+Basalt	4c	20.65c	695c	10.35c	18.34c

GML = ground magnesium limestone
Means within the same column followed by the same letters are not significantly different at P>0.05 (n=5)

Table 4
Effects of GML and biochar with or without bio-fertiliser application

Treatments	Grain yield (ha^{-1})	Straw yield (ha^{-1})	Number of filled grains (%)	Number of panicle plant^{-1}	Size of panicle (cm)	Soil pH (After 30 DAS)
Control	3.20e	8.24d	76.61d	11c	20.03d	4.08c
GML	4.04d	8.70c	81.65c	15b	21.61c	5.39b
Biochar	4.54c	9.38b	82.64c	16b	22.00c	5.18b
GML+Bio-fertilizer	5.44a	10.20a	85.89a	18a	24.40a	5.66a
Biochar+ Bio-fertilizer	5.04b	9.98a	83.61b	17a	23.11b	5.40a

*GML = Ground magnesium limestone 4 t ha^{-1}, bio-fertilizer 4 t ha^{-1}, biochar 5 t ha^{-1}.
Means within the same column followed by the same letters are not significantly different at P>0.05 (n=5)

applying GML and biochar with or without bio-fertiliser. The results of this field trial are shown in Table 4. The best treatment os still GML in combination with bio-fertiliser. The GML and biochar treatment gave reasonable rice yield of about 4 t ha^{-1}.

CONCLUSION

Rice has been planted on acid sulfate soils in Southeast Asia for many years. Due to the stress of low pH and the presence of high Al and/or Fe in the water of the fields, rice yields in the area are often below the national average. High acidity and Al and/or Fe toxicity are the result of pyrite oxidation due to its exposure to the atmospheric conditions when the areas are opened up for agriculture production. The infertility of the soils can be alleviated for sustainable rice production by applying ground magnesium limestone or basalt in combination with bio-fertiliser fortified with phosphate-solubilising bacteria. The bacteria not only excrete organic acids that inactivate Al and/or Fe via chelation, but also increase water pH to above 6. The

latter reaction results in the precipitation of Al as inert Al-hydroxides. Rice plants are also able to excrete organic acids via their roots under the stress of high Al and/or Fe that further reduce the availability of both metals in the water. In the end, rice will be able to grow well, giving reasonable yield.

ACKNOWLEDGEMENTS

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Effects of Soaking Periods and Adhesive Concentrations on the Properties of Phenol Formaldehyde Resin Treated Oil Palm Wood

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ABSTRACT

Oil palm (*Elaeis guineensis* Jacq.) is one of the most important and under-utilised non-wood biomass in Malaysia. This study examined the effect of low molecular weight phenol formaldehyde (Lmw-PF) resin impregnation on the mechanical and physical properties of oil palm trunk wood. The oil palm wood was treated using the following steps: by drying, resin impregnation, soaking and re-drying of specimens using different resin concentrations and treatment times. The determination of the modulus of elasticity, modulus of rupture and dimensional stability of treated oil palm wood were carried out using British standards. Results indicated that both resin concentration and soaking time significantly enhanced dimensional stability of the treated oil palm wood. Water absorption and thickness swelling of the treated wood displayed reduction with different soaking periods and resin concentrations with the best results of 7.37% and 5.08% respectively. Soaking had a significant effect on the bending properties of treated oil palm wood and showed 1.5 and 1.8 times improvement in modulus of elasticity and modulus of rupture respectively. It can be thus concluded that resin impregnation followed by soaking of oil palm wood is a viable method to improve its overall physical and mechanical properties as well as its dimensional stability.

Keywords: Durability, impregnation, oil palm wood, physical and mechanical properties, resin concentrations, soaking periods

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) was introduced to Malaysia from Africa as an ornamental plant. However, it found its way into commercial plantations and finally grew to become one of the most important commercial crops in Malaysia. Malaysia is the second largest producer and exporter of palm oil in the world, after Indonesia, with about 5.40 million hectares of planted area in 2014 (MPOB, 2014). Initially, the development of this crop was slow, but became an important crop for both countries. These vast plantations also produce huge amounts of underutilised lignocellulosic material. The average age for oil palm replanting is approximately 25-30 years. The production palm oil is only about 10% of the total biomass produced in oil palm plantations. The rest of the biomass is lignocellulosic materials, which consist of oil palm fronds (OPF), oil palm trunks (OPT) and empty fruit bunches (EFB). It is estimated that 14.4 million cubic metres of oil palm wood is produced annually due to replanting (Choo et al., 2013).

The outer portion of the trunks has better physical and mechanical properties compared with the inner portions (Choo et al., 2011; Balkis et al., 2012). However, oil palm trunk wood lacked in properties such as strength, durability, dimensional stability and machining when compared with other typical solid wood. Therefore, the treatment of oil palm wood (OPW) with a synthetic adhesive such as phenol formaldehyde would be considered as an alternative soluble to enhance the overall properties of this under-utilised species. Various studies have been carried out to improve the properties of OPW (Huang et al., 2014; Wahab et al., 2014; Zaidon et al., 2014). Impregnation and densification, similar to *compreg* (Amarullah, 2010; Chong et al., 2010; Faizatul et al., 2010; Bakar et al., 2013a, 2013b; Khairunnisha et al., 2014) were also applied to OPW to enhance its attributes.

In a typical resin impregnation process, adhesives can only penetrate into cell lumens and not cell walls. However, soaking which results in the diffusion of resin can enable the resin to penetrate into cell walls. This would further improve the overall dimensional stability and mechanical properties of OPW. Although there have been several studies carried out to evaluate properties of resin-treated OPW, there is little or no information on OPW treated with low molecular weight phenol formaldehyde (Lmw-PF) via a soaking process.

Therefore, the objective of this paper was to evaluate the mechanical properties and dimensional stability of OPW samples treated by impregnation and soaking using low molecular weight phenol formaldehyde (Lmw-PF).

MATERIALS AND METHODS

Mature oil palm trunks aged 25 years in Universiti Putra Malaysia's Agriculture Park were harvested and cut into 2 m long sections. The oil palm logs were sawed using polygon sawing method. As shown in Figure 1, the polygon sawing method was applied s to get the best homogenous

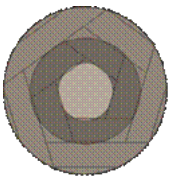


Figure 1. Polygon sawing as introduced and reported by Bakar et al., 2006

tangential lumbers from the outer parts of oil palm trunks (Bakar et al., 2006). A total of 45 samples with dimensions of 170 mm x 120 mm x 40 mm were used in this experiment.

The process consisted of five steps (Bakar et al., 2013b) - drying, resin impregnation, soaking, resin semi-curing, and curing (Figure 2). First, the lumbers were dried in a kiln at 15% moisture content (MC) and were then placed in an impregnation cylinder. Vacuum was applied for 15 minutes and the tank was then filled with Lmw-PF resin (10%, 15% and 20% concentrations) followed by an application of pressure at a level of 120 psi for 30 min. Treated samples were removed from the tank and soaked in Lmw-PF resin with concentration levels of 10% 15% and 20% for 6, 12, 18 and 24 hours respectively. Control samples did not undergo the soaking process. Next, samples were placed in an oven with a temperature of 150°C for 3 hours.

Cell wall penetration

The percentage of resin penetration into cell walls was based on the dry volume of

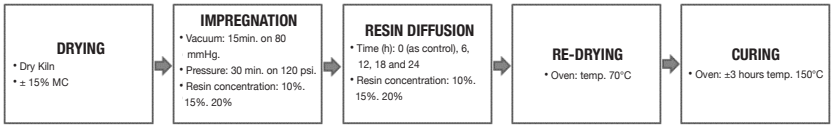


Figure 2. Five step impregnation and diffusion treatment process adapted from Bakar et al., 2013b

penetrated resin and the volume gain of treated samples. The calculation for cell wall penetration was derived from Hill (2006) as shown in Equation 1.

$$Vr = \frac{Wr}{Dr} \qquad \text{[Equation 1a]}$$

$$Vg = Vf - Vi \qquad \text{[Equation 1b]}$$

$$CWP = \frac{Vg}{Vr}\ x\ 100 \qquad \text{[Equation 1]}$$

Where, V_r is the volume of resin (cell wall + lumen cell), W_r is the weight of resin, D_r is the density of solid resin, V_g is the volume gain (volume of resin in cell wall), V_f is the final volume (sample volume after treatment), and V_i is the initial volume (sample volume before treatment).

Water absorption (WA) and thickness swelling (TS)

The water absorption and thickness swelling of the samples with dimensions of 20 mm x 20 mm x 20 mm were calculated according to British Standard BS 373:1957

(1957). The amount of absorbed water was calculated using Equation 2.

$$Water\ absorption\ (\%) = \frac{W_f - W_i}{W_i}\ x\ 100$$

[Equation2]

where, W_f is the sample after a 24-hour immersion in water and Wi is the sample weight before immersion (g). The thickness swelling was calculated using Equation 3.

$$Thickness\ swelling\ (\%) = \frac{T_f - T_i}{T_i}\ x\ 100$$

[Equation 3]

where, T_f is the thickness of the sample after a 24 hour immersion in water and Ti is the thickness of the sample immersion (mm).

Static bending

According to British Standard BS 373:1957 (1957), sample sizes for the static bending test should be 20 mm x 20 mm x 300 mm. However, the sizes of the samples in this study were modified to 20 mm x 20 mm x 150 mm because of limitations in lengths. The Modulus of elasticity and modulus of rupture were calculated using Equations 4 and 5 respectively.

$$Modulus\ of\ Elasticity\ (MPa) = \frac{P_t L^3}{4Dbh^3}$$

[Equation4]

where, P_t is the load below the proportional limit (N), L is the span of the test specimen (mm), D is the deflection at mid-span resulting from Pt (mm), b is breadth or width of the test specimen (mm) and h is height or depth of the test specimen (mm).

$$Modulus\ of\ Ropture\ (MPa) = \frac{3P_m L^3}{2bh^3}$$

[Equation 5]

where, P_m is the maximum (breaking) load (N), L is the span of the test specimen (mm), b is breadth or width of the test specimen (mm) and h is height or depth of the test specimen (mm).

Data analysis

Data was analysed using the Statistical Analysis System (SAS) and mean separation was carried out using the Least Significant Difference (LSD) method. All statistical analysis was based on $p \leq 0.05$.

RESULTS AND DISCUSSION

Table 1 shows ANOVA for the properties of the *impreg* OPW. It can be observed that all properties were significantly affected by resin concentration and soaking period. The interaction between diffusion period and resin concentration only influenced the WA and TS.

Cell wall penetration of the samples

Figure 3 shows that almost all samples which were soaked had higher cell wall penetration (CWP) compared with samples treated with just impregnation. Soaked samples showed resin CWP ranging from 7.86% to 35.15%. The highest CWP value of 35.13% was found in samples which were soaked for 24 hours and 20% resin concentration, while the lowest corresponding value of 7.86% was obtained for samples that were not soaked and 10% resin concentration. The

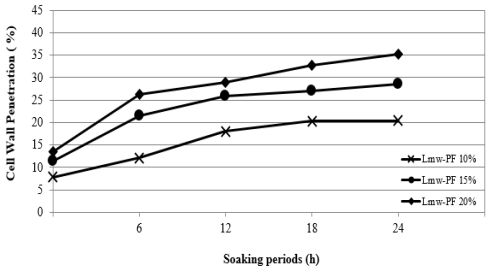


Figure 3. Cell wall penetration of *impreg* wood with different diffusion periods and different resin concentrations

CWP also increased with increased resin concentration. Irrespective of the soaking period, the mean CWP was 19.22% at 10% resin concentration and 34.49% at 20% resin concentration.

During the soaking process, resin can easily move from zones with higher concentration (in cell lumens) to zones with lower concentration (in cell walls) via diffusion (Hunt & Grant, 1967). Therefore, the additional soaking process after resin impregnation with Lmw-PF resin in this study showed that resin penetrated and bulked into cell walls.

Resin concentration also influenced resin penetration into cell walls. Figure 3 indicates that the higher the resin concentration, the higher the cell wall penetration. This could be because higher resin concentration contains more resin solids with an increased potential to allow resin penetration into cell walls.

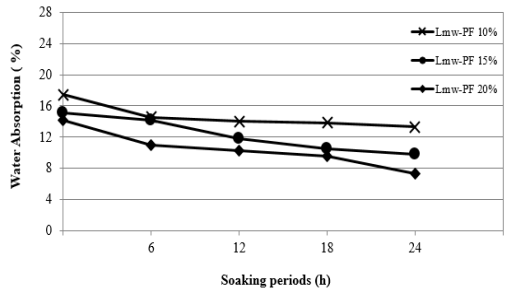
Water absorption and thickness swelling of the samples

Table 1 shows the effect of soaking periods and resin concentrations on the water absorption (WA) and thickness swelling (TS) of OPW. It is clear that WA and TS of the samples were significantly influenced by soaking time and resin concentration. Mean WA values ranged from 7.37% to 17.41%. As can be seen in Figure 4, samples soaked for 24 hours at 20% resin concentration had the lowest WA of 7.37%, and samples that

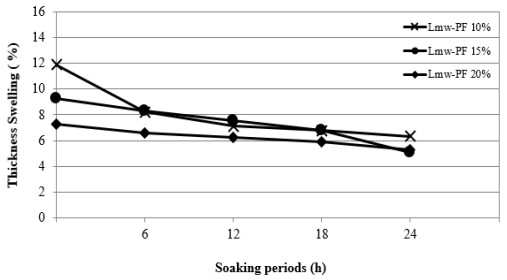
Table 1
Summary of ANOVA for properties of Impreg OPW

	Source	df	Pr> F				
						Static bending	
			CWP	WA	TS	MOE	MOR
	Diffusion period	4				0.0001*	0.0001*
	Resin concentration	2	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
	Dif. period x resin concentration	8	0.1527^{ns}	0.0214*	0.0004*	0.1766^{ns}	0.1526^{ns}

* Significantly different at p≤0.05
^{ns} Not significantly different at p>0.05;
df degree of freedom
CWP Cell wall penetration
WA Water absorption
TS Thickness swelling
MOE Modulus of elasticity



(a)



(b)

Figure 4. Dimensional stability of *impreg* OPW with different diffusion period and different resin concentration. (a) Water absorption (b)Thickness swelling

did not undergo soaking treatment at 10% resin concentration had the highest WA of 17.41%. Regardless of soaking periods, the WA was 14.64%, 12.27% and 10.48% for samples treated with 10, 15 and 20% resin concentrations respectively.

The lower WA in some samples is related to their enhanced dimensional stability. This could be due to resin replacing water in cell walls. According to several researchers (Kajita and Imamura, 1991; Furuno et al., 2004), Lmw-PF resin easily penetrates into cell walls and fully fills cell lumens and improves dimensional stability and decay resistance.

Table 1 shows that the thickness swelling (TS) of treated OPW was significantly affected by both resin concentration and soaking treatment. The mean TS values ranged from 5.08 to 11.89%. In general, the trend was similar to WA where the higher the soaking periods and resin concentrations, the lower the TS. Samples soaked for 24 hours at 15% resin concentration also had the lowest TS (5.08%) and the highest TS (11.89%) was shown by samples that were not soaked treated at 10% resin concentration (Fig. 4b). Regardless of soaking periods, samples treated using 10%, 15% and 20% resin concentration had mean TS of 8.07%, 7.39% and 6.26% respectively.

This result suggests that the additional soaking process after resin impregnation improves the dimensional stability of the material. The very low TS of treated OPW is considered as the effect of resin penetration into OPW cell walls and also cell lumens. Thus, this treatment can be used as an effective method to improve the dimensional stability of OPW. Abdullah et al. (2010) observed that increased WPG in OPW showed resistance against water absorption. The results showed that the thickness swelling of treated OPW was further reduced by the soaking treatment after impregnation leading to better dimensional stability.

Static bending of the samples

According to Figure 5, it is clear that higher resin concentrations result in higher Modulus of elasticity (MOE) and Modulus

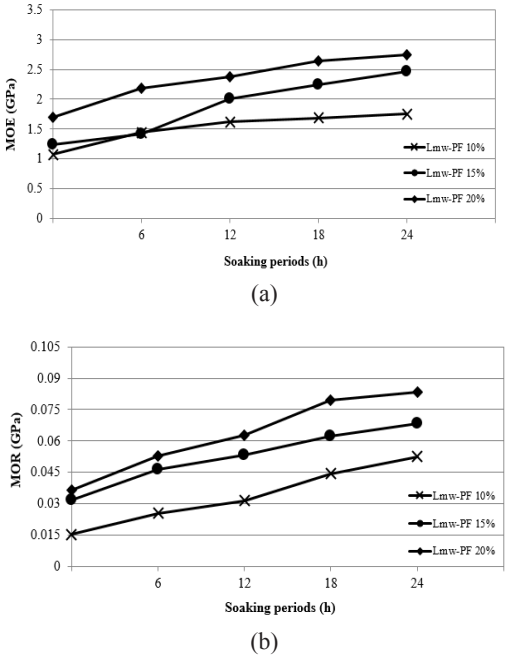


Figure 5. Static bending values of *impreg* OPW with different diffusion periods at different resin concentrations. (a) MOE (b) MOR

of rupture (MOR) values than that of lower resin concentrations. All samples that had undergone soaking treatment also had higher MOE and MOR values compared with samples treated only with impregnation. The highest MOE value of 2.74 GPa was found for samples soaked for 24 hours with 20% resin concentration and the lowest value of 1.07 GPa was found in samples that were did not undergo soaking treatment with 10% resin concentration. The highest MOR (0.08 GPa) was at a soaking period of 24 hours with 20% resin concentration and the lowest (0.02 GPa) was found in samples that did undergo any soaking period and at 10% resin concentration. The mean MOR value was 0.03, 0.05 and 0.06 GPa for 10%, 15% and 20% resin concentration respectively.

The mean MOE and MOR values of soaked OPW was 1.5 and 1.8 times higher respectively compared with samples that were not subjected to the soaking treatment. The penetration of resin into cell walls increased the stiffness value (MOE) and the value of rupture (MOR) of soaked OPW. This is consistent with a study carried out by Bakar et al. (2013b) where MOE increased with higher resin concentrations. Deka and Saikia (2000) also discovered that MOE and MOR of treated soft-wood (*Anthocephalus cadamba* Miq.) increased due to resin that fully saturated the cell walls.

CONCLUSION

The soaking treatment of OPW with Lmw-PF improved both the physical and mechanical properties of the samples. The results of this study clearly showed that the physical and mechanical properties of OPW specimens improved with increasing diffusion periods and resin concentrations. Cell wall penetration and bending strength increased with the impregnation and diffusion process compared with samples treated only with the former (impregnation process). Higher resin concentrations were expected to fully fill cell lumens and increased penetration into cell walls due to the higher content of resin solids. Meanwhile, water absorption and thickness swelling was lower in samples that had undergone soaking treatment. Spaces that should have been filled by water molecules were occupied and bulked by resin, thus improving the dimensional stability of OPW. It can be concluded that impregnation

further improves the properties of OPW specimens.

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Hypo-Osmotic Swelling Test Modification to Enhance Cell Membrane Integrity Evaluation in Cryopreserved Bull Semen

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ABSTRACT

The objective of this study was to enhance the hypo-osmotic swelling test evaluation when it reads under light microscope using 5% formaldehyde-fixed sperm solution buffer (FSSB). Twenty four ejaculates were collected from six crossbred bulls using electro-ejaculator (EE). Tris-egg yolk extender was used to cryopreserve the semen. Concentration, volume, motility, morphology and viability rates of fresh semen were evaluated and samples were cryopreserved in liquid nitrogen. After two weeks of liquid nitrogen treatment (freezing), the motility, morphology and viability rate of the semen were evaluated. In order to carry out a hypo-osmotic swelling test, post-thaw semen was divided into four aliquots based on period of incubation (30 or 60 minutes) adding FSSB to half the samples. The components of FSSB were 5% formaldehyde and 1% eosin-nigrosin stain in PBS. Results showed that F_{60} (61.48 ±0.89%) resulted in higher percentage ($P<0.05$) of total membrane intact compared with F_{30} (54.40 ±1.34%) and N_{30} (53.96±1.17%), but did not differ ($P>0.05$) with N_{60} (60.90±0.70%). In conclusion, adding 10 µl of FSSB after 60 minute of incubation with hypo-osmotic swelling solution (HOSS) will enhance evaluation of the hypo-osmotic swelling test (HOST) under light microscope.

Keywords: Hypo-osmotic swelling test, bull, sperm, cryopreservation

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INTRODUCTION

Super-vital staining and hypo-osmotic swelling tests (HOST) are used to validate the membrane integrity of spermatozoa; HOST however, is the most preferred technique because it is easy, simple, frugal, and quick (Tartagni et al., 2002).

Sperm cell swelling was first discussed by Drevius and Eriksson (1966), and its deformities were illustrated by developing a HOST for human (Jeyendran et al., 1984) and bull sperm (Revell & Mrode, 1994) based on sperm cell permeability to allow it to swell under hypo-osmotic situation. Therefore, HOST can determine the viability of sperm by increasing cell volume to obtain the balance between inside and outside solutions.

Swelling of the spermatozoon usually begins at the tail as the membrane at this area was loosely held, and then moves toward mid piece and head, while the dead, non-bioactive sperm has a normal shape because of their leaky membrane (Jeyendran et al., 1984). Hence, coiled tail, shortening of the tail and swelling head are shown in the test. This study was conducted to determine the effect of using formaldehyde and eosin-nigrosin stain on bull sperm. .

MATERIALS AND METHODS

Animal Selection

Six fertile bulls (four Brangus–Simmental crossbred and two Sahiwal-Friesian crossbred) were selected for this study. They were between three and five years old, and weigh between 570 kg and 650 kg. They were kept in a separate pen at Universiti Putra Malaysia farm. All bulls were fed with *Brachiaria decumbens* and additional 3 kg/BW palm kernel cakes concentrate. The water and mineral block were provided *ad libitum*.

Semen Collection and Processing

Semen from all bulls were collected using an electro-ejaculator (EE) (Ideal® Instruments Neogen Corporation, Lansing, Michigan, USA) as described by Sarsaifi et al. (2013). Freshly collected semen were evaluated and accepted at a concentration $\geq 600 \times 10^6$/ml, motility $\geq 75\%$, morphology and viability $> 85\%$. After evaluation, semen was diluted to final concentration of 20×10^6 sperm per 0.25ml straw using the formula below:

a. Number of Straws = Concentration of semen x Volume x Motility%* / N**

b. The total volume = number of straws x volume of one straw

c. Volume of extender = total volume – volume of semen

* Percentage of motility to cancel the percentage of immotile sperm

** N = number of sperm per straw (in this study was 20×10^6sperm/ml)

Tris-egg yolk extender was prepared according to Amirat-Briand et al. (2010).

It was divided into two fractions, the first fraction contained 0% glycerol (Tris: 2.24 g, citric acid: 1.48 g, fructose: 1 g, and 20% egg yolk in 100 ml extra pure of distilled water) while the second fraction had the same ingredients except for 12.8% glycerol. The first fraction of extender (without glycerol) was added to the semen and maintained at between 2°C and 5°C for three hours. The second fraction of extender (with 12.8% glycerol) was added at chill temperature and then immediately concussed and loaded in 0.25 ml French straws (Berndtson & Foote, 1969) and frozen in liquid nitrogen using the following steps: a) all straws were placed approximately 6.5 cm over the liquid nitrogen for five minutes, b) the distance was lowered to approximately 3.0 cm over liquid nitrogen for five minutes, and c) the distance lowered again to about 1.0 cm above the liquid nitrogen for three minutes. All straws were plunged into liquid nitrogen immediately after that and stored in a cryogenic tank. Straws were thawed after 14 days for further evaluation.

Hypo-Osmotic Swelling Solutions Preparation

All chemicals used in this study were purchased from Sigma Co. (Sigma Chemical Co., St. Louis, MO, USA). Hypo-osmotic swelling solution (HOSS) was prepared as described by (Jeyendran et al., 1984; Revell and Mrode, 1994). It contains 0.9 g fructose and 0.49 g sodium citrate dissolved up to 100 ml in distilled water. For preparation of 5% formaldehyde-fixed sperm solution buffer (FSSB), 5.0 ml of formaldehyde was added and mixed with 95.0 ml of phosphate buffered saline (stock solution). Then, 1.0ml from the stock solution was removed and added 1.0ml of eosin-nigrosin stain to achieve a final concentration of 5% formaldehyde and 1% eosin-negrosin stain.

Semen Evaluation

Sperm motility and concentration were evaluated using computer assisted sperm analyser (CASA; Hamilton Thorne Bioscience). Frozen straw is first thawed in a water bath at 37°C for 30 sec. About 10 µl of semen was placed on a dry warm dual sided sperm analysis chamber and covered with a cover glass. After one to two minutes the slide was placed in CASA, and at least three microscopic fields were read from each chamber.

The viability (Memon et al., 2012) and morphology (Chemineau et al., 1991) of sperm were evaluated using eosin-nigrosin stain. For this, 5 µl of semen was mixed with 15 µl of eosin-nigrosin for one minute at room temperature. Then, the smear was allowed to dry on hotplate stage (Copens Scientific (M) Sdn. Bhd.) maintained at 37°C and subsequently examined under a light microscope (Nikon Japan, ECLIPSE E200) at 40X magnification. Stained sperm head was classified as dead sperm while white sperm head was indicated as live. Sperm abnormalities were also determined where at least 200 sperms were evaluated at 100X.

Hypo-Osmotic Swelling Test

This experiment was divided into four different groups. For each group, 10 µl of post-thawed semen was incubated with 80 µl of HOSS in water path at body temperature. The 1st group (F_{30}) and 2nd group (N_{30}) were incubated for 30 minutes, while 3rd group (F_{60}) and 4th group (N_{60}) were incubated for 60 minutes. At the end of incubation period, 10 µl of FSSB was added to F_{30} and another 10 µl of FSSB was added to F_{60} to stop reaction, fix the shape of sperm and to give the medium some colours (F refers to that group that had FSSB at the end of incubation period, while N means none. On the other hand, the subscript $_{30}$ refers to the incubation period of 30 minutes while the subscript $_{60}$ refers to the incubation period of 60 minutes). For groups N_{30} and N_{60}, immediately at the end of incubation period, 10 µl of the mixture was placed on a warm microscopic slide and covered with cover slip and evaluated under microscope (400X). Groups F_{30} and F_{60} underwent similar process but they were evaluated after 5 minutes of adding FSSB. Sperm with intact membranes swelled and their tails curled while sperms with damages in their membranes remain unchanged. At least 200 sperm with intact and non-intact membranes were recorded.

Statistical Analysis

Data was analysed as mean ± standard error of the mean (SEM). Descriptive statistic and one way ANOVA were used to analysis HOST, fresh and cryopreserved semen while t-test pairs analysis was used to examine semen parameters to compare motility, morphology and viability before freezing and after thawing using SPSS version 22 for windows (SPSS Inc.; Chicago, IL, the USA).

RESULTS

Fresh and cryopreserved semen analysis

Table 1 shows that the concentration of fresh sperm was greater in bull B231 1270.25 X 10^6 ± 66.91 sperm/ml, and it

Table 1
Sperm parameters of fresh and cryopreserved bull semen (Mean±SEM)

Bull ID	N	Concentration X 10^6 Spermatozoa/	Fresh semen			Post-thawed semen			
			Volume/ ml	Motility%	Morphology %	Viability%	Motility%	Morphology %	Viability%
T104	4	995.00 ± 81.80^{ab}	8.88 ± 2.42	86.25 ± 2.39^{ab}	97.00 ± 0.41^{a}	94.00 ± 0.58^{a}	41.00 ± 2.61	94.75 ± 0.32	65.13 ± 2.29
B9001	4	775.00 ± 47.87^{b}	9.25± 1.60	77.00 ± 1.00^{b}	93.00 ± 1.39^{ab}	89.25 ± 1.20^{ab}	33.25 ± 1.80	89.63 ± 0.92	55.75 ± 3.71
B229	4	1175.00 ± 125.00^{ab}	5.87 ± 0.72	84.25 ± 2.95^{ab}	93.75 ± 1.36^{ab}	91.12 ± 1.33^{ab}	53.50 ± 6.30	92.87± 0.52	71.75± 2.25
B231	4	1270.25± 66.91^{a}	8.50 ± 0.29	86.25 ± 2.39^{ab}	86.87 ± 0.77^{b}	87.37 ± 0.97^{b}	47.75 ± 5.57	86.75 ± 0.48	70.75 ± 1.88
B206	4	1176.25 ±33.25^{ab}	3.78 ± 0.13	86.75 ± 1.18^{a}	94.75 ± 0.25^{ab}	87.50 ± 0.65^{ab}	51.75 ± 1.80	90.88 ± 0.37	69.25 ± 1.11
T902	4	845.00 ±90.97^{ab}	5.00 ± 0.71	77.25 ± 0.95^{b}	96.13 ± 0.37^{ab}	87.00 ± 0.41^{ab}	47.00 ± 4.10	92.12 ± 0.43	69.25 ± 1.31
Total[1]	24	1039.42 ± 47.81	6.88 ± 0.63	82.96 ± 1.13*	93.60 ± 0.76*	89.37 ± 0.62*	45.71 ± 2.06	91.17 ± 0.56	66.98 ± 1.39

[1]Parameter of fresh semen with asterisk (*)compared with the same parameter of post-thawed semen.
$^{a, b}$ and ^{ab} – different superscripts in the values of each bull in the column 'concentration', 'motility', 'morphology' and 'viability' differ significantly at P<0.05

was significantly high (P<0.05) compared with bull 9001 775 X 10^6 ± 47.87 sperm/ml. There were no significant differences (P> 0.05) in the volume of fresh semen. The motility rate of fresh semen was greater in bull B206 and it was highly significant (P< 0.05) compared with bull 9001 and T902. Sperms with good integrity and viability were recorded in bull T104 97 ± 0.41% and 94 ± 0.58% respectively. They were significantly higher (P<0.05) compared with bull 231.

Results show that the concentration, volume, motility, morphology and viability of fresh crossbred bulls semen were 1039.42 X10^6 ± 47.81(sperm/ml), 6.88 ± 0.63 ml, 82.96 ± 1.13%, 93.60 ± 0.76% and 89.37 ± 0.62% respectively. Results showed that motility, morphology and viability of frozen semen were reduced where (P<0.01) 45.71 ± 2.06%, 91.17 ± 0.56% and 66.98 ± 1.39% respectively compared with fresh semen.

Cell membrane integrity

Membrane integrity of post-thawed semen was read via two incubation periods, 30 and 60 minutes with HOSS and with or without adding FSSB. According to Table 2, the ratio of membrane integrity in F_{60} 61.48 ± 0.89 % was high (P< 0.01) compared with F_{30}54.40 ± 1.34% and N_{30}53.96 ± 1.17%. However, there were no significant differences between F_{60} and N_{60} that underwent the same incubation period. Moreover, adding FSSB gave some darkness to the solution and enabled the coiled and swollen sperm to be easily located (Figure 1).

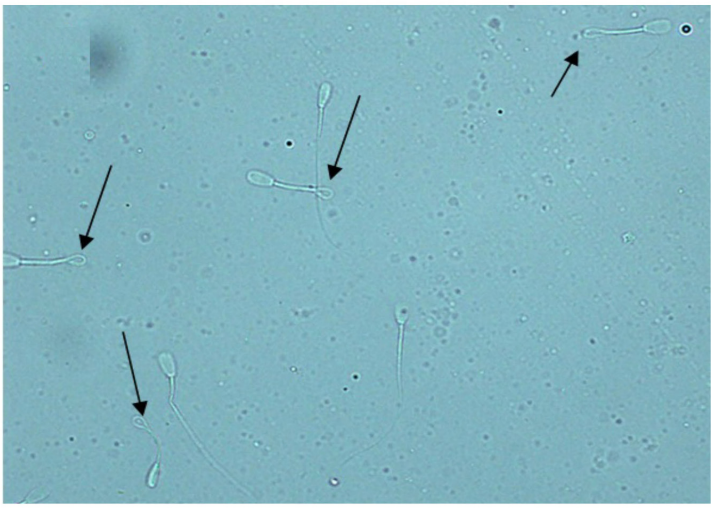
DISCUSSION

In the present study, some semen parameters were different based on the age of bulls (Al-Kanaan et al., 2015). Significant differences were recorded in semen volume, motility,

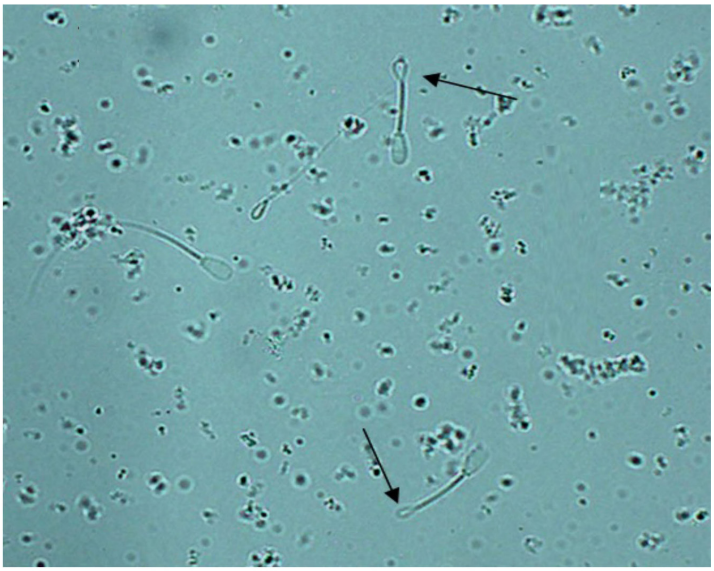
Table 2
Cell membrane integrity using hypo-osmotic swelling test of cryopreserved bull semen

Group	N	Mean	Std. Error	95% Confidence Interval for Mean		Minimum
				Lower Bound	Upper Bound	
F_{30}			1.34	51.63	57.16	43.50
N_{30}	24	53.96[a]	1.17	51.53	56.38	45.00
F_{60}	24	61.48[b]	0.89	59.65	63.31	52.00
N_{60}	24	60.90[b]	0.70	59.45	62.34	52.00

[a] and [b] – different superscripts within column differ significantly at P<0.01
F_{30}: 10 µl of thawed semen incubated with 80 µl of HOSS for 30 min at 37°C, and then FSSB was added at the end of incubation period.
N_{30}: 10 µl of thawed semen incubated with 80 µl of HOSS for 30 min at 37°C, without adding FSSB.
F_{60}: 10 µl of thawed semen incubated with 80 µl of HOSS for 60 min at 37°C, and then FSSB was added at the end of incubation period.
N_{60}: 10 µl of thawed semen incubated with 80 µl of HOSS for 60 min at 37°C, without adding FSSB.



(a)



(b)

Figure 1. Frozen bull sperm under hypo-osmotic condition; coiled sperm tail indicating intact membranes of sperm. a) without adding FSSB; b) after adding FSSB

concentration, and output between older and younger bulls. These findings are consistent with previous works (Brito et al., 2002) on genetic impact (Carabano et al., 2007), and season (Al-Kanaan et al., 2015). However, this study was conducted in a tropical region which is limited by two seasons .

Semen response to the freezing varies among males of the same species and in different species, as shown in Table 1 when motility rate was compared between bull 9001 (33.25 ± 1.80%), and bull B231 (53.50 ± 6.30%). This difference may be due to age (Foote, 2002; Snoj et al., 2013), or genetic

material variations (Gürler et al., 2015a; Snoj et al., 2013). However, differences were not due to nutrition variation, since all bulls were reared under similar conditions.

The protocol of freezing processes, three steps of freezing in liquid nitrogen, using tris-egg yolk extender obtained a motility rate 45.71 ± 2.06%, with 66.98 ± 1.39 of viability rate; this is very near to the universal acceptance of semen motility and viability after cryopreservation (Hu et al., 2011; Lessard et al., 2000; Watson, 2000).

The quality of freezing straw is limited by many factors, such as genetic material of the bull, motility of sperm before and after freezing, number of sperms that can pass the freezing stress with normal cell membrane, acrosome, and DNA material (Foote & Kaproth, 1997). In addition, the collection of semen and its procedure, age of sperm before using, shipping, and the skill of insemination are important to ensure the fertility rate of cryopreserved semen (Rycroft & Bean, 1992). All these factors are important to reduce the motility, viability, and morphology rate significantly of post thawing semen compared with fresh semen (Yimer et al., 2014, 2015; Zhang et al., 2015). Our findings are consistent with those of earlier studies.

One of the most important parameters to detect male fertility is morphology of the sperm (Lasiene et al., 2013), and abnormalities of sperm indicate a genetic disorder in spermatogenesis (Veeramachaneni & Sawyer, 1996). Therefore, it might decrease the fertility of semen (Nagy et al., 2013; Saacke, 2008; Serafini et al., 2015). In the present study, sperm morphology of frozen-thawed semen was significantly reduced compared with fresh sperm as previously highlighted (Al-Makhzoomi et al., 2008).

Sperm have three layers and are rich with polyunsaturated fatty acids, plasma, mitochondrial, and acrosome membranes. They are highly susceptible to oxidative stress during cryopreservation processes (Moussa et al., 2002). Furthermore, when sperms are frozen, they are exposed to increasingly hypertonic condition as a result of the freezing water conditions and intracellular solutes are at maximum concentration. These may rupture the cell membrane and affect the motility of the sperm. Cell membrane rupture is clearly associated with sperm viability loss (De Leeuw et al., 1993). El-Nour et al. (2001) used immotile sperm for ICSI that were divided into two groups: one group was tested using HOST (HOST-group) and the other was not tested (no-HOST). It was found that the pregnancy rate of HOST-group (40%) was higher than the no-HOST group (14%). Hence, if the sperm pass HOST, it tends to increase chances of pregnancy (El-Nour et al., 2001; Stanger, 2010). In addition, HOST is easy, inexpensive and simple (Hossain et al., 1998; Tartagni et al., 2002). All these explain the value of HOST.

This study was designed to focus on the reading method of the HOST to make it easier and more precise for searchers via adding FSSB immediately at the end of incubation period of sperm in HOSS.

Results showed that addition of FSSB after 60 minutes of incubation with HOSS showed positive impact to enhance the test of membrane integrity. The reason for adding formaldehyde at 5% is to stop the reaction of sperm with HOSS solution and keep the final shape of sperm at the end of the incubation period. In addition, under microscope, some sperm were seen moving that leads to misreading of the exact number of sperm that are active biologically. Thus, formaldehyde at 5% will prevent the sperm from moving and conserve their shape if the osmolarity is changed. Moreover, adding 1% eosin-nigrosin stain provides some darkness to the solution that will enable easy spotting of coiled and swollen sperm.

HOST is good to test male fertility (El-Nour et al., 2001; Liu et al., 1997; Perez-Llano et al., 2001; Stanger et al., 2010). The present study and previous ones (Revell and Mrode, 1994; Zubair et al., 2013) depended on HOST (Jeyendran et al., 1984) to prepare the HOSS using sodium citrate in tri form and fructose at osmolarity 150 mOsm/kg, and tested on bull semen. In our study, adding FSSB to the post thawed semen that was incubated for 60 minutes seems to increase the total percentage of membrane intact and this was consistent with a study conducted on Nili-River bulls (Zubair et al., 2013). the authors had tested different osmolarities of HOSS (70, 90, 100, 120, 140, 150, 190, 230, 280, and 300 mOsm/kg) at similar incubation period (60 minutes) and found that 150 mOsm/kg showed high percentage of membrane integrity.

However, our observations are in contrast with those of Revell and Mrode (1994), The authors had attempted to look for the best osmolarity in best incubation period of membrane osmolarity resistant correlated with pregnancy rate (49 days non-return rate after AI), and found that the best osmolarity for fresh bull semen was 150 mOsm/kg and 125 and 100 mOsm/kg for frozen semen in 20 minutes of incubation for both fresh and frozen semen. The difference between their findings and the present study may due to number of bulls that were tested, genotype and diet of animals and number of samples from each bull. A handfull of studies found that a diet rich in poly unsaturatted fatty acid can affect the strecture of sperm membrane (Gürler et al., 2015b; Khoshvaght et al., 2016) that can lead to a change in the permeability of cell membranes.

CONCLUSION

This study showed that supplementation of HOST with FSSB at the end of incubation period has a beneficial effect by enhancing the membrane integrity test reading under light microscope 40 X. In terms of incubation period, 60 minutes resulted in better membrane integrity.

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Effects of Shading and Fertiliser on the Growth and Antioxidant Content of Olives (*Olea europaea* L.)

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ABSTRACT

The olive tree not only provides fruits but also wood and nutrition such as oleuropein. Olive leaves can be made into tea by 'withering' (drying) the fresh leaves. An experiment was conducted to observe the growth and evaluate the antioxidant content of olives using different types of fertilisers. The plants were grown under partial shading and full exposure to sunlight (50% and 0% - no shading); NPK fertiliser or commercial compost was used to enrich the planting media. The results showed that plants that grew in the shade are taller and have better foliage, while use of commercial compost resulted in better growth of branches and leaves. Older leaves contained higher levels of oleuropein and ascorbic acid. Exposure to sunlight increased ascorbic acid content in older leaves compared with that of shaded plants.

Keywords: Fertiliser, shading, growth performance, olive, antioxidant

INTRODUCTION

Olive (*Olea europaea* L.) is a plant that originated from the Mediterranean region and was introduced to tropical regions such as Indonesia. The country with its tropical humid climate is a suitable place to grow olives, but it is believed that the growth as well as productivity of the olive tree would be different. Furthermore, micro-climatic conditions due to the planting system may have some influence. Olives are commonly planted in a monoculture system, but can be grown in a mix-farming system due to scarcity of land. In the monoculture system, the plants are grown in full exposure of sunlight. In the mix-culture system, the

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plants grow under many different trees that might reduce their exposure to sunlight, such as growing under a shade.

Differences in environmental conditions of both systems may affect plant growth, i.e. its height, number of leaves, or number of branches. Environmental condition is also affected by soil fertility. The main nutrients needed by plants are nitrogen (N), phosphorus (P), potassium (K) (López-Granados et al., 2004).

The olive tree relies on secondary metabolites to interact with its environment for adaptation and defence. Phenolic compounds are among the known substances that protect the tree against biotic and abiotic stresses (Battacharya et al., 2010). The most abundant phenolic compounds in olive are phenolic acids, phenol alcohol, flavonoids, and secoiridoids (Servili et al., 2004), and oleuropein is derived from secoiridoid (De La Torre-Carbot et al., 2005; Silva et al., 2006).

Olives have medicinal values and the substance extracted from the fruits and twigs is considered part of a healthy diet due to their secondary metabolite. Apart from its role in plant defence, oleuropein has antioxidant, anti-inflammatory, anti-cancer, antiviral, and antimicrobial properties (Omar, 2010; Durlu-özkaya & Özkaya, 2011). Oleuropein is found not only in the olive fruit, but also in the leaves and seeds (Ryan et al., 2002), with the highest concentration found in fruits (pastes and pulps) and in leaves (Silva et al., 2006). However, the fruits are largely processed for olive oil (Vossen, 2013). Olive leaves are another source of oleuropein, and can be processed into tea leaves, through withering and drying of fresh leaves. This must be supported by increased production of the leaves.

Ascorbic acid is as an antioxidant and a co-factor of certain enzyme involved in photoprotection of plants (Smirnoff, 1996; Khan et al., 2012; Gallie, 2013). Therefore, plant performance can be evaluated through its phenol and ascorbic acid content. It is assumed that oleuropein as well as ascorbic acid levels would vary in olive trees planted under full sunlight and under shady conditions.

There is limited information on the effect of tropical humidity on growth and antioxidant content of olives, especially the effect of shading and fertiliser use. Therefore, this research examined the growth of olive trees in various conditions, i.e. shaded growth in combination with inorganic or organic fertilisers, and the effect of treatments on their oleuropein and ascorbic acid content.

MATERIALS AND METHODS

Experimental site and plant material

The experiment was conducted in Depok, West Java, Indonesia between September 2014 and March 2015. Two year-old olive trees were planted in pots of 45 cm in diameter, one plant per pot. The distance between the pots was 70 x 70 cm.

Experimental design

This research used split plot design to measure the growth of the olive trees as well

as oleuropein and ascorbic acid content of the leaves; shading as the main plot and fertiliser regime as the sub plot. In order to analyse oleuropein and ascorbic acid concentration in fresh olive leaves, different types of leaves were placed in the sub-plots. The shading has two levels: 50% shading (N1) and full exposure to sunlight (N2). Two types of fertilisers were used: NPK 20:10:10 (P1) and commercial compost (P2). The soil was fertilised using NPK fertiliser , on day-0 with 105 g/pot and on day-56 with 50 g/pot. Compost (2 kg/pot) was mixed with the soil before being used as media. Young leaves (D1, the first to the sixth leaves from the tip) and old leaves (D2, the seventh to the 12th leaves) were collected.

Growth variable measurement

Growth of the plants was observed every three weeks for four months. Plant height was measured from the base of the stem above the ground to the tip of the shoot. Stem diameter was observed at 3 cm above ground using vernier callipers.

Olive tea leaves processing by withering fresh leaves

Fresh leaves were collected to be made into tea every week beginning from week 2 after treatment (WAT) up to 15 weeks. The method followed the processing procedure for *Camelia sinensis* leaves into green tea (Shi & Schlegel, 2012) with temperature modification. The leaves were dried under sunlight for 1-2 hour(s), then in room temperature for 1-2 day(s). Leave rolling was executed at 40°C for no longer than 15 minutes, followed by further drying in an oven at 40°C for 90 minutes.

Analysis of biochemical parameters of olives

Determination of oleuropein content in olive leaves. The oleuropein level was analysed using High Performance Liquid Chromatograph (HPLC) following the method described by Bouaziz et al. (2008). Tea leaves were crushed into powder and fresh leaves were minced in a blender for 2 minutes and stored in a dry place until the time for extraction. It was replicated two times. Six grams of each sample was used for the extraction process. After the final centrifugation at 10,000 rpm for 10 minutes, the supernatant was stored at 0°C in darkness before chromatographic analysis. The mobile phase used acetonitrile: water (7: 3) as solvent A; phosphoric acid (0.1% in water) as solvent B. The flow rate of eluates was 0.6 mL/min and injection volume was 20 µL for 50 minutes. Temperature was set at 40°C and detected at 280 nm.

Measuring ascorbic acid level in olive leaves. About 0.5 g fresh leaves (young and old leaves) or tea leaves were macerated with 5 mL of metaphosphoric acid 5% in a mortar and filtered using filter paper up to 10 mL . The sample was titrated with 2.6-dichlorophenol indophenols (2.6-DCIP) reagent 0.025% until a pink end point that persists for 15 seconds was obtained (Rao & Sresty, 2000).

Data Analysis

ANOVA was used to analyse data using SPSS version 19.0, followed by Duncan's Multiple Range Test (DMRT) to determine the difference among treatments. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Shading effects on microclimate

This research was conducted under two shading conditions, 50% shading and non-shading. The environmental parameters (temperature, light intensity, and humidity) were observed in both conditions. Table 1 shows data collected for several days during mid-day between 12pm and 1pm. Shading treatment changed the microclimate around the plants. In general, there was no noticeable difference in the temperature under shading and under full sunlight; the temperature was considered mild. The relative humidities (RHs) at mid-day ranged between 39% and 53%, where the shaded area had slightly higher RH. Shading treatment also reduced light intensity by between 60% and 65%. In the Mediterranean regions, average temperatures during winter time ranged between 2°C and 18°C and during summer between 10°C and 46°C, with RH averages at 40% and 80% (Chiraz 2013; Orlandi et al., 2013). The climate differences between Mediterranean and the Western part of Indonesia may affect metabolic activities in the plants, although olive trees can grow well in other regions due to their adaptive ability to a wide range of temperature; but the generative phase of olives needs chilling temperature to induce the initiation of flower-buds and to break dormancy (Fabbri et al., 2009; Orlandi et al., 2013).

Table 1
Environmental parameters as influenced by shading

Environmental Parameters	50% Shading	Non-Shading
Temperature (°C)	28.8 - 30	30.3 – 34.3
Light intensity (lux)	6,870- 8,600	19,800 – 21,567
Relative Humidity (%)	41.3 – 52.4	38.9 – 45.1

Effects of shading and fertilizer on growth performance

The olive plants grew from the 3rd week and were observed up to 15th week after treatment (WAT). Analysis of variance on data collected at 15th WAT shows no interaction between shading and the types of fertiliser on all growth parameters, but each single factor did have its effects. Fifty percent shading significantly increased the height of the plant and the number of leaves compared with those under full sunlight. There were more branches and leaves in plants in the media with compost with NPK fertiliser (Table 2). The olive plants that are partially shaded suffered etiolation that increased their height and amount of leaves. The colour of the leaves is also different as well as the branch growth. Olive plants

Table 2
Effects of shading and types of fertiliser on the growth of olive plants

Treatments	Plant Height (cm)	Number of Leaves	Number of Branches
Shading (N)			
50% shading (N1)	147.83[a]	2553.90[a]	59.80[a]
Non-shading (N2)	130.83[b]	2300.50[b]	55.80[a]
Fertiliser (P)			
NPK fertilizer (P1)	133.00[a]	2240.50[b]	54.20[b]
Compost (P2)	145.66[a]	2613.90[a]	61.40[a]

Numbers followed by the same letter in the same column are not significantly different with DMRT test, α=5%. Data were collected at the 15th week after treatment

that are grow in the shade have longer and more flexible branches and the leaves have a lighter green.

When the plants are treated with compost, growth of foliage and branches is better than with NPK. Compost is an organic matter derived from livestock, crop residues, or waste products. These materials will be decomposed and become a source of nutrient for plants (Brinton, 2000). Compost has more nutrients compared with synthetic fertiliser but it needs a longer time to be absorbed by plants due to its decomposition process (Wei & Liu, 2005). Compost influences plant growth directly and indirectly. It indirectly improves the soil condition physically, chemically and biologically. Compost directly provides nutrients by increasing chlorophyll content, improving photosynthesis and hormonal growth responses (Hafez et al., 2015). The NPK fertiliser (20:10:10) as much as 105 g/plant was expected to improve the growth of plants, particularly the foliage. Nitrogen is needed to form chlorophyll and in photosynthetic activity while phosphate is needed for energy storage through the transfer to ATP and ADP, while potassium enhances process of water uptake which has a consequence on cell enlargement (Freihat & Masadeh, 2006). But in this case, the amount and ratio of NPK fertiliser used was not able to increase foliage compared with using compost.

Oleuropein content

Table 3 shows oleuropein concentrations in tea leaves. Statistically, there is no link

Table 3
Oleuropein concentration in olive leaves tea from the plants grown under different growing conditions

Oleuropein concentration in olive leaves tea (mg g^{-1})*			
Shading (N)		Fertilizer (P)	
50% shading (N1)	29.17[a]	NPK fertilizer (P1)	33.93[a]
Non-shading (N2)	34.43[a]	Compost (P2)	29.68[a]

Numbers followed by the same letter in the same column are not significantly different with DMRT test, α=5%. *Means of two replications

between shading and fertiliser on oleuropein content. It is indicated that partial shading or the types of fertiliser used do not influence the oleuropein content in tea leaves.

This study proved that there is a high content of oleuropein (in average 31.8 mg g^{-1}) in processed leaves compared with those previously reported. Jemai et al. (2009) reported that oleuropein concentration in dried olive leaves from Tunisia processed at 40°C was 24.4 mg g^{-1}, while Afaneh et al. (2015) obtained 10.0 mg g^{-1} when the leaves from Palestine was processed at 25°C and 1.7 mg g^{-1} at 50°C. It suggested that in general, the climatic conditions in the research site may not be favourable for the normal growth of olive plants, and that they produced phenolic compounds, in this case oleuropein, more abundantly. The concentration of a particular phenolic compound within a plant tissue is dependent on the season and may also vary at different stages of its growth and development (Lynn & Chang, 1990; Ozyigit et al., 2007), and it is influenced by internal as well as external stress. According to Vuong et al. (2013), phenolic compounds are easily degraded by high temperature and light, and are also easily oxidised; that may describe the lower content of oleuropein in leaves originating from the above mentioned Mediterranean countries. Additionally, the three successive drying-phases applied in this research may be more effective in ensuring high oleuropein in the leaves.

In fresh leaves, the oleuropein content was not influenced by the treatments applied The difference is only between young and older leaves (Table 4). Older leaves contain more oleuropein than younger ones. In normal conditions, the concentration of antioxidant and other secondary metabolites in older plant tissues/organs is higher than in younger ones, while under stress conditions the concentration will be even manifold (Abdallah et al., 2013; Majer & Hideg, 2012). This is due to the function of secondary metabolites as self-defensive agent against predators or pathogens, and because the metabolites are synthesised when the cell growth rate starts to decline after the maturity phase (Mazid et al., 2011).

Table 4
Oleuropein concentration in fresh olive leaves

Type of leaves (D)	Oleuropein concentration in fresh olive leaves (mg g^{-1})*
Young leaves (D1)	10.17[b]
Older leaves (D2)	12.91[a]

Numbers followed by the same letter in the same column are not significantly different with DMRT test, α=5%. *Means of two replications

Ascorbic acid content

Shading and leaf type influence ascorbic acid levels. Table 5 shows that the level of ascorbic acid in N2D2 (non-shaded older leaves) was significantly higher than in young leaves of both shaded and not shaded plants (N1D1 and N2D1). Old leaves from plants exposed to full sunlight (N2D2) and those under shade (N1D2) have comparable levels of ascorbic acid, although the fomer tends to be more abundant.

When growth rate starts to decline in older leaves, the plant will accumulate more

Table 5
Effects of shading and type of leaves on ascorbic acid concentration in fresh olive leaves

Treatments	Ascorbic acid concentration (mg/g)*
N1D1 (young leaves in 50% shading)	0.16[b]
N1D2 (older leaves in 50% shading)	0.19[ab]
N2D1 (young leaves in non-shading)	0.14[b]
N2D2 (older leaves in non-shading)	0.24[a]

Numbers followed by the same letter in the same column are not significantly different with DMRT test, α=5. *Means of three replications

carbon to synthesise secondary metabolites as defence mechanism. Ascorbic acid is synthesised by using carbon such as D-galactose or D-glucose as a precursor (Loewus, 1999). It has an important role as co-factor in several enzymes and functions as antioxidant that protects photosynthetic apparatus from high light intensity (Pignocchi & Foyer, 2003; Smirnoff, 1996).

Results showed that ascorbic acid is more responsive than oleuropein in different environmental conditions, particularly when exposed to sunlight. It is assumed that under full exposure to sunlight, the synthesis of ascorbic acid will be increased to reduce the detrimental risk caused by ROS. According to Gallie (2013), ascorbic acid can detoxify free radicals as a result of full exposure to the sun. High light intensity can increase the formation of mono oxygen that can cause damage to membrane and pigments in PSI and PSII.

The ascorbic acid concentration in older leaves when exposed to sunlight (N2D2) was higher; this indicates that in olive plants, the defence mechanism through ascorbic acid is more efficient in older leaves than in younger ones. It is supported by the oleuropein content which is also higher in older leaves.

Olive tea leaves can be part of a healthy diet due to their high oleuropein content. Additionally, the tea leaves have ascorbic acid at the level of 85 mg per 100 g or 0.085% in average (data not presented). The awareness of the importance of ascorbic acid to human nutrition has facilitated the development of technologies that increase the ascorbic acid content of plants through manipulation of the biosynthetic or recycling pathways (Gallie, 2013).

CONCLUSION

Partial shading (50% shade) improved plant growth . Use of commercial compost resulted in more leaves and branches. Partial shading and compost encouraged the growth of leaves which are a good source of antioxidants. Oleuropein content in dried leaves and in fresh leaves was relatively constant, regardless of shade and the type of fertiliser used. Older leaves contained higher amount of oleuropein and ascorbic acid than the younger ones. It is suggested that under climatic conditions in the Western part of Indonesia (constant temperature

over the year) where mix-farming is also commonly practised, olive trees can grow well and the leaves containing high levels of antioxidants.

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Enhancing Solubility of Curcumin in Turmeric Oleoresin for Improving Productive Performance of Broiler Chickens

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ABSTRACT

This study examined the effect of enhancing solubility of curcumin, from Turmeric Oleoresin (TO), in boosting the productive performance of broiler chicken. Solid dispersion (SD) technique was used to enhance the solubility of curcumin for use in the broiler chicks' diet. The curcumin solubility (TOPSD) was prepared by mixing TO with carrier (Polyethylene glycol, PEG400) and adsorbent (Banana powder, BP) at the ratio of 1:1:3 by SD technique. High performance liquid chromatography (HPLC) was used to determine solubility of the mixture. The result showed that solubility rate of crude curcumin noticeably increased with carrier and adsorbent ($P<0.05$). This mixture was used to improve productive performance of broiler chickens. Two hundred and forty Arbor Acres chicks were randomly allotted to 10 groups. Each group was replicated 6 times (4 chicks per replication). The effects of sex (120 males and 120 females) and five levels of TOPSD (0, 0.2, 0.4, 0.6 and 0.8 % of diet) were examined in treatments by 5×2 factorial randomly. All chicks were raised for six weeks. Food and water were provided ad libitum. The results showed that increased levels of TOPSD reduced average daily feed intake (ADFI), improved feed conversion ratio (FCR) ($P<0.05$), but without any effect ($P>0.05$) on average daily gain (ADG) when compared with the control group. The sex of the chicks and treatment combination (the level and sex) had no ($P>0.05$) effect on productive performance.

Keywords: Turmeric, oleoresin, curcumin, solubility, broiler

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INTRODUCTION

Curcumin is the principle curcuminoid isolated from the rhizome of turmeric plant (*Curcuma longa*) and various members of the ginger family (*Zingiberaceae*). It is a medicinal plant widely used and cultivated in tropical regions. The active

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ingredients found in Turmeric (*Curcuma longa*) are curcumin, demethoxycurcumin, bisdemethoxycurcumin (Wuthi-Udomler et al., 2000) and tetrahydrocurcuminoids (Osawa et al., 1995). Curcumin is known to have antifungal (Wuthi-udomler et al., 2000), immunomodulatory (Antony et al., 1999), antioxidative (Osawa et al., 1995), antimutagenic (Soni et al., 1997), anti-inflammatory (Ammon et al., 1993), and nematocidal (Kiuchi et al., 1993) properties.

Dietary supplementation of curcumin is limited because of its low solubility in alkaline pH and being subject to hydrolysis when exposed to light, which result in poor absorption in animals (Kochhar, 2008). Research shows that curcumin is water insoluble, and has poor over-all solubility, wettability, and bioavailability. The solid dispersion (SD) technique has been used to increase solubility and absorption of poorly soluble drugs by dispersing the drug in a water soluble carrier in a solid state (Lefebvre et al., 1985). The objectives of this research were to: 1) enhance curcumin solubility by mixed Turmeric Oleoresin (TO) with carrier (Polyethylene glycol 400, PEG400) and adsorbent (Banana powder, BP) at the ratio of 1:1:3 (TOPSD) and 2) to determine the appropriate level of TOPSD for supplement in the broiler chicken diets to improve their productive performance.

MATERIALS AND METHODS

Solid dispersion (SD) preparations

Crude curcumin from Turmeric Oleoresin (TO) was mixed with carrier (ethyl acetate, PEG400) and adsorbent (BP) at the ratio of 1:1:3 (TOPSD) using SD technique. Ethyl acetate was removed from all mixed samples in hot air oven at 70°C for 30 minutes and dried in hot air ovens at 40°C for 6-12 hours. The samples were pulverised to 0.05-0.25 mm particle size using mortar and pestle. All samples were analysed for their solubility, quantity, and recovery.

Solubility of curcumin

Ten milligrams of curcumin from TOPSD was transferred into a 10 ml volumetric flask. The samples were dissolved in water. A magnetic stirrer with paddles was rotated at 200 rpm for 5, 15, 30, 60, and 120 min at 37 ± 0.5°C respectively. The supernatants were filtered through a 0.2 µm pore size millipore membrane at the same temperature. An aliquot of 20 µl was injected into high performance liquid chromatography (HPLC). The curcumin quantity and recovery were determined by HPLC using Ultrasphere® C_{18} as an analytical column. Methanol, 2% acetic acid, and acetonitrile at the ratio of 23:36:41(v/v) were used as mobile phase with 420 nm UV detection. All experiments were conducted in triplicates.

Animals and diets

Two hundred and forty Arbor Acres chicks (120 males and 120 females) were randomly allotted to a basal diet (21% crude protein and 8.6% fat) containing TOPSD at 0, 0.2, 0.4, 0.6 and 0.8 % respectively. The chicks were divided into 10 groups with

6 replications (4 chicks per replication) according to 5×2 factorial randomly. All chicks were raised for 6 weeks. Food and water were provided to broiler chickens ad libitum. Body weight and feed intake were recorded weekly between three and six weeks of age. Growth performance was calculated as average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR).

Statistical analysis

Curcumin quantity, curcumin recovery and curcumin solubility from TOPSD samples were compared using PROC TTEST. Data obtained from ADFI, ADG, and FCR were subjected to analysis of variance (SAS, 2001). Treatment means were compared using Duncan's New Multiple Range Test. The values were displayed as least square means (±SE).

RESULTS AND DISCUSSION

Quantity, recovery and solubility of curcumin

The crude TO samples were sticky and black in colour. Adding carrier and adsorbent altered the colour of crude (TO) to light yellow and in powdery form as shown in Figure 1. The crude (TO) contained 16.49% curcumin. Quantity and recovery of curcumin in TOPSD were 2.2% and 67.2% respectively (Table 1). Curcumin from TOPSD showed higher ($P<0.05$) solubility than crude TO (Figure 2). The SD technique increased the solubility and maximised the surface area of curcumin. This might be due to the role of PEG 400 and BP that increased active molecular carriers on the surface of curcumin. Pornanek & Uriyapongson, (2014) conducted experiments to improve recovery and solubility of curcumin from Turmeric Oleoresin (TO) by carrier (Polyethylene glycol 400, PEG 400) and adsorbent (Magnesium oxide, MgO) using SD technique. The ratio of 1:1:3 (TO: PEG400: MgO) suggests the highest

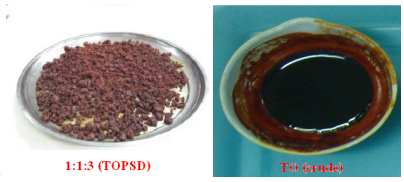

Figure 1. Characteristic of TOPSD and TO

Table 1
Quantity of curcumin and curcumin recovery from TO and TOPSD

TO: PEG400: BP	Curcumin (mg/100mg)	Curcumin recovery (%)
1:0:0 (TO)	16.49±0.01[a]	96.98±4.74[a]
1:1:3 (TOPSD)	2.28±0.01[b]	67.20±4.74[b]

Means in the same column with different lowercase letters differ ($P<0.05$)

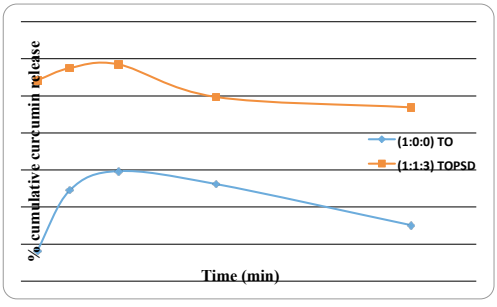

Figure 2. Solubility profile of curcumin from TO and TOPSD in water

recovery rate (81.47%) and the highest solubility in water. Other studies indicated that the solubility of curcumin increased linearly because PEG400 is highly hydrophilic (Modasiya1 & Patel, 2012). Higher solubility of curcumin was also indicated by excellent wettability, which could be observed clearly from the solid dispersion as it rapidly left the surface, and was dispersed in the bulk of dissolution media (Tonnesen, 2002). Moreover, the wetting property of the TOPSD was responsible for enhancing solubility (Leuner & Dressman, 2009; Craig, 2002).

Influence of TOPSD on growth performance of broilers

Effects of dietary supplementation of TOPSD on growth performance of broilers are shown in Table 2. The ADG of all trials were not significantly different (P>0.05). Supplementation of TOPSD at 0.6% and 0.8% in the diet resulted in lower ADFI than the TOPSD-fed broiler at 0%, 0.2% and 0.4% (P<0.05). The FCR of the broiler fed with 0.2, 0.4, 0.6 and 0.8% TOPSD were significantly lower (P<0.05) than that broiler fed with TOPSD at 0% of diet. Sex of the chicks and the treatment combination between the level and sex had no (P>0.05) effect on growth of broiler chickens. Enhancing solubility of curcumin in turmeric oleoresin (TOPSD) showed lower FCR. An improved FCR in TOPSD-fed group at level of 0.2%, 0.4%, 0.6% and 0.8% may be due to optimum antioxidant activity of curcumin to stimulate protein synthesis via the enzymatic systems in

Table 2
Effects of TOPSD levels on productive performance of broiler chickens

Items	TOPSD (%)												SEM	P-value		
	0		0.2		0.4		0.6		0.8							
	M	F	M	F	M	F	M	F	M	F				Trt	sex	trt*sex
Initial weight (g/h)	43.33	44.13	44.13	43.16	43.33	43.80	43.16	43.80	43.50	43.80	0.17			0.99	0.49	0.56
Final weight (g/h)	1485.00	1505.00	1592.08	1575.00	1560.00	1579.58	1497.00	1514.58	1486.25	1532.70	11.76			0.05	0.46	0.94
Weight gain (g/h)	1460.86	1460.87	1547.95	1531.83	1516.66	1535.78	1454.33	1457.75	1442.75	1488.90	11.75			0.06	0.46	0.95
ADFI (g/h/d)	63.48^{a}	63.81^{a}	63.13^{a}	63.10^{a}	62.14^{ab}	62.20^{ab}	61.59^{b}	61.48^{b}	61.35^{b}	61.57^{b}	0.18			0.01	0.51	0.86
ADG (g/h/d)	34.32	34.78	36.85	36.47	36.11	36.56	34.62	35.01	34.35	35.45	0.27			0.05	0.47	0.95
FCR	1.85^{a}	1.83^{a}	1.71b	1.73^{b}	1.72^{b}	1.70^{b}	1.78^{b}	1.75^{b}	1.77^{b}	1.74^{b}	0.01			0.01	0.46	0.92

ADFI means average daily feed intake, ADG means average daily gain, and FCR means feed conversion ratio, (g/h) = gram per head, (g/h/d) = gram per head per days, ^{a,b,c} means within a low with different superscript letters are significantly different at P<0.05. 0 = diet with no TOPSD, 0.2 = diet with 0.2 % TOPSD /kg diet, 0.4 = diet with 0.4% TOPSD /kg diet, 0.6 = diet with 0.6% TOPSD /kg diet, 0.8 = diet with 0.8% TOPSD /kg diet, M= male, F=female

the chicken. This is consistent with the findings of Osawa et al., 1995. Similar findings about alteration in performance parameters of broiler fed turmeric powder were reported by other researchers (Wuthi-Udomler et al., 2000; Samarasinghe et al., 2003; Durrani et al., 2006). Turmeric powder improves the liver function of broiler by decreasing the activity of alanine aminotransferase and alkaline phosphatase (Emadi & Kermanshahi, 2007a). It also enhances the antioxidant status of heat stressed broilers by improving the activity of glutathione peroxidase and superoxide dismutase and decreasing the concentration of malondialdehyde (MDA) (Zienali et al., 2011).

Adding 0.5% turmeric to the diet of the broiler chicks improved their FCR (2.08) compared with the control group (2.47) (Al-Sultan, SI. 2003). The broiler chicks that were fed dietary turmeric powder (TP) showed weight gain, energy efficiency ratio, yield of production, and lower FCR than those which were on basal diet ($P<0.05$) (Suvanated et al., 2003). Therefore, it is possible that the enhanced growth of broiler chicken is directly attributed to the role of turmeric in their feed which improved their digestive system (Platel & Srinivasan, 2000) and increased villus length and weight in the duodenum, jejunum and ceca of broiler chickens at 42 days of age (Rajput et al., 2012).

CONCLUSION

The aqueous solubility of curcumin was improved by adding TO into PEG400 and BP at 1:1:3 ratio. The treated curcumin had a higher solubility rate than crude curcumin from TO. Supplementation of the TOPSD in broiler chickens lowered FCR and reduced ADFI, while ADG of all trials was not significantly different compared with the control group.

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Translocation and Elimination of Cu in *Avicennia marina*

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ABSTRACT

Heavy metal pollution is a big problem in the aquaculture sector. Phytoremediation is one of the innovative approach to clean up the polluted water. The purpose of this research was to study the translocation of heavy metal (Cu), and its elimination using the mangrove plant, *Avicennia marina*. The study was conducted in Tapak Tugurejo, a coastal area in the northern part of Semarang City, Indonesia, where the water was polluted by heavy metals discharge (Cu) from industries nearby, at the upstream of the Tapak River. Samples of *A. marina* parts (roots, leaves, litter), sediment and water were collected and analysed to determine total Cu concentration. Results showed the plants of *A. marina* has the ability to translocate Cu metal in their tissues, respectively Cu concentrations in litter > leaf > root. Therefore, litter has the ability to eliminate metals in the environment through the defoliation process. The results also showed that Concentration Factor (CF) of Cu between water and sediment was 500.5 to 897.7, while the Bio Concentration Factor (BCF) between sediment and roots was in the range of 0.03 to 0.13. The Translocation Factor (TF) in roots and leaves ranged between 0.4 and 1.1. Hence, translocation of Cu metals was evident in the roots and leaves of *A. marina*, and the absorbed Cu was then eliminated via the litter .

Keywords: *Avicennia marina*, elimination, Cu, translocation, litter

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INTRODUCTION

Mangrove ecosystem plays an important role in coastal areas. Mangrove swamps not only protect the environment against erosion and t strong winds they also have the ability to absorb metals present in the coastal area. The mangrove roots are a natural filter of pollutants as they

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trap sediment and particles carried by downstream current to the ocean (Kumar et al., 2011). Kr'bek et al. (2011) studied the role of mangroves as the bioaccumulator of heavy metals. MacFarland & Burchett (2000) and MacFarlane et al. (2007) found a strong linear relationship between metals contained in the sediments with those in the tissues of mangrove plants (roots). This shows that the plants have the natural ability to accumulate contaminated sediments.

Avicennia marina is one of the mangrove species which is prevalent in the north coast of Java. Hastuti et al. (2013) argued that *A. marina* is a mangrove species that dominate the coastal areas of Semarang and Demak, Indonesia.

According to Usman et al. (2013) *A. marina* has the potential to accumulate Cu from sediment, as shown by high Cu accumulation in roots and leaves with Bio Concentration Factor (BCF) and Translocation Factor (TF) values > 1. Einollahipeer et al. (2013) showed that tissues of roots, stems and leaves of *A. marina* can be used as a good bio-indicator of Cu, with a BCF value of 0.60. Based on its BCF value, *A. marina* has potential to phytoremediate heavy metals (Lotfinasabasl & Gunale, 2012).

It is in at the roots of mangrove plants that heavy metal is concentrated (Tam & Wong, 1996).Mobility and solubility of metal also affect accumulation of heavy metals in plants. According to Sinha (1999) the ability of plants to accumulate heavy metals is as follows i.e. Mn>Cr>Cu> Cd>Pb. The ability to accumulate heavy metals differs from one species to the other. Heavy metal concentration in roots, branches and leaves of a plant species also differ.

It is assumed that the litter also contain considerable amount of trace metals. In addition, the litter may pose the danger of bringing the accumulated metal back into the waters. This topic however, has not been explored in depth by scholars. Therefore, the aim of this paper is to discuss heavy metal accumulation in litter, and their translocation in the roots, leaves and litters of *A. marina*.

MATERIALS AND METHODS

Study area

This research was conducted in Tapak Tugurejo, a coastal area of Semarang city, Indonesia. The area (110°17'15 " to 110°22'4" E and 6°56'13 " to 6°59'14" S) is filled with by mangrove vegetation. Tambak Aji factory is situated at the headwater of Tapak river which passes through the Tapak Tugurejo area. The pollutants from the factory is channelled to the Tapak River directly affecting Tapak Tugurejo area (Marjanto, 2005).

Sampling and sample preparation

The study examined heavy metal (Cu) concentration in water, sediments and the mangrove plant, *A. marina*, namely its roots, leaf, and litter for a period of 12 weeks (January-March) with two-week interval. 1 mol. L^{-1} HNO_3 solution was used to wash the glass tubes for at least 12 hours and then

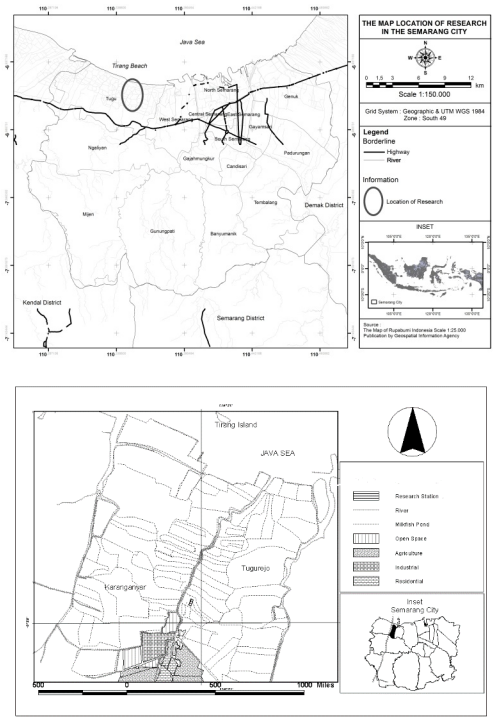

Figure 1. The Map of Tapak Region of Tugurejo, Semarang, Indonesia

rinsed 5 times thoroughly with deionised water. Each sample was replicated 8 times to guarantee the accuracy of results. Samples of *A. marina* were taken from young leaves where most Cu are accumulated Martuti & Irsadi (2014). The submerged roots were cut and litter was collected using a net trap. The water samples were collected in 150 ml polyethylene cans while the sediments were taken from. 1 cm, 5 cm and 15 cm depths respectively before they were mixed thoroughly.

Water samples were filtered with 0.45 mm Whatman GF/C filters to separate suspended particulate matter. A 0.5% (v/v) of HNO_3 was added to the filtered water for the precipitation of samples. The sediments were left to dry at room temperature for at least three days. The dried solid sample then pulverized and sieved through a 1 mm stainless steel mesh. The roots were rinsed repeatedly with de-ionised water to remove dirt before they were cut to smaller pieces and dried at 110°C for 24 hours. They were later ground into fine powder. The same method was used for the leaves and litter. Strong acids were used to oxidise the organic matter and sediments, which will release metallic elements (Blekeer, 2007). About 100 mg of dry sample of grinded roots was put in a destruction tube to which 2 ml of 4.1 (v/v) HNO_3 :37% Concentrated HCL was added to the mixture and left for 15 minutes before it was placed in an oven set at 140°C for seven hours. About 8ml of de-ionised water was added to the digested sample in the destruction tube and was swirled repeatedly. The mixture was then poured into the polystyrene tube and stored at 4°C. All the samples, water, sediments and *A. marina* (leaves, roots, and litter), were analysed using atomic absorption spectrophotometer (Plus 932, Australia) to measure the concentration of Cu.

The concentration of metals in roots, leaves, and litter were measured in order to determine the translocation of Cu in the mangrove plant. The ratio of Cu concentration in the sediment to Cu in the water was expressed as the Concentration Factor (CF). Likewise, the ratio of Cu in the sediments to Cu in the roots was expressed as the Bio Concentration Factor (BCF). The ratio of Cu concentration in the leaves to Cu in the roots was expressed as the Translocation Factor (TF).

RESULTS AND DISCUSSION

The presence of dissolved metals in seawater and sediments depends on the quality of the water. Increased activity in the water environment would lead to rising concentration of heavy metals. Results of laboratory analysis of the Cu are shown in Table 1. Copper was present in the pond in Tapak Tugurejo region, Semarang City, Indonesia.

Table 1
Cu concentration in water and sediment and the Concentration Factor (CF)

Week	Concentration*		
	Water (mg/L)	Sediment (mg/kg)	CF
0	0.05 ± 0.008	25.03 ± 4.77	500.5
2	0.03 ± 0.005	19.65 ± 2.23	577.9
4	0.03 ± 0.005	18.00 ± 4.80	720.0
6	0.02 ± 0.007	20.65 ± 3.52	897.7
8	0.03 ± 0.007	17.20 ± 3.97	521.2
10	0.02 ± 0.005	16.09 ± 3.41	670.4
12	0.03 ± 0.004	15.20 ± 1.77	524.2

*All values are mean ± standard deviation

Concentration of Cu ranged between (0.02 ± 0.005) and (0.05 ± 0.008) mg / L (Table 1). All the Cu concentration values exceeded the maximum permissible level for marine biota of 0.008 mg / L set by the Indonesian Ministry of Environment, i.e. the Decree of the Minister of Environment of Indonesia Number 51 Year 2004 on Standard Quality of Sea Water.

The Cu contamination happened in Tapak area most probably because it was located in the downstream of the Tapak River. The ponds are also vulnerable to metal pollution since they were connected to the mangrove ecosystem in the estuary of Tapak River. Chaiyara et al. (2013) contends argued that the presence of metal concentrations in water and sediment in an area depends on the presence of human activity in the upstream, such as mining, industrial, and residential.

The concentration of Cu in the sediment ranged between (15.20 ± 1.77) and (25.03 ± 4.77) mg/kg with CF of 500.5–897.7 (Table 1). Typically, there is high Cu concentration in pond sediment. This demonstrates the ability of pond sediment to accumulate Cu from the water. Sany et al. (2012) confirmed a close link between the concentration of heavy metals in sediments and those in water. Chaiyara et al. (2013) explained that the water dilution process resulted in higher concentration of Cu in sediment compared with the water layers. Sediment is important for mangrove ecosystems because of its ability to store heavy metals from the environment. The presence of heavy metal in the sediment is highly dependent

on the contamination level of the water (Abohassan, 2013).

Measurements of Cu in *A. marina* from milkfish pond showed the presence of the metal in the roots, leaves, and litter as shown in Figure 2. The concentration of Cu in the roots ranged between 0.87 ± 0.32 mg/kg and 2.21 ± 0.99 mg/kg. The leaves of *A. marina* were able to accumulate Cu between 0.88 ± 0.18 mg/kg and 2.70 ± 0.34 mg/kg. Litter in the *A. marina* which is the result of defoliation of the mangrove plant showed Cu between 2.35 ± 0.54 mg/kg and 5.72 ± 1.74 mg/kg.

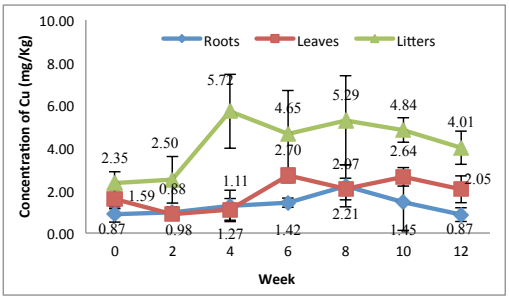

Figure 2. Cu Concentrations in Roots, Leaves, and litter in *A. marina* (all values are mean + standard deviation)

It can be seen from Figure 2 that the concentrations of Cu varied between plant tissues of *A. marina*. The highest concentration was found in the litter followed by leaves and roots respectively. On the other hand, the concentrations of Cu in all plant tissues tended to increase between 0 and fourth week and declined on the 8th week.

The BCF calculation between the root *A. marina* and pond sediment showed

Table 2
BCF and TF of Cu in A. marina

Week	BCF	TF
0	0.04	0.5
2	0.05	1.1
4	0.07	1.1
6	0.07	0.5
8	0.13	1. 1
10	0.09	0.6
12	0.06	0.4

ranged between 0.04 and 0.13 (Table 2). This small value was due to the low value of Cu concentration in sediment. Higher BCF value is associated with higher value of Cu concentration in sediment. The difference between this study and McFarlane (2002) could be explained by the difference of concentrations in the sediment. Most likely, the absorption of Cu by the root is proportional to the concentration of Cu in the sediment following first order kinetics. The breathing roots *A. marina* are on the surface of the sediment and parts of the root are above water a form of adaptation of plants on tidal conditions. Generally, the plants absorb elements through the roots, either from sediments or water, and then translocate the metal to other parts of plant and localise or accumulate it in certain tissues (Hardiani, 2009). The process of evapotranspiration process is a mechanism whereby contaminants transfer from the roots to the shoots of plants (Tangahu et al., 2011)

The ability of *A. marina* in accumulating Cu is influenced by the presence of metal in the water and sediment. Defew et al. (2004) described the presence of heavy metals in

the aquatic environment which experience precipitation, dilution and dispersion, which are absorbed by the organisms living therein. The findings of this study confirm those of previous studies on concentration of heavy metal in mangrove habitat which followed a decreasingly consecutive order, i.e. sediments>root>stem>leaf>fruit>seawater (Saifullah et al., 2004).

Table 2 shows metal transfer (TF) from roots to leaves ranged between 0.4 and 1.1. This demonstrates the potential of *A. marina* as a bio accumulator. The root of *A. marina* plays an effective role to transfer Cu to the stem tissues and leaves with a TF value of 1.47 (Einollahipeer et al., 2013). The results of this study are consistent with those of Lotfinasabasl and Gunale (2012), that higher concentration of heavy metals is accumulated in the leaves than in the root of *A. marina*. The high concentration of metals is found in the leaves because after metal penetrates the root's endodermis, metal or other extraneous substance is transported on transpiration system to the top of the plant through transporting tissues (xylem and phloem) to other parts of the plant (Priyanto & Prayitno, 2007).

Elevated Cu concentration in the leaves of *A. marina* leaves is due to their ability to accumulate metal. According to Parvaresh et al. (2011), the leaf is one of several tissues of mangrove plants which is able to accumulate metal. In addition, Nazli & Hashim (2010) argued that not only the roots but also leaves of the mangrove have the ability to accumulate heavy metals. The ability of the roots and leaves of mangrove to accumulate heavy metals is relatively higher compared with other plant species. Kamaruzzaman et al. (2011) stated that the presence of Cu in plant tissue is also expedient for its growth, particularly in leaf tissue where the process of photosynthesis occurs. Findings of this study are consistent with those of Martuti & Irsadi (2014) that the young leaves of *A. marina* have a higher Cu metal content than the old ones. However, their study did not measure the Cu content in litter. Therefore, the findings of this study is important. Further research is needed to explain why Cu content in litter is higher than in the old leaves.

The relatively high concentration of copper in the *A. marina* litter is a product of adaptation whereby the plant defends itself against contaminated environments by excreting copper through the leaves, which will then be discarded through defoliation. As confirmed by Barutu et al. (2014), the amount of accumulated metal in the leaves is the result of localisation by the plant, which concentrates metal in the organs of both intracellular and extracellular, such as the leaves. The process is a form of active plant excretion through the gland in the canopy. Meanwhile, passive mechanism includes the accumulation in the leaves as indicated by defoliation of old leaves.

Excretion is an important plant mechanism when dealing with environmental toxicity. Excretion is actively conducted via gland in the crown and passively through the accumulation of old leaves followed by litter discharge (Fitter & Hay, 1992). According to Lotfinasabasl & Gunale (2012), litter

can restore metal to the environment. Abohassan (2013) substantiated that litter is able to release <3.5% of the absorbed metal back to the environment. This condition is almost identical when the plants have high saline concentration; the mechanism by which *A. marina* survives is by excreting the salt through defoliation. MacFarlane & Burchett (2000) confirmed that the metals Zn and Cu found in the tissues of plants will be excreted through the salt gland on the lower surface of leaves.

Metal excretion process through plant litter indicates that metal accumulated will be returned to the environment. Restoring metals into the environment through litter is an adaptation mechanism of plants under extreme environmental conditions. Saenger & McConchie (2004) stated that litter can return heavy metals into the environment in a bioavailable form and the amount of metal released is relatively low. As the litter usually spreads in relatively large area due to winds or waves, the release of metal from the litter to the environment is expected to be relatively low.

CONCLUSION

Heavy metal (Cu) accumulation has been discussed extensively in this paper. Its accumulation in parts of *A. marina* has been found to be more than ten-fold higher than that in water, while the accumulation in sediment is the highest, with minimum CF of 500. The translocation of Cu in the roots, leaves and litter in *A. marina* indicated by the value of BCF and TF in the range of 0.04-0.13 and 0.4-1.1 respectively.

The results showed that Cu was translocated in the ionic form of Cu from the roots to the leaves of *A. marina*, and later eliminated through the litter. The highest concentration of Cu in *A. marina* was found in its litter, followed by leaves and roots. Although litter may release the accumulated heavy metals into the environment in a bioavailable form, the amount of metal released is relatively low due to the large spread of its fall. Thus *A. marin*a is important for reducing heavy metal (Cu) concentration in the environment.

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Utilisation of Local Crops as Alternative Media for Fungal Growth

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ABSTRACT

Potato Dextrose is the most commonly used media for the culturing of fungi. In this study, local crops were used as a substitute for potato. The growth of yeast (*Saccharomyces cerevisiae*) in broth media and molds (*Aspergillus flavus* TISTR 3366, *Bipolaris oryzae* DOAC 1760, *Fusarium semitectum* DOAC 1986 and *Penicillium* sp.) on agar media was examined. Four crops (cassava, potato, sweet potato and taro) were utilised as nutrient source in fungal media to result in four types of dextrose media while commercial potato dextrose media was used as the control. *S. cerevisiae* recorded the highest level of growth with 2.76×10^7 cells/mL when cultured in Sweet Potato Dextrose Broth at 25% sweet potato, 2% dextrose, initial pH 4.6 and agitated at 250 rpm at 27°C. Additionally, for the mold growth, Sweet Potato Dextrose Agar demonstrated significantly higher mycelial growth than commercial Potato Dextrose Agar while Taro Dextrose Agar showed similar positive result, except for *F. semitectum* DOAC 1986. This study showed that sweet potato and taro have a strong potential for use as alternative nutrient substitutes in fungal media production for yeast and mold growth.

Keywords: Culture media, fungi, growth, Potato Dextrose Agar, Potato Dextrose Broth, sweet potato, taro

INTRODUCTION

Fungi require nutrients (such as a carbon, nitrogen, vitamins, mineral elements, as well as the availability of enzymes) and certain environmental conditions (such as suitable pH value, suitable temperature, oxygen) in order to grow and reproduce. Potato has been used for fungal growth from early 20th century (Edgerton, 1908; Duggar, Severy, &

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Schmitz, 1917) and in fungal media since then (Beever & Bollard, 1970; Booth, 1971). Potato Dextrose media (PDM), made from dextrose and potato infusion, have been recognised as principal media for fungal cultivation. Fungi can break down starch in potato into soluble sugars, which can serve as a source of both carbon and energy. Furthermore, potato is a complex medium that provides nitrogen, enzymes, vitamins and mineral elements for fungal growth (Laurie, Faber, Adebola, & Belete, 2015). The high carbon: nutrient ratio of PDM hence allows efficient growth of fungi.

In most developing countries (such as Thailand), potato is generally more expensive than other cash crops. The average price per kg of potato, (obtained from the biggest wholesale farmers' market in Thailand, www. taladsimummuang.com), is higher than other crops; approximately US$ 0.99, 0.71, 0.39 and 0.34 per kg for potato, taro, cassava and sweet potato respectively (original prices are in Baht and converted into US$ with the exchange rate of 1US$ = 35.16 Baht). The cultivation of fungi using commercial PDM is costly. Therefore, researchers have attempted to develop alternative media from cassava (Weststeijn & Okafor, 1971; Rachael & Adebolu, 2014), cocoyam (Amadi & Moneke, 2012; Rachael & Adebolu, 2014), corn (Adesemoye & Adedire, 2005; Hoa & Wang, 2015), millet (Adesemoye & Adedire, 2005; Hoa & Wang, 2015), sorghum (Adesemoye & Adedire, 2005), sweet potato (Amadi & Moneke, 2012; Rachael & Adebolu, 2014; Hoa & Wang, 2015) and yam (Weststeijn & Okafor, 1971; Amadi & Moneke, 2012; Rachael & Adebolu, 2014; Hoa & Wang, 2015).

In Thailand, cassava (*Manihot esculenta*), sweet potato (*Ipomoea batatas*) and taro (*Colocasia esculenta*) are widely cultivated. These cash crops not only have high carbohydrate content but are also as nutritious (they are rich in proteins, vitamins and mineral elements) as potato (Huang, Chen, & Wang, 2007; Laurie et al., 2015). Thailand is the world's biggest exporter of cassava (83% of global market share in 2009) and an important source of revenue for the country (Poramacom et al., 2013). Sweet potato is an important source of food in developing countries and is one of the seven major world staple crops. Amadi & Moneke (2012) showed that sweet potato possessed good mycelia growth when compared with yam, cocoyam and potato. Taro is a staple food in tropical and subtropical regions of the world; however, studies have yet to prove the utility of taro as fungal media.

In this study, the growth of yeast (*Saccharomyces cerevisiae*) in broth media and molds (*Aspergillus flavus* TISTR 3366, *Bipolaris oryzae* DOAC 1760, *Fusarium semitectum* DOAC 1986 and *Penicillium* sp.) on agar media was examined for possible fungal media production by using these as substitutes for potato in Potato Dextrose Media.

MATERIALS AND METHODS

Sample Collection

Cassava (*Manihot esculenta*), Potato (*Solanum tuberosum*), Sweet Potato (*Ipomoea batatas*) and Taro (*Colocasia esculenta*) were obtained from shops in Sakon Nakhon Province, Thailand.

Media formulation

Four different media were formulated, namely Cassava Dextrose Media [Cassava Dextrose Broth (CDB), Cassava Dextrose Agar (CDA)], Potato Dextrose Media [Potato Dextrose Broth (PDB), Potato Dextrose Agar (PDA)], Sweet Potato Dextrose Media [Sweet Potato Dextrose Broth (SDB), Sweet Potato Dextrose Agar (SDA)] and Taro Dextrose Media [Taro Dextrose Broth (TDB), Taro Dextrose Agar (TDA)]. The growth of yeast and molds was examined in broth media and agar media respectively. The standard method for preparing potato infusion (PDB) involved boiling 200 g of diced potato (washed and peeled) in distilled water for 30 minutes and subsequently filtered through a muslin cloth. Then, 20 g of dextrose (UNIVAR) was added to the filtrate. The volume of the mixture was measured at 1,000 mL with distilled water. The broth media were acidified with sterile 10% tartaric acid (UNIVAR) to obtain a pH value of 4.6 and were sterilised in the autoclave for 15 minutes at 121°C. This procedure was repeated in formulating CDB, SDB and TDB by substituting potato with cassava, sweet potato and taro respectively. The control medium comprised commercial Potato Dextrose Broth (Difco™) (commercial PDB).

In the agar media, the same procedure was applied but the amount of diced crop was increased to 250g, and 20g of agar (BIOMARK LABORATORIES) was added per litre. Each agar media was acidified with 10 mL of sterile 10% tartaric acid to each litre of the medium. Commercial Potato Dextrose Agar (Difco™) (commercial PDA) was used as the control medium.

Test organisms

The test yeast was *Saccharomyces cerevisiae* (Saf-instant brand, France), and the test molds were *Aspergillus flavus* TISTR 3366, *Bipolaris oryzae* DOAC 1760, *Fusarium semitectum* DOAC 1986 and *Penicillium* sp. (obtained from the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Thailand). The test organisms were maintained on PDA slants at 4°C and subcultured to fresh PDA plates regularly every month. The stocks of test organisms were subcultured and incubated at 25°C for 48 hours and 7 days for yeast and molds respectively.

The growth of yeast in broth media assay

The suitability of the formulated media (CDB, PDB, SDB and TDB) was assessed by culturing each of them with *Saccharomyces cerevisiae*. One hundred and fifty millilitres of each broth media was placed in sterilised

250 mL Erlenmeyer flasks at an initial pH value of 4.6 and inoculated with 3.0 mL of inoculum (absorbance 0.34 at wavelength 600 nm). Inoculated flasks were shaken in an orbital shaker (Thermo Scientific Forma Incubated and Refrigerated Benchtop Orbital Shakers: Model 4586) at 200 rpm for 48 hours at 25°C. The yeast cells were counted using a counting chamber (BOECO). Various concentrations of crops (50, 100, 150, 200, 250 and 300 g per litre), various concentrations of dextrose (0, 10, 15, 20, 25, 30 and 40 g per litre), various initial pH values (3.5, 4.0, 4.5, 4.6, 5.0, 5.5 and 6.0), various agitation speeds (100, 150, 200 and 250 rpm) and various temperatures (20, 25, 27, 28, 30 and 35 ºC) were investigated in a step-by-step fashion to determine optimal conditions of the test yeast to maximise the benefits of selcted crop broth media for yeast growth. Each experiment, with three replicates, was carried out.

The growth of molds on agar media assay

The selected formulated media (CDA, PDA, SDA and TDA) were evaluated by culturing them with the test molds. The test molds were subcultured to fresh PDA plates using a cork borer and incubated at 25°C for 7 days. A sterile cork borer (dipped into alcohol and flamed), with an outside diameter of approximately 6.25 mm was used to bore holes on the edge of pure starter cultures of the molds. The mycelia agar plugs were then removed using a sterilised wire needle and transferred top down onto the center of the formulated media. Each of the four molds from the pure cultures was inoculated on the plates of formulated media CDA, PDA, SDA, TDA and commercial PDA in the same manner. The diameter of growth was measured using a vernier caliper on day 7. The whole inoculation process was repeated three times for each formulated media with each of the test molds.

RESULTS AND DISCUSSION

The growth of yeast in broth media assay

Analysis of variance (ANOVA) was used to analyse results and mean differences were considered significant at $p<0.01$ by Bonferroni multiple comparison. The growth of *Saccharomyces cerevisiae* in formulating broth media is presented in Figure 1. SDB showed significantly high level of growth of *S. cerevisiae* with 6.65×10^6 cells/mL and is followed by commercial PDB, PDB, TDB

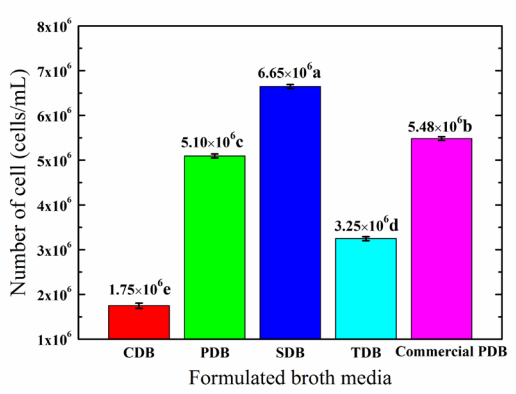

Figure 1. The growth of *Saccharomyces cerevisiae* in formulating broth media and commercial PDB over 48 h

Note. A pair of averages with different letters is considered significantly different at $p<0.01$

and CDB with 5.48×10^6, 5.10×10^6, 3.25×10^6 and 1.75×10^6 cells/mL, respectively. Laurie et al. (2015) reported that sweet potato has a higher carbohydrate and energy content than potato, and its mineral content (calcium, iron, magnesium, phosphorus, potassium and zinc) and vitamins (thiamin, niacin, riboflavin and vitamin B6) are similar with potato. The SDB could promote the highest growth of *S. cerevisiae* because carbohydrate, energy, minerals (such as iron, magnesium, phosphorus) and vitamins (especially thiamin) are key factors for fungal growth. The growth of *S. cerevisiae* in TDB was rather low. Although taro had high carbohydrate, mineral and vitamin content (Huang et al., 2007), a high amylopectin content of 72 – 83 % (Elisabeth, 2015) resulted in a sticky characteristic in the TDB which affects the oxygen absorption of yeast. The lowest growth of *S. Saccharomyces cerevisiae* was observed in CDB due to the sticky characteristic of the broth, like that of the TDB (Kwoseh et al., 2012). Trace amounts of vitamins (Laurie et al., 2015) and a high content of hydrogen cyanide (HCN) were also reported in cassava (Charles et al., 2005). Boiling of cassava will create cyanide residue in the water (Cooke & Maduagwu, 1978) as it releases HCN from its tissue (Burns et al., 2012). Although autoclaving reduced the cyanide content, it was not completely eliminated (Chove & Mamiro, 2010). It is highly toxic for all aerobic organisms including fungi because it prevents oxygen uptake (Latif & Müller, 2015).

The SDB was selected for optimising culture conditions comprising concentrations of sweet potato, concentrations of dextrose, initial pH value, agitation speeds and temperature. The growth of *S. cerevisiae* in SDB in various concentrations of sweet potato, and in various concentrations of dextrose is shown in Figures 2(a) and 2(b), respectively. The ideal concentration of sweet potato was 250 g per litre (Figure 2(a)). This concentration produced almost three times the number of cells than the standard concentration of potato infusion in Potato Dextrose Media of 200 g per litre. A concentration of dextrose at 20 g per litre demonstrated a significantly better level of growth than glucose restricted (<20 g per litre) and high glucose (>20 g per litre) cultures (Figure 2(b)). The concentration of nutrients in the culture media affected yeast growth. Glucose restriction and high glucose content could induce oxidative stress in the yeast (Francesca et al., 2010) and is capable of damaging important cellular constituents such as DNA, lipids and proteins.

The optimal pH range for yeast growth can vary from 4 to 6, depending on temperature, oxygen concentration, and strain of yeast (Narendranath & Power, 2005). The test yeast in SDB at 25% sweet potato, 2% dextrose, 200 rpm and 25°C presented the highest level of growth with an optimum pH value of 4.6 (Figure 2(c)). Noé Arroyo-López et al. (2009) discovered a similar maximum specific growth rate of *S. cerevisiae* T73 at a pH value of 4.76.

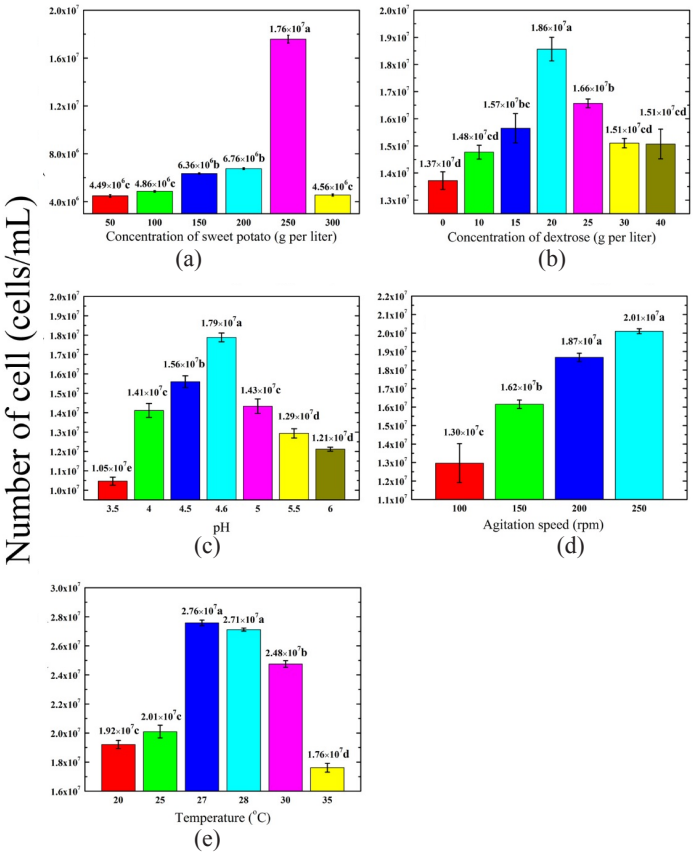

Figure 2. The growth of *Saccharomyces cerevisiae* in SDB at various culture conditions [(a) concentrations of sweet potato, (b) concentrations of dextrose, (c) initial pH values, (d) agitation speeds; and (e) temperatures] over 48 h

Note. A pair of averages with different letters is considered significantly different at $p<0.01$

In aerobic organisms, the two major functions of respiration are oxidation of reduced cofactors and the generation of metabolic energy in the form of ATP. Oxygen is used as the terminal electron acceptor for mitochondrial respiration and for assimilatory oxygenation reactions (Weusthuis et al., 1994). It is an important factor in yeast metabolism, which could clearly be seen in the increase in agitation speed from 100 to 250 rpm that led to an increase in the yeast population (Figure 2(d)). However, agitation speeds of 200 and 250 rpm did not affect the number of yeast cells.

The growth of *S. cerevisiae* in SDB at 25% sweet potato, 2% dextrose, an initial pH value of 4.6 and at 250 rpm at various temperatures is shown in Figure 2(e). The best growth of the test yeast was achieved at 27°C as reported by Pérez-Ramírez et al. (2012) who found that the maximum level of biomass production of *S. cerevisiae* in coconut water medium was obtained at 27°C, while temperatures higher or lower than 27°C caused a decrease in biomass

values. Nevertheless, in this study, the test yeast illustrated the best level of growth at 28°C with no significant difference at 27°C. The lowest level of growth was recorded at 35°C. High temperature stress causes many changes in the yeast cells that can ultimately affect protein structures and functions, accumulate denatured and aggregated biomacromolecules, and give rise to growth inhibition or cell death. It can also disorder the integrity of cell membranes, increase membrane permeability, and affect the plasma membrane fluidity (Zhang et al., 2015).

The growth of molds on agar media assay

The growth of the four test molds on four samples of formulated agricultural crop agar media and commercial PDA over seven days are presented in Figure 3. *Aspergillus flavus* TISTR 3366 grew significantly fastest on SDA and TDA with a growth diameter of 80.35 and 80.26 mm respectively. Rachael and Adebolu (2014) reported a similar best mycelial growth of *A. flavus* in SDA whereby the latter also promoted significantly higher mycelial growth of *Fusarium semitectum* DOAC 1986 and *Penicillium* sp. than on the other formulated agar media, including commercial PDA. *Bipolaris oryzae* DOAC 1760 showed the highest level of growth on TDA. A comparison of mold growth on the formulating agar media with commercial PDA revealed that all of the test molds grew significantly higher on SDA than on commercial PDA, while most of the test molds grew significantly higher on TDA than they did on commercial PDA, except for *F. semitectum* DOAC 1986. However, both SDA and TDA stimulated higher mycelial growth than PDA in all of the test molds. Similar results of fungal growth enhancement were found in oyster mushroom (*Pleurotus cystidiosus*) with Sweet Potato Dextrose Agar (Hoa & Wang, 2015). Sweet potato was excellent in encouraging either the growth of the test yeast or the growth of the test molds. The sticky characteristic of the taro on fungal growth has an effect on the broth media but it does not affect the agar media. The lowest growth of mycelium in all test molds was found on CDA. This was to be expected because of the low vitamin content in cassava. The HCN was also toxic for both the test yeast and molds. Commercial PDA showed higher levels of all test molds growths than PDA.

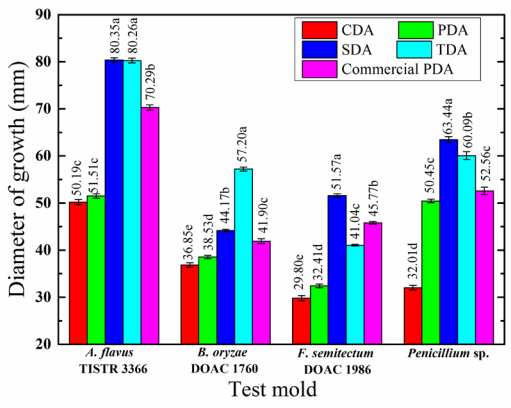

Figure 3. The mycelial growth of test molds on formulating agar media and commercial PDA over 7 days

Note. A pair of averages in each test molds with different letters is considered significantly different at $p<0.01$

CONCLUSION

Our findings showed that sweet potato can be a good alternative source for potato in Potato Dextrose Media, whereas taro can only be used as a substitute for the potato on agar media for yeast and mold cultivation, especially with regard to biomass production. Crops with high content of amylopectin, that cause sticky characteristic, are not appropriate for use in liquid media. Tropical and subtropical countries which has good laboratory facilities can use sweet potato and taro as substitutes for potato in fungal media production.

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Comparisan of Ossicle Shape and 12S rRNA Gene Sequencing Techniques for Species Identification of *Gamat*-based *beche-de-mer* from Langkawi Island, Kedah

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ABSTRACT

Due to the issues of species substitution and product mislabelling of *beche-de-mer* worldwide, this study aimed to identify the species of seven *gamat*-based *beche-de-mer* specimens from Kuah, Langkawi Island, Kedah, Malaysia based on ossicle shapes and non-protein-coding 12S mitochondrial rRNA gene sequences. In general, ossicles were well extracted from the specimens. At least eight ossicle shapes were observed i.e. large I-shaped rod, perforated plate, table, C-shaped rod, button, rosette, I-shaped rod and X-shaped rod. Except for button, I-shaped rod and X-shaped rod, the other five ossicle shapes are common in *Stichopus horrens*. However, the species status could not be resolved at this level due to the presence of uncommon ossicle shapes and the physical features of the specimens could not be used as supporting data as they were different from the live or unprocessed sea cucumber. In this study, 12S mitochondrial rRNA gene sequences were analysed using the Basic Local Alignment Search Tool programme for Nucleotides (blastn), resulting in the species identification of the *beche-de-mer* specimens as *S. horrens*, known locally as *gamat emas* (golden sea cucumber) with 96-99% similarity (an average of 98%). The phylogenetic trees based on the Neighbour-Joining method, Maximum Parsimony method and Maximum Likelihood method indicated that all 12S mitochondrial rRNA gene sequences of the *beche-de-mer* specimens clustered with the reference samples of *S.*

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horrens from Pangkor Laut, Pangkor Island, Perak, Malaysia, supporting the BLASTN results and confirming their species status as *S. horrens*. Furthermore, 10 partial 12S mitochondrial rRNA gene sequences of the reference samples and the *beche-de-mer* specimens of *S. horrens* were registered with the GenBank (Accession No.: KX879628-KX879637). Overall, the findings suggested that the species identification of the *beche-de-mer* specimens using 12S mitochondrial rRNA gene sequence gave better inference than ossicle-shape identification. The outcomes of this study benefit enforcement agencies in their work of monitoring and overcoming the issues of species substitution and product mislabelling of *beche-de-mer* or commercial dried sea cucumber in Malaysian markets as well as in global markets.

Keywords: 12S mitochondrial rRNA gene, *gamat*-based *beche-de-mer*, ossicle shape, phylogenetic trees, *Stichopus horrens*

INTRODUCTION

Sea cucumber (Phylum Echinodermata: Class Holothuroidea) is a marine heritage of Malaysia due to its high value in traditional medicine and the *trepang* or *beche-de-mer* industry (Hashim, 2011). The marine-dwelling organism was categorised as *gamat* and *timun laut* (Kamarudin et al., 2009, 2015). The term *gamat* refers to all species from the family Stichopodidae e.g. *Stichopus herrmanni* (the curryfish) and *Thelenota anax* (the amberfish), while the term *timun laut* refers to all non-*gamat* species e.g. *Bohadschia vitiensis* (the brown sandfish) and *Holothuria (Microthele) fuscopunctata* (the elephant trunkfish). In terms of species richness, Kamarudin et al. (2015) reported the presence of 10 *gamat* species in Malaysia including eight *Stichopus* species and two *Thelenota* species. Furthermore, Choo (2008) reported that at least six *gamat* species are of commercial importance in Malaysia. According to Conand et al. (2014), *Thelenota ananas* (the prickly redfish) and *S. herrmanni* – both *gamat* species were recorded in Malaysia – were included in the International Union for Conservation of Nature (IUCN) Red List for aspidochirotid holothuroids as endangered or at risk of extinction and vulnerable or at risk of extinction, respectively.

In Sabah, Malaysia, *timun laut* and *gamat* species have been exploited as food in the *beche-de-mer* industries. The term *beche-de-mer* refers to sea cucumber that is cooked and dried for commercial purposes (Purcell, 2014). An estimated 139 tonnes of sea cucumber was landed in Sabah from the year 2000 until 2005 (Annual Fisheries Statistics, Sabah, 2000-2005). In contrast to *timun laut*, the *gamat* species is popular as the main ingredient in *gamat*-based traditional products in Peninsular Malaysia e.g. body fluid extracts (*air gamat*) and lipid extracts (*minyak gamat*). *Gamat* is usually cooked and dried into *beche-de-mer*, locally known as *ibu gamat*, before being used as the main ingredient in traditional products. *Ibu gamat* is sold in Malaysian markets. Langkawi Island, Kedah and Pangkor Island, Perak are the two main production

sites of *gamat*-based traditional products in Peninsular Malaysia (Kamarudin et al., 2015). However, between the two islands, Langkawi Island, Kedah is more popular with the *gamat*-based traditional products. Gamat Asli Enterprise is one of the most established producers and distributors of *gamat*-based products in Langkawi Island, Kedah and it has been operating since 1960s (Wariman, 2002). Nowadays, in Malaysia, *gamat*-based products are also manufactured using modern technologies.

A number of 19 sea cucumber species are commercialised in Malaysia (Choo, 2008). Preceding *beche-de-mer* marketing, sea cucumber is gutted, boiled, roasted and finally preserved through drying, smoking or freezing. Pickling and canning are other options that may be used in the extensive processing. Processing usually leads to deformation of the body of the sea cucumber and subsequently, causes difficulties in species identification and confirmation of the processed sea cucumber or *beche-de-mer* in markets through their morphology. There have been cases where sea cucumber-based products were not labelled with their species names and other details such as the name, address and contact information of the manufacturer as well as quantity information. Rasmussen and Morrissey (2008) mentioned that the presence of intentional species substitution and mislabelling of sea cucumber products have been reported worldwide. Wen et al. (2011) reported that seven samples of commercial sea cucumber products from Guangzhou, China (63.6%) were incorrectly labelled. In Malaysian markets, Kamarudin et al. (2015a, 2015b) reported the presence of unlabelled *beche-de-mer* products that could be related to the issue of intentional species substitution. In fact, the global issues have been affecting the trading of processed sea cucumber. In order to address the issues, rapid, reproducible and reliable techniques for identifying the animal origin of biological specimens have been discovered and developed since 1992 (Bartlett & Davidson, 1992; Wen et al., 2011).

Therefore, the aim of this study was to identify the species of *gamat*-based *beche-de-mer* specimens from Kuah, Langkawi Island, Kedah, Malaysia based on ossicle shapes and non-protein-coding 12S mitochondrial rRNA gene sequences. Toral-Granda (2005) has suggested that ossicle, the small part of calcified material from the sea cucumber's body, is informative and useful in identifying the species of sea cucumber in any form e.g. fresh, salted and dried forms. In fact, the ossicle continued to be an important characteristic for morphological identification of sea cucumber species (Kamarudin & Mohamed Rehan, 2015). However, the absence of common ossicle shapes and the presence of uncommon ossicle shapes will cause uncertainty in species status. For that reason, besides the ossicle shape identification technique, 12S mitochondrial rRNA gene sequencing technique was included in this study in order to obtain a more accurate conclusion and comparison. In summary, the outcomes of this study suggest that

the species identification of the *beche-de-mer* specimens using 12S mitochondrial rRNA gene sequence gave better inference as compared to identification based on ossicle shape. Despite that, both techniques are recommended to be used together for accurate species identification and confirmation of processed sea cucumber, in this case the commercial *gamat*-based *beche-de-mer* specimens from Kuah, Langkawi Island, Kedah, Malaysia. National enforcement agencies around the world are recommended to use both techniques for monitoring and overcoming the issues of species substitution and product mislabelling of *beche-de-mer* in global markets.

Figure 1. Specimens of *gamat*-based *beche-de-mer* from Kuah, Langkawi, Kedah, Malaysia supplied by Gamat Asli Enterprise

MATERIALS AND METHODS

Study site and sampling

Seven specimens of *gamat*-based *beche-de-mer* (i.e. in dried form) or *ibu gamat* distributed by Gamat Asli Enterprise were bought in Kuah, Langkawi Island, Kedah, Malaysia [Global Positioning System (GPS) position: 4° 21' 32.53"N, 100° 46' 24.91"E)]. The specimens were labelled LKIG 1 to LKIG 7 (Figure 1) and stored in a -20°C chest freezer for long-term storage with proper cataloguing at the Faculty of Science and Technology (FST), Universiti Sains Islam Malaysia (USIM), Nilai, Negeri Sembilan.

Ossicle extraction and shape observation

The methods outlined by Kamarudin and Mohamed Rehan (2015) were used with little modification. A small piece of tissue (i.e. 20 mg) from each *beche-de-mer* specimen was cut with a sterile blade and then placed on a glass microscope slide. Several drops of liquid household bleach were applied onto the tissue portion to dissolve away the soft tissue. The mixture was left at room temperature for 30 min until a white pellet of ossicles was formed in the liquid solution. The ossicle shapes were observed at 400x magnification using Olympus culture microscope model CKX41. During the observation, the images of the ossicle shapes were captured and recorded for morphological identification. The main focus in this study was to identify the shapes of ossicles; therefore, the definite microscopic size of each ossicle type was not entirely counted (Figure 2).

Total genomic DNA extraction

A 200 mg tissue from each *beche-de-mer* specimen was disrupted and homogenised using the QIAGEN TissueRuptor prior to

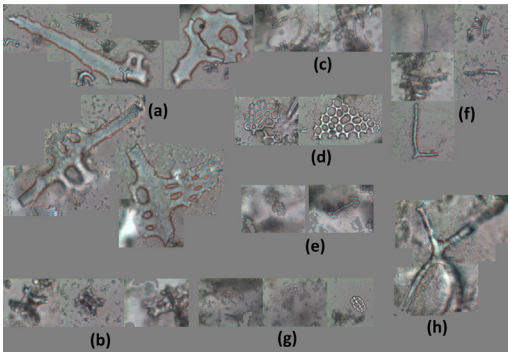

Figure 2. Ossicle shapes in the *gamat*-based *beche-de-mer* specimens from Kuah, Langkawi, Kedah, Malaysia. (a) Large I-shaped rods, (b) tables, (c) C-shaped rods, (d) perforated plates, (e) rosettes, (f) small rods, (g) buttons, and (h) X-shaped rod

the total genomic DNA extraction using the DNeasy mericon Food Kit by QIAGEN. The protocol was modified in order to obtain a better yield of total genomic DNA. The approximate yield of the total genomic DNA was determined using 1% agarose gel with FloroSafe DNA Stain through horizontal gel electrophoresis. The total genomic DNA extracts were stored in a -20°C chest freezer for long-term storage.

Polymerase chain reaction (PCR)

Non-protein-coding 12S mitochondrial rRNA gene was amplified through standard PCR procedures using the 2x TopTaq Master Mix Kit by QIAGEN [~360 bp of fragment length based on Palumbi et al. (1991)].

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

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

The PCR run was programmed for 35 cycles. Cycle parameters for the PCR run were set for 2 min at 95°C for initial denaturation, 30 s at 95°C for denaturation, 30 s at 50.3°C for annealing, 45 s at 72°C for extension, repetition of step 2-4 for another 34 cycles and 5 min at 72°C for final extension.

PCR product purification and DNA sequencing

The PCR products were purified using the 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). The purified PCR products were sent for DNA sequencing at the First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor, Malaysia.

Phylogenetic analyses and GenBank submission

Sequenced 12S mitochondrial rRNA gene PCR products were displayed using Chromas programme version 2.5.1 (Copyright© 1998-2016 Technelysium Pty Ltd). Online Basic Local Alignment Search Tool programme for Nucleotides (blastn) was then accessed to assign each DNA sequence obtained from this study to a particular sea cucumber species or genus. For the multiple sequence alignment of forward reaction sequences, ClustalX programme version 2.1 (Thompson et al., 1997) was used prior to the phylogenetic tree reconstruction.

The reconstruction of a Neighbour-Joining (NJ) tree, Maximum Parsimony (MP) tree and Maximum Likelihood (ML) tree were computed using Molecular Evolutionary Genetics Analysis 6 programme (MEGA6) (Tamura et al., 2013). In addition, a 12S mitochondrial rRNA gene sequence of *Holothuria (Mertensiothuria) leucospilota* from Teluk Nipah, Pangkor Island, Perak (HL1, GenBank Accession No.: KX768273) was included as the outgroup while three 12S mitochondrial rRNA gene sequence of morphospecies *Stichopus horrens* from Pangkor Laut, Pangkor Island, Perak (SHP1-SHP3) were incorporated as the commercial species standard or reference samples. In contrast to the specimens of *beche-de-mer* from Kuah, Langkawi, Kedah, the total genomic DNA of the reference samples was extracted from their fresh tissues i.e. unprocessed samples. Regarding the GenBank submission, Sequin version 15.10 programme was used to prepare sequence data in the submission entry in order to obtain the accession numbers from the GenBank, National Center for Biotechnology Information (NCBI), U. S. National Library of Medicine.

RESULTS AND DISCUSSION

The ossicles from the commercial *beche-de-mer* specimens from Kuah, Langkawi Island, Kedah, Malaysia were successfully extracted even though the specimens underwent extensive processing including gutting, boiling, roasting and subsequent preservation procedures for storage prior to marketing and consumption. Despite the fact that the processes caused body deformation and this led to difficulties for clear species identification and confirmation on the basis of morphology, the ossicles were still observable. At least eight ossicle shapes were microscopically observed without the size measurement i.e. large I-shaped rod, perforated plate, table, C-shaped rod, button, rosette, I-shaped rod and X-shaped rod (Figure 2, Table 1). According to Selenka (1867), the large I-shaped rod, perforated plate, table, C-shaped rod and rosette (Figure 2(a)-2(e)) were common ossicle shapes in *S. horrens*. All the common ossicles were

Table 1
List of ossicle shapes in the gamat-based beche-de-mer specimens from Kuah, Langkawi, Kedah

Specimen	Large I-shaped rod	Table	C-shaped rod	Perforated plate	Rosette	I-shaped rod	Button	X-shaped rod
LKIG 1	√	√	√	√	x	x	√	x
LKIG 2	√	√	√	x	√	√	x	x
LKIG 3	√	√	√	√	√	√	√	x
LKIG 4	√	√	x	√	x	√	x	x
LKIG 5	√	√	√	√	√	x	√	x
LKIG 6	√	√	√	√	√	√	x	√
LKIG 7	√	√	√	x	√	√	√	√

present in LKIG 3, LKIG 5 and LKIG 6 while the other four specimens showed the absence of one or two common ossicle shape(s) (Table 1). The absence could have been due to the effects from the *beche-de-mer* processing methods. Besides that, some ossicle shapes could be overlooked during the microscopic observation.

Interestingly, three ossicle shapes i.e. button, I-shaped rod and X-shaped rod were also recorded in this study (Figure 2(f)-2(h)). The button shape was observed in LKIG 1, LKIG 3, LKIG 5 and LKIG 7; the I-shaped rod in LKIG 2, LKIG 3, LKIG 4, LKIG 6 and LKIG 7; and the X-shaped rod in LKIG 6 and LKIG 7. Kamarudin and Mohamed Rehan (2015) also recorded the presence of the I-shaped rod in the tentacles and respiratory trees of *S. horrens* specimens from Pangkor Island, Perak, Malaysia as well as the X-shaped rod in the tentacles and gastrointestines of the species. However, the button shape was not listed. As a result, the species status of the *commercial beche-de-*mer specimens was hard to be confirmed at this level due to the absence of the common ossicle shapes and the presence of the uncommon ossicle shapes. It was also difficult to identify the specimens due to their deformed physical appearance that was different from the live or unprocessed sea cucumbers.

The blastn results for the non-protein-coding 12S mitochondrial rRNA gene sequences of the *beche-de-mer* specimens demonstrated that the specimens were identified as *S. horrens* with 96-99% similarity i.e. an average of 98% similarity. All the partial 12S mitochondrial rRNA gene sequences of the *beche-de-mer* specimens of *S. horrens* were registered with the GenBank (Accession No.: KX879630 - KX879637). In Peninsular Malaysia, *S. horrens* or the dragonfish is well known locally as *gamat emas* or the golden sea cucumber (Kamarudin et al., 2009; 2015). In terms of the multiple sequence alignment of the forward reaction sequences against the outgroup using the ClustalX programme version 2.1 (Figure 3), the results showed that 21 out of 356 base positions were found to contain variable bases within the sequences of the *beche-de-mer* specimens. Eighteen base positions presented transversion, two base positions showed transition and one base position demonstrated an insertion. Interestingly, there were 13 variable bases in the sequence of LKIG7 specimen consisting of one transition (i.e. adenine to thymine), one insertion of adenine and 11 transversions. Despite that, the blastn result identified it as *S. horrens* with 96% similarity.

Regarding the phylogenetic analyses, the tree reconstruction based on the distanced-based NJ method with clustering algorithm, along with the character-based MP method and the character-based ML method both with optimality criterion, involved 11 nucleotide sequences including 10 in-groups and an out-group to root each tree. All positions containing gaps and missing data were eliminated. As a result, there were a total of 346 positions in the final dataset. The optimal NJ tree with

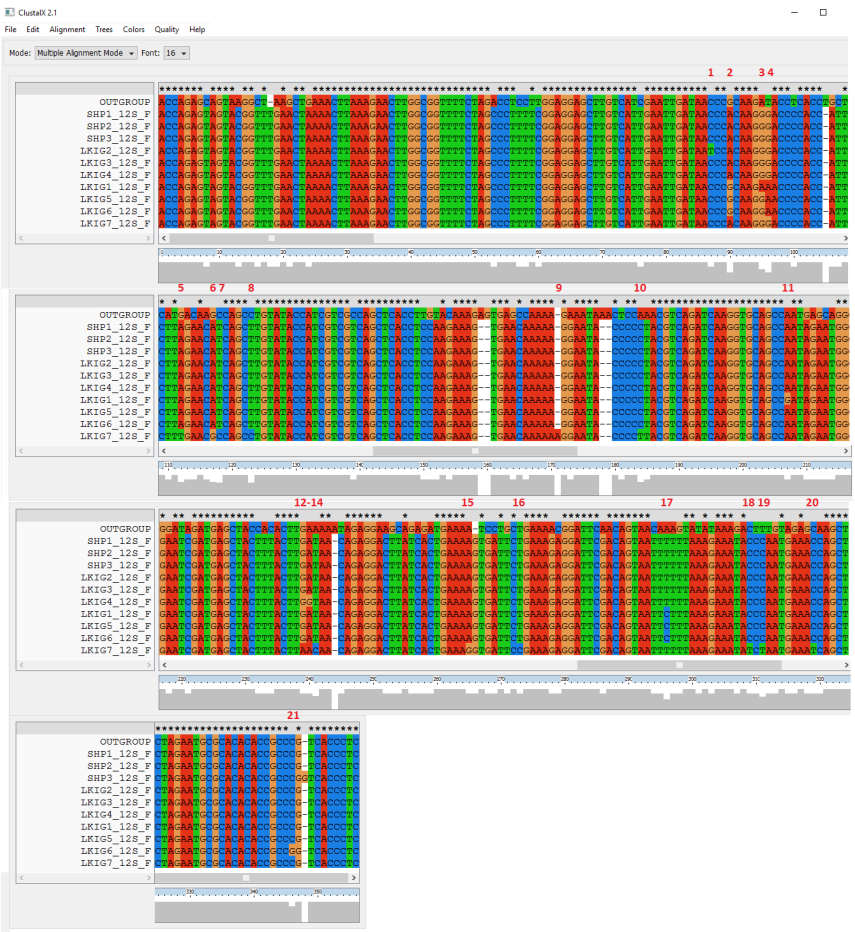

Figure 3. Multiple sequence alignment of forward reaction sequences of *gamat*-based *beche-de-mer* specimens from Kuah, Langkawi, Kedah, Malaysia using ClustalX programme version 2.1 (Thompson et al., 1997). Twenty-one out of 356 base positions, highlighted in red numbers, were found to contain variable bases

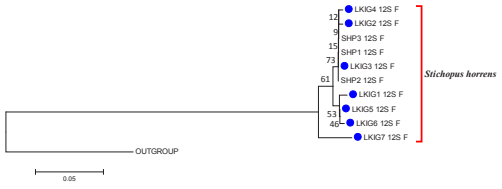

Figure 4. The phylogenetic inference of *gamat*-based *beche-de-mer* specimens from Kuah, Langkawi, Kedah, Malaysia using the Neighbour-Joining method (Saitou & Nei, 1987)

the sum of branch length=0.38155689 is shown in Figure 4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as the trees' evolutionary distance used to infer the phylogenetic tree. Evolutionary distance was computed using the Maximum

Composite Likelihood method (Tamura et al., 2004) and is expressed as the number of base substitutions per site. For the MP tree (Figure 5), the bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [pg. 126 in ref. (Nei & Kumar, 2000)], with search level 1, in which the initial trees were obtained by the random addition of sequences (i.e. 10 replicates). Moreover, the ML tree with the highest log likelihood (-844.0290) is shown in Figure 6. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. Like the NJ tree, the ML tree was drawn to scale, but with branch lengths measured in the number of substitutions per site.

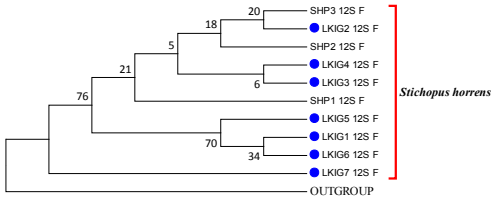

Figure 5. The phylogenetic inference of *gamat*-based *beche-de-mer* specimens from Kuah, Langkawi, Kedah, Malaysia using the Maximum Parsimony method

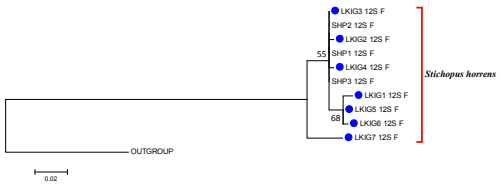

Figure 6. The phylogenetic inference of *gamat*-based *beche-de-mer* specimens from Kuah, Langkawi, Kedah, Malaysia using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993)

The phylogenetic trees indicated that all 12S mitochondrial rRNA gene sequences of the *gamat*-based *beche-de-mer* specimens clustered with the reference samples of *S. horrens* from Pangkor Laut, Pangkor Island, Perak, Malaysia (Figures 4-6). All the partial 12S mitochondrial rRNA gene sequences of the reference samples of *S. horrens* were also registered with the GenBank (Accession No.: KX879628 - KX879630). Previously, Kamarudin and Mohamed Rehan (2015) had verified the species status of the reference samples as *S. horrens* using the cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene sequencing technique. It was claimed that in size, *S. horrens* from Langkawi Island, Kedah was physically smaller than *S. horrens* from Pangkor Island, Perak due to environmental pollution and stress (Ibrahim, 2004); however, the phylogenetic analyses carried out in this study showed that the in-groups were of a single species i.e. *S. horrens* without genetic difference by geography. Hence, the results of the phylogenetic analyses suggest the species of the commercial *gamat*-based *beche-de-mer*

specimens from Kuah, Langkawi Island, Kedah, Malaysia to be *S. horrens*.

Since the *gamat*-based *beche-de-mer* specimens in this study underwent body deformation, their physical features could not be used to identify their sea cucumber species as they were not completely similar to the original morphological features. Therefore, the ossicle shape was used for species identification. However, species identification of the specimens based on ossicle shape itself could not be fully resolved. Even though there were common ossicle shapes of *S. horrens* in the microscopic observation, an additional approach was still required to support and then confirm species identity due to the presence of additional ossicle shapes. This study also suggested that species identification of the processed sea cucumbers using 12S mitochondrial rRNA gene sequences gave a more accurate conclusion compared to identification by ossicle shape, suggesting that 12S mitochondrial rRNA gene sequencing can be a suitable additional approach for identifying sea cucumber species. This study also discovered the potential of the 12S mitochondrial rRNA gene sequencing technique for use by enforcement agencies to resolve the issues of species substitution and product mislabelling of processed sea cucumber in Malaysia as well as throughout the world. Effective maternal inheritance, apparent haploid genome, considerable non-recombination, continuous replication and greater rate of substitution compared to the 'single-copy' nuclear has made mitochondrial DNA, including the 12S mitochondrial rRNA gene, the main interest in genetic studies (Nabholz et al., 2008). By taking into account the observation of five common ossicle shapes of *S. horrens* in the specimens, both the non-protein-coding 12S mitochondrial rRNA gene sequencing along with the ossicle shape observation can be used to provide better resolution of species status of *beche-de-mer* specimens.

In general, the specimens of *gamat*-based *beche-de-mer* or *ibu gamat* distributed by the Gamat Asli Enterprise are *S. horrens*, the Langkawi *gamat* or *gamat emas*, which contain high therapeutic value, including the capabilities to hasten wound healing and rejuvenate tissues (Hashim, 2011). The company is one of the most established producers and distributors of gamat-based products in Langkawi Island (Wariman, 2002). However, sea cucumber ranching being carried out by the Langkawi Development Authority (LADA) in Teluk Yu, Temoyong, Langkawi and Tuba Island, Langkawi uses *Holothuria (Metriatyla) scabra* instead of *S. horrens* (Sharif & Osman, 2016). *H. scabra* is regarded as one of the *timun laut* species (Kamarudin et al., 2015) but some people in Malaysia also regard it as a *gamat* species. According to Choo (2008), *S. horrens* and *H. scabra* are among Malaysia's commercial species of sea cucumber. However, the sandfish is regarded as "endangered, or at a high risk of extinction" based on the IUCN Red List for aspidochirotid holothuroids (Conand et al., 2014). The use of *H. scabra* in sea ranching

could be due to its well-studied and well-developed hatchery and culture techniques that are used worldwide.

CONCLUSION

Ossicles from the commercial *beche-de-mer* specimens from Kuah, Langkawi Island, Kedah, Malaysia were extracted with at least eight ossicle shapes i.e. large I-shaped rod, perforated plate, table, C-shaped rod, button, rosette, I-shaped rod and X-shaped rod. The first five shapes are common in *S. horrens*. Nonetheless, the absence of one or two common ossicle shape(s) and the presence of additional uncommon shapes required the use of an additional species identification approach. In terms of species status and genetic relationship based on the non-protein-coding 12S mitochondrial rRNA gene sequence, the blastn results suggested that the *beche-de-mer* specimens were *S. horrens* (i.e. *gamat emas*) with an average of 98% similarity. In total, 10 partial 12S mitochondrial rRNA gene sequences of *S. horrens* resulting from this study were registered with the GenBank, NCBI, U. S. National Library of Medicine (Accession No.: KX879628-KX879637). The phylogenetic trees of NJ, MP and ML demonstrated that all 12S mitochondrial rRNA gene sequences of the *beche-de-mer* specimens clustered with the reference samples of *S. horrens* from Pangkor Laut, Pangkor Island, Perak, Malaysia, confirming their species status as *S. horrens*. Besides this, the findings suggest that species identification of the *beche-de-mer* specimens using 12S mitochondrial rRNA gene sequence gave better inference compared to the ossicle shape. Despite that, species identification by means of the non-protein-coding 12S mitochondrial rRNA gene sequencing along with the ossicle shape observation gave better resolution in species identification and status confirmation. For monitoring and overcoming the issues of species substitution and product mislabelling of processed sea cucumber in Malaysian markets and also in global markets, this study showed the potential of 12S mitochondrial rRNA gene sequencing along with ossicle shape observation for use by enforcement agencies for the purpose of monitoring and overcoming the issues of species substitution and product mislabelling of *beche-de-mer* or commercial sea cucumber in Malaysian markets as well as in global markets.

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the Sea Cucumber (Echinodermata: Holothuroidea) Database at http://sites.google.com/site/malaysianseacucumber/.

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The Role of Heritability and Genetic Variability in Estimated Selection Response of Soybean Lines on Tidal Swamp Land

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ABSTRACT

Selection response is affected by genetic variability and heritability. High selection response is achieved by broad genetic variability and or high heritability. The objective of this study is to estimate the selection response of soybean lines. Forty soybean lines derived from "Sinabung" × MLGG 1087 cross were grown in tidal swamp land in Barito Kuala, South Kalimantan. Broad genetic variability was shown by seven agronomical characters, such as days to flowering and maturing, number of branches per plant, number of reproductive nodes per plant, number of filled pods per plant, weight of 100 grains, and grain yield. Narrow genetic variability was shown by plant height. High heritability was shown by days to flowering (0.923), days to maturity (0.896) and weight of 100 grains (0.762); where their selection responses were 4.34 days, 3.53 days and 1.21 g, respectively. Moderate heritability was shown by plant height (0.435) and number of filled pods per plant (0.226) with the selection response of 6.41 cm and 1.82 pods. Low heritability was shown by number of branches per plant (0.121), number of reproductive nodes per plant (0.160) and grain yield (0.056) that lead selection response of 0.10 branch, 0.58 nodes and 0.03 t/ha grain. In this study, the characters with high genetic variability but low heritability produced low selection response. It indicates that the role of heritability was greater than genetic variability.

Keywords: Acid soil, genetic variability, heritability, selection response, soybean, tidal swamp land

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INTRODUCTION

Soybean ranks as the third most important food crop in Indonesia, after rice and maize. Soybean is used to make tempeh and tofu. The average demand of soybean is estimated to be 2.2 million tons every year

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(Kementerian Pertanian, 2015). In 2015 total area where soybean was harvested was 614,095 ha, a decrease of 1,590 ha or 0.26% from 2014 (BPS, 2016). Soybean demand in 2019 is expected to be 3.25 million tons (Bapennas, 2013) and due to constraints in available land suboptimal land such as swamp area covering 33.4 million ha, which includes of 20.19 million ha of tidal and 13.28 million ha of water logged areas are seen as the alternative (Alihamsyah et al., 2003).

Tidal swamp land is suboptimal land (acidic Organosols) where issues such as macronutrients deficiency and micronutrients toxicity (von Uexkull & Mutert, 1995) need to be addressed. Soil amelioration and developing tolerant plant are two techniques to overcome some of the challenges, of which developing and cultivating tolerant plant is the more affordable. The objective of the research in the present work was to compare the role of genetic variability and heritability in estimated selection response of soybean lines on tidal swamp land.

MATERIALS AND METHODS

The study was carried out from February to May 2012 on tidal swamp land type C in Wanaraya, Barito Kuala, South Kalimantan. Tidal swamp land type C is a type of swamp land, where there is no water logging even in high tidal phase and water table level that is less than 50 cm below the soil surface (Alihamsyah et al., 2003). The soil properties for the site was acidic as indicated by very low pH and high Al saturation. Exchangeable aluminum (Al_{ex}) and hydrogen (H_{ex}) were 9.65 me/100 g and 0.46 me/100 g, respectively resulting very high Al saturation of 79.09% (Table 1). Organic C is very high in this peat soil. These soil properties, especially Al saturation, are appropriate for determining tidal swamp land-tolerance for soybean planting. The design was randomized completely block with three replications. Forty populations of F7 derived from crossing between "Sinabung" and genotype of MLGG 1087 - "Sinabung" is a high yielding variety in optimal condition, while MLGG 1087 is a land race that adaptive in tidal swamp land.

Before planting, weed control measures were adopted to eradicate the weeds, and the minimum tillage was applied by plowing and leveling the soil. Every soybean line

Table 1
Soil properties

Soil properties	Value
pH (H2O)	4.40
pH (KCl)	4.37
N (Kjedahl) (%)	0.70
C organic (Kurnis) (%)	8.05
P2O5 (Bray I) (ppm)	81.50
SO4 (ppm)	144.00
Fe (ppm)	471.83
Zn (ppm)	2.44
Cu (ppm)	1.89
K (me/100 g)	0.25
Na (me/100 g)	0.40
Ca (me/100 g)	0.97
Mg (me/100 g)	0.47
CEC (me/100 g)	103.00
Al exchangeable (me/100 g)	9.65
H exchangeable (me/100 g)	0.46

was grown in a plot of 1.6 m × 3 m size with planting space of 0.4 m × 0.15 m. Fertilizers of 125 kg/ha Urea, 250 kg/ha SP36 and 150 kg/ha KCI were applied two times, at sowing time with dosages of 50 kg/ha Urea, 250 kg/ha SP36 and 150 kg/ha KCI, while the rests were applied when the plants began to bloom. Two and four weeks after planting, manual weeding was done in order to control weed growth. The presence of pests and diseases were intensively monitored and harvesting was carried out when plants reached physiological maturity.

The temperature at the experiment site ranged between 26.8-27.5°C with an average minimum temperature 24.1 °C and maximum temperature 32.2°C. The relative humidity and rainfall ranged between 80.6-85.3% and 126.3-402.8 mm with the highest relative humidity and rainfall recorded in February and April respectively (Table 2).

The data was analysed by PKBT-STAT 1.0; expected mean squares, genoytpic coefficient of variance (GCV) was calculated (Singh & Chaudhary, 1979). Genetic variability criteria were classified based on genotypic standard deviation ($\sigma_{\sigma_g^2}$) according to Anderson and Bancroft in Wahdah et al. (1996). The broad genetic variability was achieved when GCV ≥ 2 $\sigma_{\sigma_g^2}$, whilst narrow genetic variability was achieved when GCV $< 2\sigma_{\sigma_g^2}$. Selection response was estimated according to Falconer (1989) as $SR = i \cdot h^2 \cdot SD$; where SR = selection response, i = selection intensity, h^2 = heritability, SD = standard deviation.

Table 2
Weather data from February to May 2012

	Minimum temperature (°C)	Maximum temperature (°C)	Average temperature (°C)	Relative humidity	Rainfall (mm) (%)
February	24.2	31.3	26.8	85.3	155.7
March	24.1	31.9	26.9	84.3	263.6
April	24.2	33.0	27.2	84.4	402.8
May	24.1	33.1	27.5	80.6	126.3
Average	24.1	32.3	27.1	83.6	237.1

RESULTS AND DISCUSSION

Analysis of variance for phenotypic performance of the observed agronomical characters showed that six characters were significantly different (Table 3). Six characters that differed significantly were days to flowering, days to maturity, plant height, number of reproductive nodes per plant, number of filled pods per plant and weight of 100 grains. To measure genotypic performance, the data was studied by eliminating environmental factor. Hence, the non significant calculation based on analysis of variance on number of branches per plant and grain yield does not indicate no genetic variability.

Table 3
Analysis of variance of agronomical characters of 40 soybean lines

Character	MS_g	MS_e
Days to flowering (day)	21.49**	0.58
Days to maturity (day)	15.11**	0.56
Plant height (cm)	210.20*	63.43
Number of branches per plant	0.89^{ns}	0.63
Number of reproductive nodes per plant	12.83*	8.17
Number of filled pods per plant	63.38**	33.81
Weight of 100 grains (g)	2.44**	0.23
Grain yield (t/ha)	0.20^{ns}	0.17

**significant at level of 1%, *significant at level of 5%, nsnot significant at level ≤5%

The average of days to flowering reached 41 (Table 4). The earliest days to flowering was 35 days and the longest 46 days. Similar pattern was shown with regards to days taken to reach maturity, and which averged 84 days. There were two soybean lines where maturity was lower than 80 days, i.e. Snb/1087-210-4-8 (79 days) and Snb/1087-119-2-6 (78 days). These two lines are prospective choices for developing early maturity soybean variety.

Table 4
Range, average and standard deviation of agronomical characters of 40 soybean lines

Character	Range	Average
Days to flowering (day)	35.0 – 45.7	41.4
Days to maturity (day)	78.3 – 87.0	84.1
Plant height (cm)	28.2 – 70.1	49.5
Number of branches per plant	0.6 – 3.2	2.3
Number of reproductive nodes per plant	5.7 – 15.3	11.0
Number of filled pods per plant	7.1 – 29.3	17.1
Weight of 100 grains (g)	5.26 – 9.12	7.45
Grain yield (t/ha)	0.26 – 1.43	0.70

Plant height ranged between 30 – 70 cm with the average being 49.5 cm (Table 4). The highest number of plants was about 45 cm which might be due to the high acidity suppressing plant vegetative growth. Verde et al. (2013) reported plant height is suppressed due to high acidity. In this study number of branches ranged between 1 – 3 branches per plants. Number of reproductive nodes per plant is also often affected by branches, where more branch nodes and branch reproductive nodes are produced from greater branch dry matter per plant (Carpenter & Board, 1997).

Grain yield is the most important character in soybean, and average of grain yield was 0.70 t/ha ranging from 0.26 – 1.43 t/ha (Table 4). There were four soybean lines having grain yield higher than 1 t/ha, i.e. Snb/1087-210-4-13 and Snb/1087-238-1-1 (1.1 t/ha), Snb/1087-210-4-9 (1.20 t/ha), and Snb/1087-159-4-4 (1.43 t/ha). Grain yield is a complex trait that has relationship with other yield components, especially number of filled pod and grain size. In this study,

number of filled pods per plant ranged 7.1 – 29.3 pods with average of 17.1 pods per plant. The highest number of filled pods per plant was reached by Snb/1087-148-2-10 (29 pods) and Snb/1087-210-4-7 (27 pods). One of the four soybean lines (line of Snb/1087-210-4-13) with highest grain yield, also had relatively high number of filled pod per plant (23 pods per plants). In this study one of the four soybean lines with the highest grain yield, Snb/1087-159-4-4, also had the highest grain size (9.12 g/100 grains). Therefore, these two lines are very prospective for developing high yielding soybean variety for tidal swamp land tolerance.

In analysis of variance, environmental factor is included in calculating the variance. Separation environmental factor is needed to ascertain genotypic performance such as genotypic coeficient variance (GCP) and heritability. Based on the genetic standard deviation ($\sigma_{\sigma_g^2}$), all of the observed characters had broad genetic variability, except plant height that had narrow genetic variability (Table 5). The broad GCV allows high selection response, as reported in Barma et al. (2012) that the crosses with high genetic variability tended to produce high selection response because genetic variability is calculated from genetic variance. The GCV values were lower than phenotypic coefficient of variation (PCV) values for all observed charcaters (Table 5). The difference between PCV and GCV values varied among the observed characters. The characters of days to flowering, days to maturity, and weight of 100 grains achieved closer values between these two parameters. The presence of wider adaptability of the closer values between PCV and GCV indicate the low environmental factor influencing the characters (Dilnesaw et al., 2013; Reni and Rao, 2013; Nidhi et al., 2015; Eka & Lal, 2016). Therefore, this closer values is important because phenotypic expression indicates its genotypic value.

The characters: days to flowering, days to maturity and weight of 100 grains

Table 5
Phenotypic and genotypic coefficient of variance and variability criteria of agronomical characters of 40 soybean lines

Character	PCV	GCV	$\sigma_{\sigma_g^2}$	Criteria
Days to flowering (day)	6.641	6.381	1.583	Broad
Days to maturity (day)	2.767	2.620	1.114	Broad
Plant height (cm)	21.407	14.126	17.004	Narrow
Number of branches per plant	36.056	12.538	0.096	Broad
Number of reproductive nodes per plant	28.439	11.367	1.310	Broad
Number of filled pods per plant	38.662	18.368	5.990	Broad
Weight of 100 grains (g)	13.202	11.525	0.181	Broad
Grain yield (t/ha)	60.219	14.194	0.024	Broad
Grain yield (t/ha)	60.219	14.194	0.024	Broad

showed the highest heritability, i.e. 0.923, 0.896 and 0.762 respectively (Table 6). Some authors also reported high heritability on days to flowering (Aditya et al., 2011), days to maturing (Kuswantoro, 2012; Ojo & Ayuba, 2016), and weight of 100 grains (Aditya et al., 2011). On the other hand, some studies reported lack of consistency in heritability values on the matter of weight of 100 grains (Aditya et al., 2011; Alt et al., 2002; Johnson et al., 2001; Kuswantoro et al., 2006, 2013; Reni & Rao, 2013). This inconsistency may be due to the different genotypes, environment, and interaction between genotypes and environments.

Plant height and number of filled pods per plant showed moderate heritability (Table 6). In optimal soil condition, some studies reported high heritability for plant height (Malek et al., 2014; Reni & Rao, 2013; Yadav et al., 2015) and number of pods (Adsul & Monpara, 2014). The difference heritability between soil acid condition and optimal may be due to the suppressing plant height by acid soil condition. Moderate heritability on plant height and number of filled pods suggests that genetic factor and environmental factors have an effect on these two characters.

Low heritability was indicated by the number of branches per plant, number of reproductive nodes per plant, and grain yield (Table 6). Low heritability on these three characters suggests environmental factors have high role in expressing these characters. However, some studies reported that number of branches per plant had high heritability (Aditya et al., 2011; Reni & Rao, 2013). However, low and moderate heritability for two different populations was obtained by Wiggins (2012), and high heritability on grain yield in Aditya et al. (2011) and Adsul and Monpara (2014). Low heritability indicates that environmental factor has a higher role than genetic factors. The inconsistent heritability value on grain yield may be due to the different populations.

High heritability values were shown by number of days taken for plants to flower, period to reach maturity and the weight of 100 grains. These three characters will affect

Table 6
Standard deviation, heritability and selection response of agronomical characters of 40 soybean lines

Character	Standard deviation	H_{bs}	$SR_{1.755}$
Days to flowering (day)	2.68	0.923	4.34
Days to maturity (day)	2.24	0.896	3.53
Plant height (cm)	8.39	0.435	6.41
Number of branches per plant	0.49	0.121	0.10
Number of reproductive nodes per plant	2.07	0.160	0.58
Number of filled pods per plant	4.59	0.226	1.82
Weight of 100 grains (g)	0.90	0.762	1.21
Grain yield (t/ha)	0.26	0.056	0.03

selection response (Table 6). Selection responses for these three characters were 4.34 days, 3.53 days and 1.21 g/100 grains. It means that one cycle of selection will decrease 4.34 days and 3.53 days for days to flowering and days to maturing respectively, and increase 1.21 g/100 grains for weight of 100 grains. These values are the highest values that can be achieved in this population because the heritability factor is of the broad heritability kind that is still included epistatic effect. This selection response is selected individually based on the single character. In grain yield, the selection response is 0.03 t/ha in one cycle. This selection response was supported by selection response of number of filled pods and weight of 100 grains, due to the relationship between grain yield with number of filled pods and weight of 100 grains.

CONCLUSION

The offspring of "Sinabung" × MLGG 1087 achieved broad genetic variability with regards to days taken to flower and reaching maturity, number of branches per plant, number of reproductive nodes per plant, number of filled pods per plant, weight of 100 grains, and grain yield (t/ha). The broad genetic variability of a character is expected to produce high selection response. In this study, not all of characters with high genetic variability produced high selection response due to the low heritability. The role of heritability in selection response was higher than genetic variability. The characters with high heritability reached higher selection response and per se.

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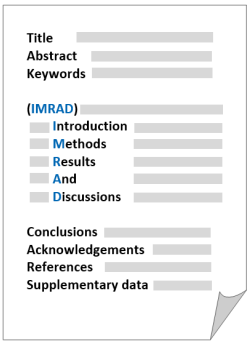

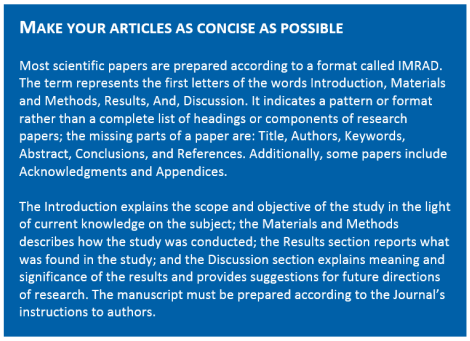

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