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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.

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

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

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

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

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

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

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

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

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

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

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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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Short Communication

Foreword

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

The 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 to benefit the science community.

This issue contains **20 articles**, consisting of **two** review papers, a short communication and **17** regular papers. The authors come from different countries, namely **Malaysia**, **Iran**, **Thailand**, **Nigeria**, **Bangladesh** and **Indonesia**.

The first review paper discusses the micropropagation of rodent tuber plant in Medan (*Typhonium flagelliforme* Lood.) by organogenesis (*Nesti, Merry, Chelen and Bahtiar*), while the second examines the signalling behaviour of abscisic acid on physiological activities in plants under stress (*Naimah, N. and Jahan, M. S.*). Rafidah, O., Firdaus-Nawi, M., Sitti Raehanah, M. S., Ina-Salwany, M. Y., Ching, F. F., Abidin, N. A. and Zamri-Saad, M. in their short communication discuss an outbreak of *vibrio alginolyticus* infection in juvenile sea cucumbers *Holothuria scabra* in Sabah.

The 17 regular papers cover a wide range of topics. In the first research paper, in-vitro antioxidant potential in leaf, stem and bark of *Azadirachta indica* (*Abdulaziz Rabiu Abdulkadir, Nashriyah Mat, and Md Sarwar Jahan*) is discussed while the next paper examines the effect of elemental sulphur application rates on soil Al3+ solubility and its concentration in maize plants (*Zea mays* L.) (*Karimizarchi, M., Aminuddin, H., Khanif, M. Y. and Radziah, O.*). The other papers focus on: adding L-Tyrosine to improve betalain production in red pitaya callus (*Fadzliana, N. A. F., Rogayah, S., Shaharuddin, N. A. and Janna, O. A.*); isolation and characterisation of ascomycetes isolated from *Eurycoma longifolia* Jack

and Malay traditional vegetables (*Nur Ain Izzati, M. Z., Nur Adni, M. and Mohd Razik, M.*); interaction between the long-tailed macaque and the dung beetle in Langkawi (*Muhaimin, A. M. D., Aifat, N. R., Abdul-Latiff, M. A. B., Md. Zain, B. M. and Yaakop, S.*); enhancement of performance of farmed buffaloes pasture management and feed supplementation in Sabah (*Zamri-Saad, M., Azhar, K., Zuki, A. B., Punimin, A. and Hassim, H. A.*); weed seed bank of parthenium weed (*Parthenium hysterophorus* L.) in Batang Kali, Selangor, Malaysia (*Karim, S. M. R., Nurzafirah, Z. and Norhafizah, M. Z.*); micropropagation of *Dendrobium signatum* Rchb.f. (*Khwanduean Rattana and Supavee Sangchanjiradet*); morphological and molecular characterisation of *Campylocarpon fasciculare* and *Fusarium* spp., the cause of black disease of grapevine in Iran (*Khosrow Chehri*); food and feeding habits and allometric relationship of *Synodontis schall* in Lower Ogun River, Akomoje, Ogun State, Nigeria (*F. I. Adeosun, I. Abdulraheem, B. T. Adesina and O. M. Amrevuawho*); comparative evaluation of growth functions in three broiler strains of chicken in Nigeria (*Adenaike, A. S., Akpan, U., Udoh, J. E., Wheto, M., Durosaro, S. O. Sanda, A. J. and Ikeobi, C. O. N.*); short-term heat exposure effect on PSII efficiency and growth of rice (*Oryza sativa* L.) (*Dongsansuk, A., Theerakulpisut, P.* and *Pongdontri, P.*); *Pleurotus ostreatus*: Its effect on Carcass, serum metabolites and meat lipoprotein content of broiler chicken (*Ekunseitan, D. A., Ekunseitan, O. F., Odutayo, O. J. and Adeyemi, P. T.*); nutritional values and amino acid profile of *Clinacanthus nutans* (belalai gajah/ sabah snake grass) from two farms in Negeri Sembilan (*Kong, H. S. and Abdullah Sani, N.*); integrated management of stem canker and black scurf of potato (*Md. Matiar Rahman, Md. Ayub Ali, Tapan Kumar Dey, Md. Monirul Islam and Laila Naher*); characterisation of phytochemical compounds and antimicrobial activity of crude alkaloid from papaya (*Carica papaya* L. var. Eksotika) leaf extract (*Keong Bun Poh and Phua Wan Jien*); and isolation and characterisation of genotype VII Newcastle disease virus from NDV vaccinated farms in Malaysia (*Aljumaili, O. A., Yeap, S. K., Omar, A.,R. and Aini, I.*).

The articles in this issue are intriguing, thought-provoking and useful in reaching new milestones in your own research. There, we urge you to recommend the JTAS to your colleagues and students.

I also take the opportunity 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 is appreciated.

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

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Review Article

Micropropagation of Rodent Tuber Plant (*Typhonium flagelliforme* Lodd.) from Medan by Organogenesis

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ABSTRACT

Rodent Tuber is an anticancer herbal plant from Araceae family which is very sensitive to environmental condition and has a low plantlet reproduction rate. This research was aimed to an obtain effective method of micropropagation on Rodent Tuber plant with high rate multiplication factors. The source of explants used the mother plant originating from Medan (Indonesia). MS medium supplemented with the combination of 0.5 mg/L of BAP and various concentrations of NAA was used. Explants were successfully induced in medium containing 0.5 mg/L of BAP and 0.5 mg/L of NAA. Growing media for plant multiplication were ½ MS and MSO. In the treatment media, BAP was given in five different concentrations, i.e. 0.5, 1, 1.5, 2, and 2.5 mg/L. The result showed that, ½ MS medium added with 1.5 mg/L of BAP was effective in inducing the production of 4.20 ± 1.03 plantlets. *In vitro* root induction of Rodent Tuber was achieved in MSO medium supplemented with 0.5, 1, 1.5, 2, and 2.5 mg/L of NAA. MSO medium supplemented with 1.5 mg/L of NAA could induce the formation of 43.20 ± 21.21 new roots. Viability percentages of Rodent Tuber from Medan acclimatization was 100%. The usage of MS basal media enriched with BAP and NAA is able to effectively increase the production of new plants and roots of Rodent Tuber plant.

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INTRODUCTION

Rodent Tuber (*Typhonium flagelliforme* Lodd.) is an Indonesian herbal plant that belongs to the Araceae family (Surachman, 2009). This plant can be found in several countries such as India (Mankaran et al*.,* 2013), Indonesia (Essai, 1986), Malaysia (Lin, 2005), Sri Lanka and Australia (Sai et al*.,* 2000). Rodent Tuber lives 1-300 m above sea level (Essai, 1986) in humid and shady areas (Nobakht et al., 2009).

Rodent Tuber is able to reach 26 cm in height. This plant has flowers that resemble a rat's tail. The flower of the rodent tuber plant is sterile i.e. it cannot reproduce sexually (Nobakht et al., 2009). Rodent Tuber is commonly propagated by the vegetative method i.e. by separating buds produced from the mother plant's tuber (Syahid & Kristina, 2007).

According to phytochemical analysis, this plant contains several bioactive compounds such as alkaloids, flavonoids (Mankaran et al., 2013), terpenoids, steroids (Nobakht et al., 2010), Ribosome Inactivating Proteins (RIP) (Indrayudha et al., 2006; 2011), antioxidants (Sukardi, 2011) and antibacterial compounds (Mohan et al., 2008). Besides that, Rodent Tuber extract has been found to be successful in inducing apoptosis of breast cancer cells T47D (Norrochmand et al*.,* 2011) and MCF-7 (Putra et al., 2011). It was also found to inhibit the proliferation of human T4-lymphoblastoids (Mohan et al., 2008; Mohan et al., 2010) and NCI-H23 non-small cell lung carcinoma (Lai et al*.,* 2008). The ability of Rodent Tuber to inhibit and kill cancer cells has made this plant one of the plant commodities used as a raw material in complementary and alternative medicine (Mohan et al., 2011; Mankaran et al*.,* 2013).

Rodent Tuber has a low reproduction rate and is very sensitive to enviromental factors. Therefore, the number of Rodent Tubers in the plant's natural habitat is very low. However, demand for this plant is very high due to its biological activity. Vegetative propagation in *in vitro* culture is an appropriate method for overcoming this problem of low numbers because this method is able to produce many plants in a short period of time (Tiwar et al., 2011). The success of propagation in tissue culture is influenced by several factors i.e. genotype of plant, formulation media and physiological conditions of the mother plant. Same plants from different locations will show different results in the same formulation media. The influence of climate and conditions of the physical environment can also have an effect on the physiological conditions of the mother plant (George & Sherrington, 1984; Wattimena et al., 1992).

Plant Growth Regulators (PGR), if used in the right concentration, are able to optimise plant multiplication (Mustafa et al., 2012). PGR is instrumental in regulating the physiological activity of a plant such as growth, development and organogenesis (George & Sherrington, 1984). One of the PGRs that is usually used

to induce the production of plants in *in vitro* culture is 6-benzylamniopurin (BAP). BAP is a cytokinin-type PGR that is effective in inducing the production and propagation of buds from bud eyes (Gunawan, 1987). The supplementation of BAP in a Murashige-Skoog (MS) medium could induce the production of buds from plants such as *Amygdalus communis* L.cv. Yaltsinki (Akbas et al., 2009), *Psoralea corylifolia* Linn (Pandey et al., 2013), *Vinca rosea* L. (Haq et al., 2013) and *Potulaca grandiflora* Hook (Jain et al., 2010).

In Indonesia, Rodent Tuber has been found in several regions such as Bogor, Pekalongan and Medan. Laurent et al., (2013) identified the genetic differences between Rodent Tubers from three different regions by analysing their RAPD molecular marker profiles. Rodent Tuber from Bogor was multiplicated in MS medium to which was added 1 mg/L of NAA and 0.5 mg/L of BAP (Sianipar et al., 2011). Besides that, Rodent Tuber from Pekalongan has also been propagated *in vitro* in MS medium supplemented with 0.5 mg/L of NAA and 0.5 mg/L of BAP (Sianipar et al., 2015). Rodent Tuber from Medan is one of the plant accessions in Indonesia. The regeneration of this plant in *in vitro* culture can be done in two ways i.e. by somatic embryogenesis and organogenesis. In our previous research (Sianipar et al., 2011), the somatic embryogenesis micropropagation method was performed on Rodent Tuber from Bogor. However, plants from different accessions usually have different optimal micropropagation conditions as well. Therefore, this research aimed to formulate a new and efficient micropropagation method for propagating Rodent Tuber plants from Medan by applying different concentrations of BAP as PGR.

MATERIALS AND METHOD

Sterilisation and Initiation of Explant

This research used bud eyes and tubers of Rodent Tuber from Medan as the initial plant materials/explant source. The explants were washed in running tap water to remove any soil particles attached to them. The explants were submerged in 200 ml solution containing 0.25 g of bactericide and fungicide for 30 min, and 2.25 mg/ml of rifampicin for 4 h. The explants were then submerged in 2% *Clorox*® bleach for 15 min, 1.5% *Clorox*® bleach for 15 min, 1% *Clorox*® bleach for 10 min and 0.1% $HgCl_2$ for 10 min. The explants were washed twice with sterile water and then cultured in growth media for initiating buds. There were three types of growth media for initiating buds, each with a different PGR composition. The media used were all Murashige-Skoog (MS) with 30 g sucrose and 100 mL coconut water added for a one-Litre media. The first medium formulation contained 0.5 mg/L *Benzylaminopurine* (BAP) and NAA in three different concentrations i.e. 0.5, 1.0 and 1.5 mg/L. The explants were stored in an incubation room with light intensity 2000 lux for 16 h with a room temperature of about 24 ± 1^0C for shoot and root multiplication.

Shoot Multiplication

The shoot multiplication media were ½ MS and MSO, and to each was added BAP in five different concentrations i.e. 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L. Each treatment was replicated 10 times. The variables observed were the number of shoots in the 10th week. The explants were then subcultured in the optimal medium.

Root Induction

The growth medium for root induction was an MSO basal medium supplemented with NAA in five different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L). The experiment was repeated 10 times. The variables observed were the length and number of roots in the 10th week.

Acclimatisation

Plantlets which had many roots were acclimated after the 10th week. The plantlets, especially their roots, were washed with water and submerged in 1 g/L of Agrimicyn bacteriocide and 0.25 g Benlate fungicide solutions for 3 min each. The plantlets were then acclimated in a medium composed of husk and compost in the ratio of 1:1 in plastic bottles in a greenhouse. Watering was done twice a day to maintain humidity. The variable observed was the viability percentage of plantlets during four weeks of pre-acclimatisation. Plants which could withstand the pre-acclimatisation period were planted in media containing soil and compost in the ratio of 1:1. These plantlets were maintained in a greenhouse. The parameters observed were the plantlet's viability percentages for a period of four weeks.

Research Design and Statistical Analysis

The experimental design of this experiment was a completely random design with eight replications. The statistical analysis of the average number of shoots was done at the 10th week. The statistical analyses used were the normality test Kolmogorov-Smirnov, followed by an analysis of variance (ANOVA) test for normally distributed data.

RESULTS AND DISCUSSION

Induction and Initiation of Explant

Rodent Tuber explants from Medan, Indonesia were cultured in MS medium supplemented with BAP and NAA. The part of explant used for initiating buds formation was the bud on the mother plant's tuber. Buds were used as explants for micropropagation because they contain meristematic somatic cells. According to Nobakht et al. (2009), the bud eye of Rodent Tuber is a potential explant for micropropagating Rodent Tuber in *in vitro* culture.

An *in vitro* culture medium of Rodent Tuber was MS combined with 0.5 mg/L of BAP and three different concentrations

of NAA i.e. 0.5, 1.0 and 1.5 mg/L. The percentages of sterile and viable explants in different media were supplemented with 0.5 mg/L BAP and NAA and 0.5 mg/L BAP and 1.5 mg/L NAA, both at 100% (Table 1).

Table 1
Percentage of sterile and viable explants in initiation media

The composition of growth medium (mg/L)		The percentage of sterile and viable explants*
BAP (mg/L)	NAA (mg/L)	
0.5	0.5	100%
0.5	1.0	66.6%
0.5	1.5	100%

* The number of explants initiated for each treatment was three explants

Sterile and viable explants were obtained from all of the three types of media during the initiation stage. The percentage of sterile explants in medium containing 1.0 mg/L NAA was quite low i.e. 66.6% because the explants were contaminated with fungi.

At the bud induction and initiation stages, the percentage of sterile and viable explants was determined by the type, concentration and time of sterilisation. Organic and inorganic components of the medium were the determining factors of differentiation and de-differentiation (for example, formation of meristem-interfascicular cambium and cork cambium from fully differentiated parenchyma cells) processes. PGRs largerly contributed to the plant's morphogenesis (George & Sherrington, 1984). In this research, the MS medium to which had been added sucrose and coconut water was able to fulfil the explant's requirements of macro- and micronutrients. The addition of BAP is important for mitosis cell division and inducing bud formation, while NAA is for cell division and root induction. Roots will be induced if the ratio of NAA to BAP is higher than one (George & Sherrington, 1984). According to Nobakht et al. (2009), the application of BAP as cytokinin, either alone or combined with NAA as auxin, is effective in increasing the proliferation rate of buds and the fresh weight of Rodent Tuber explant (Syahid & Kristina, 2007).

Multiplication

Rodent Tuber from Medan was propagated in two different types of growth media i.e. ½MS and MS. The ½MS medium contained half of the macro- and micronutrients of basal MS medium. Explants that were cultured in the ½MS medium produced a higher number of shoots. The highest increase in the number of shoots was achieved in the medium supplemented with 1.5 mg/L of BAP (Figure 1).

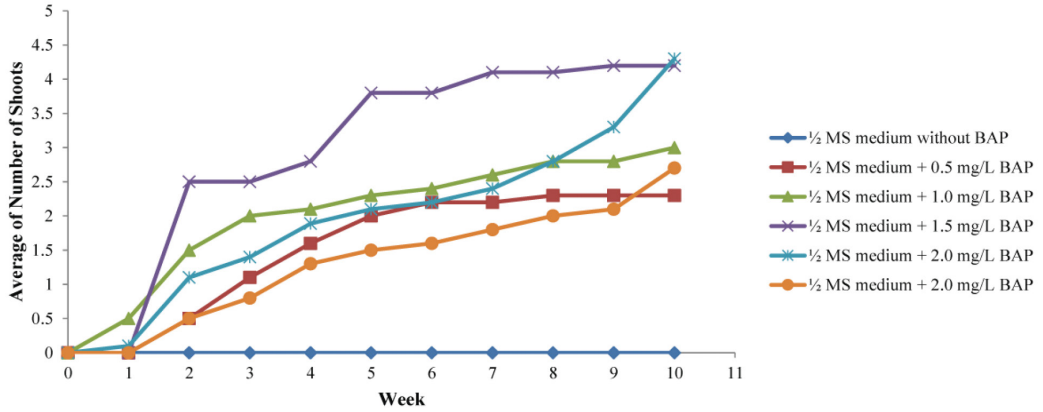

Figure 1. The increase in the number of shoots in various ½MS media supplemented with BAP

The highest number of shoots i.e. 4.20 was achieved on ½MS medium supplemented with 1.5 mg/L of BAP. A Duncan test at the accuracy level of 5% showed that the number of shoots in ½MS medium supplemented with 1.5 mg/L BAP was significantly different from that produced by the control, ½MS with 0.5 mg/L BAP, ½MS with 1.0 mg/L BAP and ½MS with 2.5 mg/L BAP. Meanwhile, the average number of shoots in the ½MS medium supplemented with 1.5 mg/L of BAP was not significantly different from that produced by the ½MS medium supplemented with 2.0 mg/L of BAP (Table 2 and Figure 4(c)).

Table 2
Multiplication of Rodent Tuber from Medan in ½MSO medium

Media	Average number of shoots (mean ± SD)*
T0=½ MS + 0 mg/L BAP	0.00±0.00^{a}
T1=½ MS + 0.5 mg/L BAP	2.30±1.49^{b}
T2=½ MS + 1.0 mg/L BAP	3.00±.15^{bc}
T3=½ MS + 1.5 mg/L BAP	4.20±1.03^{d}
T4=½ MS + 2.0 mg/L BAP	4.10±0.88^{cd}
T5=½ MS + 2.5 mg/L BAP	2.70±1.95^{b}

* Each treatment was replicated 10 times. Average numbers with the same letters indicated that there were no significant differences at p-value ≤0.05 according to the Duncan analysis.

The increasing concentration of BAP up to 2.5 mg/L lowered the number of shoots. This showed that a relatively low concentration of PGR will greatly affect the differentiation process. Like the ½MS treatment, the MSO medium supplemented with BAP was also able to induce the production of new shoots every week. The MSO medium supplemented with 2.5 mg/L BAP was the optimum medium for propagating plants *in vitro* (Figure 2).

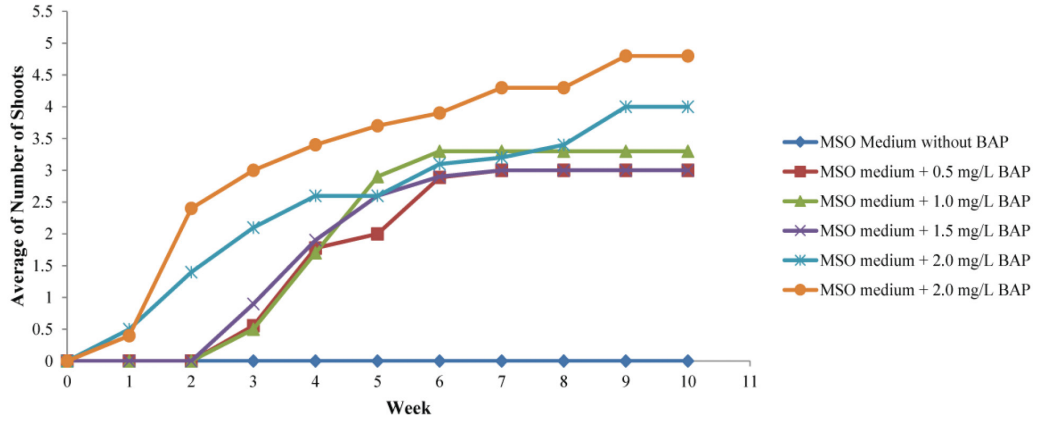

Figure 2. The number of shoots of Rodent Tuber from Medan in MS medium supplemented with BAP

Among the MS medium treatments, the highest average number of shoots was obtained by the MS medium supplemented with 2.5 mg/L of BAP. The average number of shoots in that medium reached 4.8. A Duncan test with an accuracy level of 5% showed that there were significant differences in the number of shoots between the MS medium to which had been added BAP 2.5 mg/L and the control medium, as well as between the MS medium to which had been added 0.5 mg/L of BAP and the MS medium to which had been added 1.5 mg/L of BAP. The average number of shoots between the MS medium supplemented with 1.0 mg/L of BAP and the MS medium supplemented with 2.0 mg/L of BAP was not significantly different.

Table 3
Multiplication of Rodent Tuber from Medan in MS medium

Media	Average number of shoots (mean ± SD)*
T0=MS+0 mg/L BAP	0.00±0.00^{a}
T1=MS+0.5 mg/L BAP	3.00±2.16^{b}
T2=MS+1.0 mg/L BAP	3.30±2.26^{bc}
T3=MS+1.5 mg/L BAP	3.00±1.41^{b}
T4=MS+2.0 mg/L BAP	4.00±1.76^{bc}
T5=MS+2.5 mg/L BAP	4.80±1.23^{c}

* Each treatment was replicated 10 times. Average numbers with the same letters indicated that there were no significant differences at p-value ≤0.05 according to the Duncan analysis.

The ½MS and MSO basal media were able to support the growth of the plant cultures based on the observation of the number of heathly shoots produced. However, the application of the ½MS and MS without the addition of BAP was not able to increase the number of shoots. BAP is important for inducing shoot multiplication (Wattimena et al., 1992) and it can induce cell division and differentiation to produce new buds, either directly or indirectly. The same concentration of BAP can also induce shoot production of *Musa acuminata* cv. Berangan plant (Jafari et al., 2011). The application of PGR outside the safe concentration range will destroy plant tissue and inhibit bud production and cell enlargement, so plant growth will also be inhibited (George & Sherrington, 1984; Sharman et al., 2012). The same applied to *Melissa officinalis* (Tavares et al., 1996) and *Hedeoma multifolium* (Koroch, 1997). The ½MS medium to which had been added 1.5 mg/L BAP produced the highest number of shoots compared to the other treatment with ½MS, while the MSO medium to which had been added 2.5 mg/L BAP was the optimum treatment compared to the other treatments with MSO.

Root Induction

Root induction of *in vitro* shoots was achieved in the MS medium supplemented with five different concentrations of NAA i.e. 0.5 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L and 2.5 mg/L. Root length and average number of roots were observed in the 10th week. Root length was not increased significantly in the medium supplemented with NAA. The longest root was obtained in the MSO medium i.e. 7.79 cm (Table 4). NAA was important for root induction, according to the analysis of the average number of roots (Table 5). The highest average number roots was obtained by the culture in the MS medium supplemented with 1.5 mg/L NAA i.e. 43.20 (Figure 4D). The Duncan test with an accuracy level of 5% showed that there were significant differences between the treatments.

Table 4
Average root length of Rodent Tuber from Medan

Media	Average length (cm) (mean±SD)*
T0=MS+0 mg/L NAA	7.79±2.97[b]
T1=MS+0.5 mg/L NAA	5.66±0.57[a]
T2=MS+1.0 mg/L NAA	6.11±1.39[a]
T3=MS+1.5 mg/L NAA	6.14±1.56[a]
T4=MS+2.0 mg/L NAA	5.14±0.92[a]
T5=MS+2.5 mg/L NAA	5.36±0.58[a]

* Each treatment was replicated 10 times. Average numbers with the same letters indicated that there were no significant differences at p-value ≤0.05 according to the Duncan analysis.

Table 5
The average number of roots of Rodent Tuber from Medan

Media	Average number of roots (mean ± SD)*
T0=MS + 0 mg/L NAA	19.20±10.67[ab]
T1=MS + 0.5 mg/L NAA	12.50±4.03[a]
T2=MS + 1.0 mg/L NAA	26.20±17.42[ab]
T3=MS + 1.5 mg/L NAA	43.20±21.21[c]
T4=MS + 2.0 mg/L NAA	26.40±11.92[ab]
T5=MS + 2.5 mg/L NAA	28.20±17.69[b]

* Each treatment was replicated 10 times. Average numbers with the same letters indicated that there were no significant differences at p-value ≤0.05 according to the Duncan analysis.

The addition of NAA in the MS medium affected the production of the roots of the Rodent Tuber plant from Medan but did not significantly affect the elongation of roots. NAA is a synthetic auxin that is able to stimulate cell growth, cell division and the formation of fruit and roots (Wattimena et al*.,* 1992). Auxin that has been absorbed will be metabolised through the auxin transport method and is important for stimulating the elongation of roots, formation of adventive shoots and root hairs and determining the roots' growth direction (Teale et al*.,* 2004). The application of exogenous auxin in the culture medium was essential for lateral root formation (Thimann, 1936).

The application of exogenous auxin in a culture medium is important for initiating lateral root formation (Chhun et al., 2003). NAA in plant tissue culture medium was able to induce the formation of lateral roots of red betel plant (Sianipar et al., 2016), rice mutant *Lrt 1* (Chhun et al*.*, 2003) and Mellissa officinalis (Sevik & Guney, 2013). This research generated the micropropagation method of Rodent Tuber from Medan, Nort Sumatra (Indonesia) up to the phase of acclimatisation. Although Nobakht et al. (2009) conducted micropropagation research on the same plant with a mother plant originating from Malaysia, the different climatic conditions and the physical environment had a huge effect on the physiological conditions of the mother plant as a source of the explants. This phenomenon is a problem that is common with tissue culture even in the same location as a different isolation of plants (dry/wet climate) will affect the success of micropropagation (totipotency cells) (George & Sherrington, 1984; Wattimena et al., 1992).

Acclimatisation

The plantlets were acclimated in media composed of husk and compost in the ratio of 1:1 in a greenhouse. After four weeks, the viability percentages of the Rodent Tuber plantlets during pre-acclimatisation and post-acclimatisation were observed (Figure 3 and 4E).

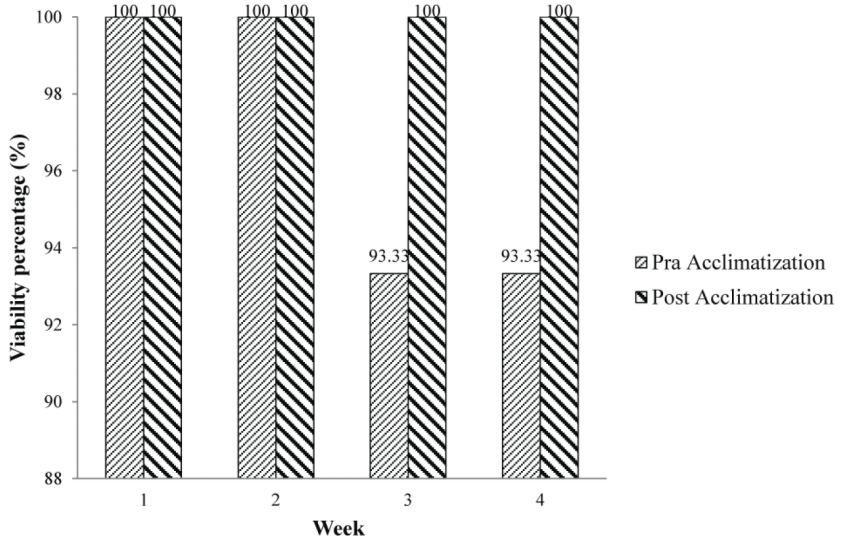

Figure 3. Acclimatisation of the plantlets of Rodent Tuber from Medan by plant tissue culture method

The viability percentage of the plantlets during pre-acclimatisation was very high i.e. reaching 93.33%. This has also been observed by Sianipar et al (2015), who showed a viability percentage of 100% from Rodent Tuber from Pekalongan. The viability percentage of Rodent Tuber from Malaysia documented by Nobakht et al. (2009) reached 90%. During post-acclimatisation, the viability percentage of Rodent Tuber from Medan even reached 100%. This was even higher than the viability percentage of Rodent Tuber from Pekalongan, which was only 58%. This result showed that different accessions/genotypes of plants have different adaptablity to environmental conditions. The role of roots in acclimatisation is crucial because roots determine the effectiveness of the absorbtion of nutrition and water from the medium. The ability of Rodent Tuber from Medan to withstand environmental conditions during pre-acclimatisation and post-acclimatisation is largerly increased if plants have a good root system during the *in vitro* culture period. The application of auxin in an appropiate concentration is vital for supporting the growth and developmental physiological response of the root meristem (Figure 4F).

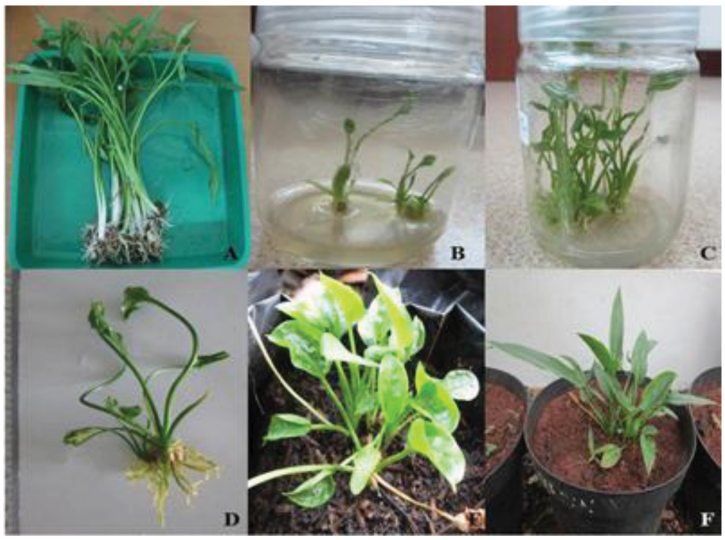

Figure 4. Micropropagation of Rodent Tuber from Medan

Note: A- Rodent Tuber from Medan used as the explant mother plant; B- Initiation in MS medium to which had been added 0.5 mg/L BAP and 0.5 mg/L NAA; C: Multiplication of shoots in the 10th week in ½MS medium to which had been added 1.5 mg/L BAP; D: Root induction in MS medium to which had been added 1.5 mg/L NAA; E: Pre-acclimatisation of plantlets; and F: Post-acclimatisation of the plant that had been propagated in *in vitro* culture previously.

CONCLUSION

Rodent Tuber from Medan was able to multiply and produce a high number of shoots i.e. 4.20 ± 1.03 shoots in a ½MS medium supplemented with 1.5 mg/L BAP in the 10th week. Rodent Tuber from Medan is unique because it can grow optimally in a ½MSO medium. The highest number of shoots i.e. 4.80 was obtained from the MSO medium supplemented with 2.5 mg/L BAP. The highest number of roots i.e. 43.20 lateral roots was achieved from the MSO medium supplemented with 1.5 mg/L NAA. Rodent Tuber from Medan has good adaptability to environmental conditions, with a viability percentage of 93.33% during pre-acclimatisation and 100% during acclimatisation.

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REFERENCES

Akbas, F., Isikalan, C., Namli, S., & Ak, B. (2009). Effect of plant growth regulators on *in vitro* shoot multiplication of *Amygdalus communis* L. cv. Yaltsinki. *African Journal of Biotechnology*, *8*(22), 6168–6174.

Chhun, T., Taketa, S., Tsurumi, S., & Ichii, M. (2003). Interaction between two auxin-resistant mutants and their effects on lateral root formation in rice (Oryza sativa L.). *Journal of Experimental Botany, 54*(393), 2701–2708.

Essai. (1986). *Medicinal herbs index in Indonesia*. Jakarta: PT Essai Indonesia.

George, E. F., & Sherrington, P. D. (1984). *Plant propagation by tissue culture*. Basingstoke, England: Exegetics Ltd.

Gunawan, L. W. (1987). *Teknik kultur jaringan*. Bogor, Indonesia: Laboratorium Kultur Jaringan Tanaman, Pusat Antar Universitas Bioteknologi Institut Pertanian Bogor.

Haq, R., Naz, S., Aslam, F., & Manzoor, F. (2013). Comparison of in vitro response of micropopragation and callogenesis of medicinal plant, L. *Journal of Agricultural Research*, *51*(1), 9–17.

Indrayudha, P., Wijaya, A., & Iravati, S. (2006). Uji aktivitas ekstrak daun dewandaru dan daun keladi tikus terhadap pemotongan dna superkoil untai ganda. *Jurnal Farmasi Indonesia, 3*(2), 63–70.

Indrayudha, P., Wijayanti, N., & Sismindari. (2011). Antiangiogenesis activity of protein fraction containing MJ C acidic ribosome inactivating protein of Mirabilis jalapa L. *Jurnal Bahan Alam Indonesia*, *7,* 277–281.

Jafari, N., Othman, R. Y., & Khalid, N. (2011). Effect of benzylaminopurine (BAP) pulsing on in vitro shoot multiplication of Musa acuminate (banana) cv. Berangan. *African Journal of Biotechnology*, *10*(13), 2446–2450.

Jain, A. K., & Bashir, M. (2010). In-vitro propagation of a medicinal plant *Portulaca grandiflora*. Hook. *World Journal of Agricultural Sciences*, *6*(3), 327–330.

Koroch, A. R., Juliani, H. R., Juliani, H. R., Jr., & Trippi, V. S. (*1997)*. Micropropagation and acclimatization of *Hedeoma multiform*. *Plant Cell Organ Culture: Journal of Plant Biotechnology, 48*(3), 213–217.

Lai, C. S., Mas R. H., Nair, N. K., Majid, M. I. A., Mansor, S. M., & Navaratnam, V. (2008). *Typhonium flagelliforme* inhibits cancer cell growth *in vitro* and induces apoptosis: An evaluation by the bioactivity guided approach. *Journal of Ethnopharmacology*, *118*(1), 14–20.

Laurent, D., Sianipar, N. F., Chelen, L., & Wantho, A. (2013). Analysis of genetic diversity of Indonesian rodent tuber (*Typhonium flagelliforme* Lodd.) cultivars based on RAPD marker. *Presented at the International Conference on Biological Science, the Faculty of Biology, University of Gadjah Mada*. Yogyakarta, Indonesia: AIP Publishing.

Lin, K. (2005). Ethanobotanical study of medical plants used by the Jah Hut People in Malaysia. *Indian Journal of Medical Sciences*, *59*(4), 156-161.

Mankaran, S., Dinesh, K., Deepak, S., & Gurmeet, S. (2013). *Typhonium flagelliforme*: A multipurpose plant. *International Research Journal of Pharmacy*, *4*(3), 45-48.

Mohan, S., Abdul, A. B., Abdelwahab, S., Zubairi, A., Sukari, M., Abdullah, R., … & Syam, S. (2010). *Typhonium flagelliforme* induce apoptosis in CEMs cells via activation of caspase-9, PARP cleavage and cytochrome c release: Its activation coupled with G0/G1 phase cell cycle arrest. *Journal of Ethnopharmacology*, *131*(3), 592–600.

Mohan, S., Bustamam, A., Ibrahim, S., Zubairi, A., & Aspollah, M. (2008). Anticancerous effect of *Typhonium flagelliforme* on human T4-Lymphoblastoid cell line CEM-ss. *Journal of Pharmacology*, *3*(6), 449–458.

Mohan, S., Bustamam, A., Ibrahim, S., Al-Zubairi, A., Aspollah, M., Abdullah, R., & Elhassan, M. M. (2011). *In vitro* ultramorphological assessment of apoptosis CEMs induced by linoleic acid-rich fraction from *Typhonium flagelliforme* tuber. *Evidence-Based and Alternative Medicine, 2011,* 1–12.

Mustafa, N. S., & Taha, R. (2012). Influence of plant growth regulator and subculturing on *in vitro* multiplication of some fig (*Ficus Carcia*) cultivars. *Journal of Applied Science Research*, *8*(8), 4038–4044.

Nobakht, G. M., Kadir, M. A., & Stanslas, J. (2009). *In vitro* mass propagation of *Typhonium flagelliforme* as affected by plant growth regulators. *African Journal of Biotechnology*, *8*(24), 6840–6843.

Nobakht, G. M., Kadir, M. A., & Stanslas, J. (2010). Analysis of preliminary phytochemical screening of *Typhonium flagelliforme*. *African Journal of Biotechnology*, *9*(11), 1655–1657.

Nurrochmad, A., Lukitaningsih, E., & Meiyanto, E. (2011). Anti-cancer activity of rodent tuber (*Typhonium flagelliforme* Lodd.) blume on human breast cancer t47d cells. *International Journal of Phytomedicine*, *3*(1), 138–146.

Pandey, P., Mehta, R., & Upadhyay, R. (2013). *In vitro* propagation of an endangered medical plant *Psoralea corylifolia* Linn. *Asian Journal of Pharmaceutical and Clinical Research*, *6*(3), 115–118.

Putra, A., Tjahjono, & Winarto. (2011). Ekstrak rodent tuber (*Typhonium flagelliforme*) fraksi diklorometanolik dan ekspresi caspase-3 dan p21 *cell-Line* kanker payudara MCF-7. *Media Medika Indonesiana, 45*(2), 95–104.

Sai, S., Keng, C., Pargini, N., & Teo, C. (2000). *In vitro* propagation of *Typhonium flagelliforme* (Lodd) blume. *In vitro Cellular and Developmental Biology – Plant*, *36*(5), 402–406.

Sevik, H., & Guney, K. (2013). Effect of IAA, IBA, NAA and GA3 on rooting and morphological features of *Melissa officinalis* L. stem cuttings. *The Scientific World Journal*, *2013*, 1–5.

Sharman, J., Khan, S., & Varman, R. (2012). *In vitro* culture establishment and shoot induction of mallotus philippenesis (Lam.) M. *ARG. Pharmacie Globale International Journal of Comprehensive Pharmacy*, *3*, 1–5.

Sianipar, N. F., Laurent, D., & Tanty, H. (2015, September). Induction, multiplication, and acclimatization of rodent tuber (Typhonium flagelliforme Lodd.) plant from Indonesia by in vitro organogenesis. In *International Conference on Technology, Informatics, Management, Engineering & Environment (TIME-E), 2015* (pp. 59-63). IEEE.

Sianipar, N. F., Rustikawati, Maarisit, W., Wantho, A., & Sidabutar, D. N. R. (2011). Embryogenic calli induction, proliferation and regeneration of rodent tuber plant (Thyphonium flagelliforme Lodd.) by single node culture. *Paper presented at the International Conference on Biological Science, the Faculty of Biology, University of Gadjah Mada BIO-UGM*. Yogyakarta, Indonesia: AIP Publishing.

Sianipar, N. F., Verlina & Rosaria. (2016). Induction, multiplication, and acclimatization of red betel plant (*Piper crocatum* Ruiz and Pav.) by in vitro organogenesis. *Jurnal Teknologi*, *78*(5-6), 35–40.

Sukardi. (2011). Identifikasi dan karakterisasi umbi *Rodent Tuber* sebagai zat antioksidan alami. *Gamma*, *6*(2), 143–151.

Surachman, D. (2009). Penggunaan beberapa taraf konsentrasi paklobutrazol dalam media konservasi rodent tuber (*Typhonium flagelliforme* Lodd.) *in vitro*. *Buletin Teknik Pertanian*, *14*(1), 31–33.

Syahid, S., & Kristina, N. (2007). Induksi dan regenerasi kalus *Rodent Tuber* (*Typhonium flagelliforme*. Lodd.) secara *in vitro*. *Jurnal Littri*, *13*(4), 142–146.

Tavares, A. C., Pimenta, M. C., & Goncalves, M. T. (1996). Micropropagation of *Melissa officinalis* L. through proliferation of axillary shoots. *Plant Cell Respiratory*, *15*(6), 441–444.

Teale, W. D., Papanov, I. A., Ditengou, F., & Palme, K. (2004). Auxin and the developing root of *Arabidopsis thaliana*. *Physiologia Plantarum*, *123*(2), 130–138.

Thimann, K. V. (1936). Auxins and the growth of roots. *American Journal of Botany, 23*(8), 561–569.

Tiwar, S., & Arnold, R. (2011). Micropropagation as an advanced technology for the conservation of plants. *International Journal of Drug Discovery and Herbal Research*, *1*(2), 95–99.

Wattimena, G. A. (1992). *Bioteknologi tanaman*. Bogor: Departemen pendidikan dan kebudayaan direktorat jenderal pendidikan tinggi pusat antar universitas bioteknologi institusi pertanian Bogor.

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Review Article

Signaling Behaviour of Abscisic Acid on Physiological Activities in Plants Under Stress

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ABSTRACT

Plants manage water stress conditions through a variety of signalling networks, which maintain physiological balances of the plants. The phytohormone, abscisic acid (ABA), regulates growth and development of plants by acting on different physiological features of plants. Various stress factors including drought result in raising the endogenous ABA concentration in plant cells. Abscisic acid modifies gene function and its transformation in plants when plants are experiencing stress conditions. Molecular mechanisms show signalling functions of ABA in plants. Several researchers have demonstrated the different roles of ABA in plants such as regulation of stomatal aperture, production of secondary metabolites and signalling cross-link with other molecules. Researchers also show significant interest in and place importance on the signalling regulation of ABA on the growth and physiology of plants. Therefore, this review highlights the signalling pathway of ABA with or without other molecules to restrain growth and physiological parameters of plants under climate change conditions.

Keywords: Arabidopsis, glutathione, guard cells, plant growth, reactive oxygen species, signalling pathway, stomatal aperture, stress factors

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INTRODUCTION

Abscisic acid (ABA) is recognised under stress conditions by plants, allowing it to amend physiological features of plants under stress to cope with multiple detrimental environmental indicators that challenge to abiotic and biotic

consequences due to climate change. Currently, biochemical, molecular, genetic and genomic methodologies have shown the fundamental charter of the ABA signalling network in plants. Reports on ABA receptors, especially, have further clarified understanding of the functional mechanisms of ABA in plants (Zhang, 2014). Additionally and more importantly, a number of ABA-related genes has been engineered for improvement of stress resistance to provide chances for the progress of new varieties with superior stress resistance. ABA regulates physiological processes, primarily biosynthesis de novo ABA, by relevant enzymes rather than the relocation of remaining pools of ABA (Hartung et al., 2002). Genetic analysis has revealed that different physiological processes such as seed germination, osmotic stress and transpiration are linked to the ABA activity, emphasising the responsibility of ABA in signalling networking in plants (Seo & Koshiba, 2002).

In the last 30 years, several reports confirmed that ABA regulates the stomatal aperture of guard cells. Recent reports have stated that ABA functions with several factors to modulate the growth of plants (Jahan et al., 2008; Khokon et al., 2011; Okuma et al., 2011; Jahan et al., 2016). The status of water maintains ABA strength in the xylem stream. ABA-induced stomatal closure controls physiological functions such as photosynthesis (Davies et al., 1993), which may affect growth, physiology and development of stressed plants (Jahan et al., 2013; Khairi et al., 2015; Khairi et al., 2016). This review cites new understanding of the current roles of ABA to up-regulate plant physiological functions under climate change conditions for sustainable growth and production of plants.

Discovery and Catabolism of Abscisic Acid

Abscisic acid is a small molecule, which encompasses various functional groups. Addicott et al. (1963) isolated two liable compounds aimed at abscission fruit (cotton) i.e. abscisin I and abscisin II. Abscisic acid was formerly called 'abscisin II'. It plays a significant role in abscission of fruit and bud dormancy and has inhibitory roles as well as promotes physiological functions in plants (Salisbury & Ross, 1992). Stability in biosynthesis and catabolism controls endogenous ABA levels in numerous plants. ABA is a compound that naturally synthesises in plants via a direct pathway, the mevalonic pathway, in chloroplast and via an indirect pathway, the methylerythritol phosphate (MEP) pathway, which appears to occur in cyanobacteria and eukaryotes (Schwartz & Zeevaart, 2010). Stress, for example drought and temperature, highlights the biosynthesis of ABA in plants. In the direct pathway, isomerisation of allofarnesene and oxidation synthesise ABA in fungi (Inomata et al., 2004). In the indirect pathway, carotenoids, chloroplast-produced pigments, which have 40 carbon molecules, appear in the ABA structure.

In the reaction centre, a molecule of violaxanthonin was seen in research to produce a molecule of xanthonin, which is unstable and therefore, transforms to ABA aldehyde (ABAld). Further oxidation methods used in research resulted in activation of ABA by two enzymes, abscisic aldehyde oxidase and molybdenum cofactor sulfurase. In wilt tomato mutants, flacca and sitiens, the transformation of ABAld to ABA was constructed in cell-free extracts due to oxidase enzyme (Taylor et al., 1988), which produces ABA (Leydecker et al., 1995). In several plant species, the hydroxylation phase in the ABA catabolic route in plants catalysed C-8′ of ABA and produced unstable 8′-hydroxy-ABA (Walton, 1983).

Transportation and Distribution of ABA

Water deficit synthesises ABA in cells sited across the vasculature; it is translocated to the guard cells and stimulates stomatal closure. In this relation, ABA exporter genes and importer genes are expressed; they determine the amount of mobile ABA present in the apoplastic space by balancing export and import of ABA content out of and into vascular tissue (Seo & Koshiba, 2002). ABA is capable of moving both up and down along the stem through the xylem and phloem tissues and parenchyma cells in plants, which do not show polarity with other growth regulators such as auxins (Salisbury & Ross, 1992). Abscisic acid is synthesised in leaves and roots and freely moves from plant to soil as well as from soil to plant. Roots grow well under soil solution, which contains plants hormones. If the ABA is not at equilibrium, ABA transport from root to soil is disrupted, which also disrupts the signalling relationship between the roots and the shoot (Sauter et al., 2001). Translocation of ABA from cell-to-cell is done through the plasmodesmata and transmembrane transport, after which it is synthesised in the cells. In addition, ABA presences in xylem sap is exported to the outside of the cells (Hartung et al., 2002). After that, ABA is moved to other cells to stimulate physiological reactions in plants.

A plant may uptake ABA from acidic soil, which increases external take-up of ABA concentration by the roots (Freundl et al., 2000). In contrast, roots lose ABA content under high soil pH condition (Degenhardt et al., 2000). This is due to the effects of the reduction of ABA content in the surrounding medium (Freundl et al., 2000). Several factors other than water stress such as salt content, phosphorus deficiency and ammonium increment stimulate ABA biosynthesis in the root but nitrate deficiency and alkaline reduces it (Wolf et al., 1990; Jaschke et al., 1997; Freundl et al., 2000). Therefore, water stress and salt accumulate a higher concentration of ABA in the roots of plants (Sauter et al., 2001). The increment of ABA in roots may increase the flow of ABA in the cells of roots through the symplastic and apoplastic pathway under stress conditions, then translocate to the

guard cells to close the stoma (Sauter et al., 2001; Jahan et al., 2016). In contrast, increasing water flow rapidly decreases ABA concentration through bypass flow of ABA to the endodermis, leading to ABA homeostasis in the xylem (Tardieu et al., 1992; Freundl et al., 1998).

The pH of cells affects the delivery of ABA. ABA biosynthesis occurs in the cytosol when the pH level is at 7.2. Therefore, most of the ABA is ionised in the cytosol and exists as ABA−, followed by ABA efflux. Then, the ABA moves through the apoplastic space (Kramer, 2006). At this stage, the pH in the apoplast remains at 5.0-6.0. This pH gradient condition suggests active transport mechanism of apoplastic ABA in cells. However, stoma are closed due to a flux of ABA in well-watered conditions by changing acidic pH to alkaline pH conditions. A little alkalinisation in xylem sap may result in stomatal closure (Wilkinson & Davies, 1997) and might influence leaf growth (Bacon et al., 1998). Apoplastic pH rises to about 7.0 when plants are under water stress conditions, which enables a moderately large percentage of ABA-H to move over extended distances across the apoplast (Wilkinson & Davies, 2002). The transportation of ABA is required to induce ABA signalling under stress conditions. The distribution of ABA is a concern to environmental conditions. Under normal circumstances, ABA concentration in leaves is higher than in roots and stems (Bahrun et al., 2002).

Intercellular Transport of ABA to Stomatal Movement

ABA is a key controller of stomatal movement in guard cells under water scarcity. Several studies have focussed on the signal transduction pathways underlying the ABA-regulated stomatal aperture. ABA is primarily localised in chloroplast and vascular tissue. From here, it is transported to sites of action in guard cells during water shortage (Seo & Koshiba, 2002). Drought-induced pH changes in different parts of the leaves of a plant (Islam et al., 2011) control a complex distribution of ABA throughout the various cells of the leaves. Then ABA gathers in guard cells and persuades stomatal closure (Jahan et al., 2016). Water stress leads to an increment of ABA content in the apoplast but not in the mesophyll cells (Wang & Jia, 1995). Interestingly, ABA translocates from mesophyll and vascular cells to the guard cells to close stomata under stress conditions, but under non-stress conditions, stomatal movement is unaffected by the ABA although both mesophyll and vascular cells contain plentiful ABA content. Drought stress increases ABA content in the mesophyll and vascular cells of leaves and ABA-induced secondary signalling metabolites (Jahan et al., 2016; Munemasa et al., 2007). More prominently, ABA build-up in guard cells seems to protect plants against drought stress (Okuma et al., 2011).

Cross Talk Between ABA and Methyl Jasmonate Acid in Signalling Pathways

Guard cells are specialised cells that control gas exchange and water movement to respond to numerous types of stress due to climate change through the closing and opening of stomatal pores (Kim et al., 2010; Okuma et al., 2011; Jahan et al., 2014). Plants synthesise ABA in response to consequences of climate change and prove the transduction of ABA to guard cells through production of second messengers, for example, cytosolic $Ca2^{+}$, nitric oxide (NO) and reactive oxygen species (ROS) in guard cells (Murata et al., 2015). A plant hormone, jasmonate, regulates stomatal opening in reaction to diverse stimuli such as senescence, wounding and pathogen attacks in plants (Turner et al., 2002). MeJA stimulates ROS intervention in guard cells like ABA under different climate change conditions such as water stress, wounding, salinity and insect attacks (Tsonev et al., 1998; Suhita et al., 2004). Similarities observed in different stomatal aperture experiments involved the NAD(P)H oxidase inhibitor, diphenyleneiodonium (Tsonev et al., 1998). Two NAD(P)H oxidases, AtrbohD and AtrbohF, link methyl jasmonate acid (MeJA) and ROS for signalling in guard cells, proving that MeJA and ABA function in the similar signalling way to persuade stomatal closure. Furthermore, both ABA and MeJA stimulate NO synthase (NOS) guard cells during NO-induced stomatal closure, which indicates a similar path of ABA and MeJA in the signalling force of stomatal closure (Desikan et al., 2002; Sasaki-Sekimoto et al., 2005). As a result, MeJA- and ABA-induced stomatal closure is due to the production of ROS and NO in guard cells through the stimulation of $Ca2^{+}$ from intracellular stores to the cytosol (Desikan et al., 2002). MeJA does not induce stomatal closure in the *abi2-1* mutant, an ABA-insensitive mutant; this suggests that a protein phosphatase 2C links with MeJA core components in ABA regulation in receptor complexes (Munemasa et al., 2007; Desikan et al., 2002; Murata et al., 2001). ABA and MeJA signalling might affect ABA receptor developments to control downstream signal mechanisms.

Abscisic Acid Signalling in Glutathione Functions on Guard Cells

Abscisic acid stimulates different physiological processes of a plant in stress conditions (Finkelstein et al., 2002). ABA features on GSH variation in guard cells of Arabidopsis (Okuma et al., 2011; Jahan et al., 2014). Water stress promotes the primary gathering of ABA that generates ROS creation, which might adjust GSH biosynthesis in plants (Murata et al., 2015). ABA signalling in GSH was studied earlier in guard cells of the Arabidopsis plants (Jahan et al., 2008; Jahan et al., 2011; Okuma et al., 2011; Munemasa et al., 2013). The Arabidopsis mutant, GLUTATHIONE S-TRANSFERASE U17 (AtGSTU17), stores a greater amount of ABA in the presence of GSH (Cheng et al., 2013). Therefore, the functions of GSH

with ABA in plants would be a critical aspect for the plants' ability to cope with climate change conditions (Cheng et al., 2013). ABA affects GSH content in ratio to GSSG, GR activity and γ-ECS transcript levels in maize genotypes (Okuma et al., 2011; Cheng et al., 2013). Glutathione peroxidase3 (*atgpx3*) controls H2O2 homeostasis in ABA-mediated stomatal movement (Miao et al., 2006), which suggests that ABA signalling with GSH is linked to the stomatal movement in guard cells of Arabidopsis (Okuma et al. 2011; Jahan et al., 2014). Therefore, GSH-deficient mutants, the *ch1-1* and cadmium sensitive mutant (*cad2-1*), exhibit a higher sensitivity to ABA than the wild types (Okuma et al., 2011; Jahan et al., 2011, 2008). In addition, Munemasa et al. (2013) confirm that GSH modulates the signalling compartment of ABA in guard cells.

It has been genetically and chemically confirmed that GSH does not affect ROS production in guard cells as well as the activation of $Ca2^{+}$ -permeable channel (ICa) (Akter et al., 2013; Okuma et al., 2011), which suggests that GSH might play a role in coupling H_2O_2 to downstream an ABA-induced signalling cascade in guard cells (Okuma et al. 2011). In a current development, Munemasa et al. (2013) stated that GSH depletion affects ROS signalling in apoplastic space rather than in the cytosol of guard cells during ABA-induced stomatal closure (Munemasa et al., 2013). Different studies have stated a similar result, that apoplastic ROS signals regulate stomatal movement (Hossain et al., 2013; An et al., 2008). Therefore, GSH-modulated ABA signalling of a GSH-deficient mutant (Jahan et al., 2008) is not conditional to the production of H_2O_2 in guard cells (Munemasa et al., 2013).

Abscisic Acid Signalling on Growth of Plants

Climate change is becoming a severe problem for plant growth in several regions of the world. Plants respond to drought conditions through intensely complicated mechanisms such as genetic and physiological processes (Chaves et al., 2003; Izanloo et al., 2008). Water stress influences plant growth and biomass production remarkably (Chaves et al., 2003; Hisao, 1973). The leaf area of *Leymus chinensis*, for instance, is significantly stimulated by rewatering compared to a short-term drought (Xu & Zhou, 2007). In another instance, tiller production of *Leymus chinensis* recovered after rewatering, suggesting that development of meristems of the tiller might play an important role in response to water stress conditions (Xu & Zhou, 2009). Water stress is closely related to the elevation of ABA in guard cells to close stomata (Jahan et al., 2016), which might reduce gas exchange through the opening of guard cells. Reactive oxygen species (ROS) increases during drought conditions because of the influence of ABA production (Jahan et al., 2008). Water application reduced ROS production in guard cells and the retrieval net photosynthetic and transpiration rate in plants (Okuma et al.,

2011; Khairi et al., 2016). Low relative water content increased ROS production (Murata et al., 2015), which is also related to the ABA signalling for plant growth. Stoma closure due to drought conditions limits photosynthesis in plants (Chaves et al., 2003). Drought reduces light-dependent photosynthesis and stomatal conductance in plants (Peñuelas et al., 2004; Xu & Zhou, 2009). This was consistent with the results obtained by Nozulai et al. (2015) and Khaiti et al. (2016) in rice plants, where reduction of water content reduced plant physiological parameters. This result also suggests that ABA might have a significant role in gas exchange in drought conditions. In addition, Izanloo et al. (2008) stated that water stress declined quantum yield in the photosystem II, indicating a functional activity of ABA in regulating light reaction in plants. A defect of the light-harvesting mutant, *ch1-1*, led to higher sensitivity to ABA than was seen in wild types (Jahan et al., 2014, 2016). Therefore, *ch1-1* mutant plants are deficient in morphological, physiologic and yield parameters compared to wild types of the plant (Jahan et al., 2014, 2016). It is suggested that ABA controls growth and development of plants through multi-signalling waves.

CONCLUSION

Considering the latest documentation, it is clear that ABA has a distinct role in the hormonal signalling pathway in controlling physiological functions of plants. There is evidence that ABA modulates physiological changes in plants in the presence of stress conditions through a signalling network with other plant hormones as well as antioxidants. However, understanding the workings of the protocols of the gene that controls ABA function in plants needs further study. Further study and experimental proof are also necessary for understanding the role of ABA in different hormonal signalling cascades for a clearer picture of the ABA signalling-web on the growth and physiology of plants.

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REFERENCES

Addicott, F. T., Lyon, J. L., Ohkuma, K., Thiessen, W. E., Carns, H. R., Smith, O. E., … & Wareing, P. F. (1968). Abscisic acid: A new name for abscisin II (dormin). *Science, 159*(3822), 1493-1493.

Akter, N., Okuma, E., Sobahan, M. A., Uraji, M., Munemasa, S., Nakamura, Y., ... & Murata, Y. (2013). Negative regulation of methyl jasmonate-induced stomatal closure by glutathione in Arabidopsis. *Journal of plant growth regulation, 32*(1), 208-215.

An, Z., Jing, W., Liu, Y. L., & Zhang, W. H. (2008). Hydrogen peroxide generated by copper amine oxidase is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Journal of Experimental Botany, 59*(4), 815–825.

Bacon, M. A., Wilkinson, S., & Davies, W. J. **(**1998). pH-regulated leaf cell expansion in droughted plants is abscisic acid dependent. *Plant physiology, 118*(4)**,** 1507–1515.

Bahrun, A., Jensen, C. R., Asch, F., & Mogensen, V. O. (2002). Drought-induced changes in xylem pH, ionic composition, and ABA concentration act as early signals in field-grown maize (*Zea mays L.*). *Journal of Experimental Botany, 53*(367), 251–63.

Chaves, M. M., Maroco, J. P., & Pereira, J. S. (2003). Understanding plant response to drought-from genes to the whole plant. *Functional Plant Biology, 30*(3), 239–64.

Cheng, M. C., Liao, P. M., Kuo, W. W., & Lin T. P. (2013). The *Arabidopsis* ethylene response factor1 regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. *Plant Physiology*, *162*(3), 1566–1582.

Davies, W. J., Trejo, C. L., & Palmer, S. J. (1993) ABA and the control of growth and physiology of stressed plants. In M. B. Jackson & C. R. Black (Eds.), *Interacting stresses on plants in a changing climate. NATO ASI series (Series I: Global environmental change)* (vol. 16). Berlin, Heidelberg: Springer.

Degenhardt, B., Gimmler, H., Hose, E., & Hartung, W. (2000). Effect of alkaline and saline substrates on ABA contents, distribution and transport in plant roots. *Plant and Soil, 225*(1), 83–94.

Desikan, R., Griffiths, R., Hancock, J., & Neill, S. (2002). A new role of an old enzyme: Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences USA, 99*(25), 16314–16318.

Finkelstein, R. R., Gampala, S. S., & Rock, C. D. (2002). Abscisic acid signaling in seeds and seedlings. *The Plant Cell Online, 14*(suppl 1), S15-S45.

Freundl, E., Steudle, E., & Hartung, W. (1998). Water uptake by roots of maize and sunflower affects the radial transport of abscisic acid and the ABA concentration in the xylem. *Planta, 207*(1), 8–19.

Freundl, E., Steudle, E., & Hartung, W. **(**2000). Apoplastic transport of abscisic acid through roots of maize: Effect of the exodermis. *Planta, 210*(2), 222–231.

Hartung, W., Sauter, A., & Hose, E. (2002). Abscisic acid in the xylem: where does it come from, where does it go to? *Journal of Experimental Botany, 53*(366), 27–32.

Hisao, T. C. (1973). Plant responses to water stress. *Annual Review of Plant Physiology, 24*(1), 519–70.

Hossain, M. S., Ye, W., Hossain, M. A., Okuma, E., Uraji, M., Nakamura, Y., ... & Murata, Y. (2013). Glucosinolate degradation products, isothiocyanates, nitriles, and thiocyanates, induce stomatal closure accompanied by peroxidase-mediated reactive oxygen species production in Arabidopsis thaliana. *Bioscience, biotechnology, and biochemistry, 77*(5), 977-983.

Inomata, M., Hirai, N., Yoshida, R., & Ohigashi, H. (2004). Biosynthesis of abscisic acid by the direct pathway via ionylideneethane in a fungus, *Cercospora cruenta*. *Bioscience Biotechnology Biochemistry, 68*(12), 2571–2580.

Islam, M. M., Hoque, M. A., Okuma, E., Jannat, R., Banu, M. N. A., Jahan, M. S., ... & Murata, Y. (2009). Proline and glycinebetaine confer cadmium tolerance on tobacco bright yellow-2 cells by increasing ascorbate-glutathione cycle enzyme activities. *Bioscience, biotechnology, and biochemistry, 73*(10), 2320-2323.

Izanloo, A., Condon, A. G., Langridge, P., Tester, M., & Schnurbusch, T. (2008) Different mechanisms of adaptation to cyclic water stress in two South Australian bread wheat cultivars. *Journal of Experimental Botany, 59*(12), 3327–3346.

Jahan, M. S., Nakamura, Y., & Murata, Y. (2011). Histochemical quantification of GSH contents in guard cells of *Arabidopsis thaliana*. *Science Asia, 37,* 291–295.

Jahan, M. S., Nozulaidi, M., Khairi, M., & Mat, N. (2016). Light-harvesting complexes in photosystem II regulate glutathione-induced sensitivity of Arabidopsis guard cells to abscisic acid. *Journal of Plant Physiology, 195,* 1–8.

Jahan, M. S., Nozulaidi, M., Khandaker, M. M., Afifah, A., & Husna, N. (2014). Control of plant growth and water loss by a lack of light-harvesting complexes in photosystem-II in Arabidopsis thaliana ch1-1 mutant. *Acta Physiologia Plantarum, 36*(7), 1627–1635.

Jahan, M. S., Nozulaidi, M. B. N., Khairi, M. B. C. L., & Khanif, Y. M. (2013). Effects of water stress on rice production: Bioavailability of potassium in soil. *Journal of Stress Physiology and Biochemistry, 9*(2), 97–107.

Jahan, M. S., Ogawa, K., Nakamura, Y., Shimoishi, Y., Mori, I. C., & Murata, Y. (2008). Deficient glutathione in guard cells facilitates abscisic acid-induced stomatal closure but does not affect light-induced stomatal opening. *Bioscience Biotechnology Biochemistry, 72*(10), 2795–2798.

Jaschke, W. D., Peuke, A. D., Pate, J. S., & Hartung, W. (1997). Transport, synthesis and catabolism of abscisic acid (ABA) in intact plants of castor bean (*Ricinus communis* L.) under phosphate deficiency and moderate salinity. *Journal of Experimental Botany, 48*(9)**,** 1737–1747.

Khairi, M., Nozulaidi, M., Afifah, A., & Jahan, M. S. (2015). Effect of various water regimes on rice production in lowland irrigation. *Australian Journal of Crop Science, 9*(2), 153–159.

Khairi, M., Nozulaidi, M., & Jahan, M. S. (2016). Effects of flooding and alternate wetting and drying on the yield performance of upland rice. *Pertanika Journal of Tropical Agricultural Science, 39*(3), 299–309.

Khokon, M. A. R., Jahan, M. S., Rahman, T., Hossain, M. A., Munemasa, S., Mori, I. C., … & Murata, Y. (2011). Allylisothiocyanate (AITC) induces stomatal closure in Arabidopsis. *Plant Cell Environment, 34*(11), 900–1906.

Kim, T. H., Böhmer, M., Hu, H., Nishimura, N., & Schroeder, J. I. (2010). Guard cell signal transduction network: Advances in understanding abscisic acid, CO2 and Ca2+ signaling. *Annual Review of Plant Biology, 61*, 561–591.

Kramer, E. M. (2006). How far can a molecule of weak acid travel in the apoplast or xylem? *Plant physiology, 141*(4), 1233–1236.

Leydecker, M. T., Moureaux, T., Kraepiel, Y., Schnorr, K., & Caboche, M. (1995). Molybdenum cofactor mutants, specifically impaired in xanthine dehydrogenase activity and abscisic acid biosynthesis, simultaneously overexpress nitrate reductase. *Plant physiology, 107*(4), 1427–1431.

Miao, Y., Lv, D., Wang, P., Wang, X. C., Chen, J., Miao, C., & Song, C. P. (2006). An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. *Plant Cell, 18*(10), 2749–2766.

Munemasa, S., Muroyama, D., Nagahashi, H., Nakamura, Y., Mori, I. C., & Murata, Y. (2013). Regulation of reactive oxygen species-mediated abscisic acid signaling in guard cells and drought tolerance by glutathione. *Frontier Plant Science, 4,* 472-477.

Munemasa, S., Oda, K., Watanabe-Sugimoto, M., Nakamura, Y., Shimoishi, Y., & Murata, Y. (2007). The *coronatine-insensitive 1* mutation reveals the hormonal signaling interaction between abscisic acid and methyl jasmonate in *Arabidopsis* guard cells. Specific impairment of ion channel activation and second messenger production. *Plant physiology, 143*(3), 1398–1407.

Murata, Y., Mori, I. C., & Munemasa, S. (2015). Diverse stomatal signaling and the signal integration mechanism. *Annual Review of Plant Biology, 66*, 369–392.

Murata, Y., Pei, Z. M., Mori, I. C., & Schroeder, J. (2001). Abscisic acid activation of plasma membrane Ca 2+channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1-1* and *abi2-1* protein phosphatase2C mutants. *Plant Cell, 13*(11), 2513–2523.

Nozulaidi, M., Khairi, M., & Jahan, M. S. (2015). Effects of different salinity levels on rice production. *Australian Journal of Basic and Applied Sciences, 9*, 524–530.

Okuma, E., Jahan, M. S., Munemasa, S., Ogawa, K., Watanabe-Sugimoto, M., Nakamura, Y., … & Murata, Y. (2011). Negative regulation of abscisic acid-induced stomatal closure by glutathione in *Arabidopsis*. *Journal of Plant Physiology, 168*(17), 2048–2055.

Peñuelas, J., Munné-Bosch, S., Llusià, J., & Filella, I. (2004). Leaf reflectance and photo- and antioxidant protection in field-grown summer-stressed *Phillyrea angustifolia*. Optical signals of oxidative stress? *New phytology, 162*(1), 115–124.

Salisbury, F. B., & Ross, C.W. (1992). *Plant physiology*. Belmont, CA: Wadsworth.

Sasaki-Sekimoto, Y., Taki, N., Obayashi, T., Aono, M, et al. (2005). Coordinated activation of metabolic pathways for antioxidants and defense compounds by jasmonates and their roles in stress tolerance in Arabidopsis. *Plant Journal, 44*(4), 653– 668.

Sauter, A., Davies, W. J., & Hartung, W. (2001). The long-distance abscisic acid signal in the droughted plants: The fate of the hormone on its way from root to shoot. *Journal of Experimental Botany, 52*(363), 1991–1997.

Schwartz, S. H., & Zeevaart, J. A. D. (2010). Abscisic acid biosynthesis and metabolism. In P. J. Davies (Ed.), *Plant hormones* (3rd Ed.) (pp. 137–55). New York: Springer.

Seo, M., & Koshiba, T. (2002). Complex regulation of ABA biosynthesis in plants. *Trends Plant Science, 7*(1), 41–48.

Suhita, D., Raghavendra, A. S., Kwak, J. M., & Vavasseur, A. (2004) Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. *Plant Physiology, 134*(4), 1536–1545.

Tardieu, F., Zhang, J., Katerji, N., Bethenod, O., Palmer, S., & Davies, W. J. **(**1992). Xylem ABA controls the stomatal conductance of field-grown maize subjected to soil compaction or drying soil. *Plant Cell and Environment, 15*(2)**,** 193–197.

Taylor, I. B., Linforth, R. S. T., Alnaieb, R. J., Bowman, W. R., & Marples, B. A. (1988). The wilty tomato mutants Flacca and Sitiens are impaired in the oxidation of Aba-Aldehyde to ABA. *Plant and Cell Environment, 11*(8), 739–45.

Tsonev, T. D., Lazova, G. N., Stoinova, Z. G., & Popova, L. P. (1998). A possible role for jasmonic acid in adaptation of barley seedlings to salinity stress. *Journal of Plant Growth Regulation, 17*(3), 153–159.

Turner, J. G., Ellis, C., & Devoto, A. (2002). The jasmonate signal pathway. *Plant Cell, 14*(Suppl 1), 153–164.

Walton, D. C. (1983). Structure-activity relationships of abscisic acid analogs and metabolites. In F. T. Addicott (Ed.), *Abscisic acid* (p. 113–46). New York: Praeger.

Wang, X. C., & Jia, W. (1995). Stress-induced stomatal closure in relation to compartmentation and redistribution of ABA in intact leaves of *Vicia faba L*. *Acta Phytophysiologica Sinica, 21*(4), 324–328.

Wilkinson, S., & Davies, W. J. (1997). Xylem sap pH increase: A drought signal received at the apoplastic face of the guard cell that involves the suppression of saturable abscisic acid uptake by the epidermal symplast. Plant Physiology, 113(2), 559–573.

Wilkinson, S., & Davies, W. J. (2002). ABA-based chemical signalling: The co-ordination of responses to stress in plants. Plant and Cell Environment, 25(2), 195–210.

Wolf, O., Jeschke, W. D., & Hartung, W. (1990). Long-distance transport of abscisic acid in NaCl-treated intact plants of *Lupinus albus*. *Journal of Experimental Botany, 41*(5)**,** 593–600.

Xu, Z. Z., & Zhou, G. S. (2007). Photosynthetic recovery of a peren- nial grass *Leymus chinensis* after different periods of soil drought. *Plant Production Science, 10*(3), 277-285.

Xu, Z. Z., & Zhou, G. S. (2009). Shimizu H. Are plant growth and photosynthesis limited by pre-drought following rewatering in grass? *Journal of Experimental Botany, 60*(13), 3737–49.

Zhang, D. P. (Ed.). (2014). *Abscisic acid: Metabolism, transport and signaling*. New York, NY: Springer Dordrecht Heidelberg.

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In-vitro Antioxidant Potential in Leaf, Stem and Bark of *Azadirachta indica*

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ABSTRACT

Azadirachta indica (Meliaceae; Neem tree) is a resourceful medicinal plant. Almost all parts of the plant are used in traditional and folklore medicine. This study was conducted to measure antioxidant activity, total phenolic content (TPC) and total flavonoid content (TFC) in a crude extract of different parts of the neem tree. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging and Ferric-Reducing Antioxidant Power Assay (FRAP) were used to evaluate antioxidant activity, while total phenolic and total flavonoid compounds were measured using the Folin-Ciocalteu method and aluminium chloride ($AlCl_3$) assay, respectively. Bark and leaf extract showed higher free radical scavenging activity, with IC_{50} value of 23.27 and 55.07 (µg/ml) respectively, while seed extract showed the lowest activity, with IC_{50} of 672.36 (µg/ml; ($P<0.05$). The bark extract revealed significantly higher antioxidant activity and phenolic content than the leaf and seed extract ($P<0.05$). Flavonoid content in leaf extract was found to be significantly higher ($P<0.05$) than in seed and bark extract; however, bark extract showed significantly lower flavonoid content than seed extract. These results suggest that the potency of *Azadirachta indica* makes it a good source of natural antioxidant compounds.

Keywords: Antioxidants, flavonoids, Folin-Ciocalteu, IC_{50}, neem, phenolics

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INTRODUCTION

Azadirachta indica is an evergreen tree that grows up to 15 m in height. It has round, large crown branches, its bark is moderately thick, dark grey outside but reddish inside and it has a colourless, sticky foetid sap. Its light green pinnate leaves are crowded at the end of the branches and are

about 20-40 cm long. *Azadirachta indica*, popularly known as neem, originated from India and belongs to the Meliaceae family of plants (Pandey et al., 2014). The United States National Academy of Sciences published a report entitled, 'Neem – A Tree for Solving Global Problems'. Life on earth ultimately depends on plants. Every plant can be utilised for different purposes by man and animals; in fact, plants have long been used in traditional medicine as well as modern medicine (Nahak & Sahu, 2010). The neem plant has been used in Unani, Ayurveda and Homeopathy as a medicine to treat fever and infestation by intestinal worms as well as for tooth cleaning and appetite boosting (Biswas et al., 2002; Ghimeray et al., 2009). According to Nahak and Sahu (2010) more than 100 bioactive compounds, the most important being azdirachtin, are present in different parts of the neem plant. Azadirachtin, a potential antecedent, controls growth as well as reproductive regulating activities (Ghimeray et al., 2009; Pandey et al., 2014). The neem plant contains a high amount of phenolic and polyphenolic compounds, depending on the geographical location and environmental factors (Kaushik et al., 2007).

The need for natural antioxidants has increased because of demand from science and industry. The pharmaceutical, cosmetic and food industries rely on natural antioxidants because of the strong biological activity of medicinal plants as well as to avoid the side effects that come with the use of synthetic antioxidants (Nahak & Sahu, 2010). Plants that contain vitamins, flavonoids and polyphenols show high antioxidant activity (Gupta & Sharma, 2006). Natural antioxidants not only improve the quality of food but also that of human health as they prevent the chain reaction triggered by free radicals (Nahak & Sahu, 2010). While much research into the neem plant has been done, none has focussed on neem plants grown in the environmental conditions of Malaysia. Therefore, the aim of this research was to study the antioxidant potential of neem plants native to peninsular Malaysia.

MATERIALS AND METHOD

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), Dimethylsulphoxide (DMSO), quercetin, Folin-Ciocalteau, Na_2CO_3 and 2,4,6-Tripyridyl-s-Triazine (TPTZ), Gallic acid, methanol, $AlCl_3$ and K-acetate were the chemicals used in this experiment.

Materials from the Plant

Fresh leaves, fruit and bark of neem plants were collected from Universiti Sultan Zainal Abidin (UniSZA), Gong Badak campus, Terengganu, Malaysia. The Faculty of Bioresources and Food Industry, UniSZA, Tembila, Terengganu, Malaysia authenticated the plant, which was kept at the university herbarium.

Extract Preparation

The plant samples were washed properly and separated into leaf, fruit and bark

and then dried. The samples were oven-dried at 43°C, then extracted with 95% ethanol. The crude extract was filtered using Whatman number 1 filter paper and further concentrated on a rotary evaporator (EYELA N-1110, Tokyo) at 45-50°C, dried and kept at 4°C for further assay. The sample and solvent mass ratio was 1:2 during extraction; this was dissolved in DMSO, methanol and/or diluted double-distilled water for the final concentration according to requirement (Abdulkadir et al., 2016a).

Total Phenolic Content Assay (TPC)

Total phenolic content of the extracts was determined according to Ainsworth (2007), with some few modifications (Abdulkadir et al., 2015a). Folin-Ciocalteu (F-C) reagent was used in the experiment, where 250 µL of extract diluted in DMSO was put in a test tube and subsequently mixed with 1.25 ml of F-C reagent diluted in distilled water with a ratio of 1:9 followed by incubation for 10 minutes. A volume of 1 ml of 7.5% Na_2CO_3 solution was added and the extract was subsequently incubated for 30 minutes in the dark prior to data taking using a spectrophotometer at 650 nm. Gallic acid solution was used as a standard. Three replicates were maintained for this experiment.

Total Flavonoid Content Assay (TFC)

TFC was used to determine total flavonoid content using a previous method (Kalita et al., 2013) with some modification (Abdulkadir et al., 2015b). Methanol solutions of quercetin of volume 0.32 mg/mL were used and further dilutions of different concentrations were read by spectrophotometer at 415 nm. A calibration curve was prepared and used. A volume of 0.5 mL of extract from each part was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. Aluminium chloride reacts with flavonoids such as flavones and flavonols and forms stable acid complexes through the C-4 keto group and or between the C-3 and C-5 OH group. Three replicates were maintained.

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) Assay

Antioxidant activity in the extract of different parts of *Azadirachta indica* in DPPH assay was tested according to Clarke et al. (2013), with some slight modification (Abdulkadir et al., 2015c) i.e. 500 µL of extract diluted in methanol was mixed with 1 ml of DPPH in methanol (0.3 mM). The tubes containing the extract were kept in the dark for 30 minutes, after which the absorbance of the solution was measured at 517 nm using a spectrophotometer (SHIMAZU). Blanks, containing methanol only, were run concurrently with quercetin solution dissolved in methanol serving as a standard. IC_{50} concentration was established as the concentration able to reduce DPPH absorbance by 50%. The different samples of extract were first tested at the single concentration of 0.3 mM followed by

subsequent serial dilution that resulted in a range of concentration values. The DPPH scavenging effect (% inhibition) = $[A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the plant extract.

Ferric-Reducing Antioxidant Power Assay (FRAP)

The FRAP assay was carried out according to Benzie and Strain (1996) with slight modification (Abdulkadir et al., 2016b). The FRAP reagents were prepared using 10 mmol TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mmol HCl, 20 mmol iron (III) chloride aqueous solution and acetate buffer (pH 3.6) with the ratio 1:1:10 (v/v), respectively. The FRAP reagents were freshly prepared before starting the experiment and warmed at 37°C in a water bath for 30 minutes before use. Fifty microliters of sample were added into 1.5 mL of the FRAP reagent. The absorbance of the reaction mixture was measured using spectrophotometer at 593 nm after 30 minutes of incubation. A volume of 2000 µM of iron (II) sulfate solution was used as a standard and further diluted to 1000, 500, 250, 125, 62.5 and 31.25 µM. The results were expressed as µmol Fe(II)/g dry weight of the plant material. Three replications were maintained and the mean values were calculated.

Statistical Analysis

Each assay was subjected to one-way analysis of variance using the Statistical Package for the Social Sciences (SPSS, version 17.). A significance level of 0.05 % was used to test differences among the samples used.

RESULTS

Total Phenolic Content

The standard curve of Gallic acid and quercetin solution was used to calculate total phenolic and total flavonoid content in the fruit, stem and leaf of the neem plant (Table 1). The data collected on GAE (Gallic acid equivalents) and QAE (Quercetin equivalents) of the extract from the different parts of the plant were interpreted. In the equation formulated, Y represented average absorbance of the sample and X, the amount of Gallic acid or quercetin acid in µg/ml**.**

Table 1
Linear equations and their R^2 values obtained from the standard calibration curve

Assay	Calibration curve	R^2
TFC	Y=0.0025X+0.0708	0.9986
TPC	Y=0.0088X+0.0932	0.9991
FRAP	Y=0.0003X+0.7706	0.9995

Table 2
Estimation of total phenolic content (TPC; mg GAE/g) in different plant parts of Azadirachta indica

Sample	TPC (mg GAE/g)
Leaf	136.45±0.99[b]
Seed	30.43±1.16[c]
Bark	285.77±4.49[a]

[a, b & c]Indicate a significant difference at ($P < 0.05$)

Table 3
Estimation of the total flavonoid content (TFC; mg GAE/g) in different parts of Azadirachta indica

Sample	TFC (mg QAE/g)
Leaf	77.01±3.11[a]
Seed	8.08±0.69[b]
Bark	2.21±0.23[c]

[a, b & c]Indicates a significant Different at ($P < 0.05$)

Phenolic content in the samples were significantly different. Bark extract contained the highest level of phenolic content, 285.77±4.49 (mg GAE/g; P<0.05), which was significantly higher than 136.45±0.99 (mg GAE/g) in the leaves of the neem plant (Table 2). Furthermore, the leaves showed significantly higher (P<0.05) phenolic content than the seed, which showed a content of 30.43±1.16 (mg GAE/g; Table 2).

Total Flavonoid Content

The total flavonoid content in different samples was calculated via linear equation obtained from the standard calibration curve of the quercetin acid (Table 1). The flavonoid content of the leaf extracts was 77.01±3.11 (mg QAE/g; P<0.05), which was significantly higher than the value of 8.08±0.69 in the seed extract (Table 3). A similar result was observed when we compared the flavonoid content between the seeds and the bark. The seeds showed a significantly higher flavonoid content than the bark, which showed a flavonoid content of 2.21±0.23 (Table 3).

DPPH Antioxidant Activity

DPPH radical scavenging was used to determine antioxidant activity in the extract; this method reduces stable radical compounds, turning the substance from purple to yellow. The inhibition percentage in the leaf extract was 90.32% in bark, which was similar to the control (quercetin) but significantly higher (P<0.05) than the value of 88.43% recorded for the leaf extract (Table 4). A similar result was observed when we compared the inhibition percentage of the seeds and the leaves. The leaves showed a significantly higher percentage of inhibition (P<0.05) than the seeds at 69.83% (Table 4). Antioxidant potential of the sample was determined by the amount of antioxidant needed to scavenge 50% of DPPH free radicals (IC_{50}) at 517 nm. The IC_{50} result showed significant difference (P<0.05). Quercetin (control) was found to have lowered IC_{50} values of 15.64 µg/ml compared to the extract used, which significantly differed between the seeds and leaves, showing slight difference of value from that of the bark extract, which had an IC_{50} of 23.27 µg/ml.

Table 4
DPPH percentage inhibition and IC_{50} in the different parts of Azadirachta indica

Sample	DPPH % inhibition (µg/ml)	IC_{50} (µg/ml)
Leaf	88.43[b]	55.07[b]
Seed	69.83[c]	672.36[a]
Bark	90.32_{a}	23.27[c]
Quercetin	90.28[a]	15.64[d]

[a, b & c] Indicates a significant difference at ($P<0.05$)

Ferric-Reducing Antioxidant Power Assay (FRAP)

The result revealed changes in absorbance of the control ferrous (II) sulphate along the concentration gradient; therefore, it could be concluded that absorbance depends on the increase of concentration. The ability of the samples used to reduce Fe^{3+} to Fe^{2+} was expressed as equivalence of ferrous sulphate (mM), and the results were calculated using the equation obtained from the standard curve of Fe (II) sulphate (Table 1). The FRAP value of 1668.44±11.10 (mM Fe (II)/g; $P<0.05$) in the extract of bark was significantly higher than that of 1131.78±34.65 (mM Fe (II)/g) and 267.89±50.04 (mM Fe (II)/g) in the leaves and seeds, respectively (Table 5). In addition, the FRAP value of the leaf extract was significantly higher ($P<0.05$) than that of the leaf extract (Table 5).

Table 5
Ferric-reducing antioxidant power of different parts of Azadirachta indica

Sample	FRAP value (mM Fe (II)/g)
Leaf	1131.78±34.65[b]
Seed	267.89±50.04[c]
Bark	1668.44±11.10[a]

[a, b & c] Indicates a significant difference at ($P<0.05$)

DISCUSSION

Total Flavonoid Content

Naturally occurring flavonoid compounds have a polyphenolic structure, which is mostly soluble in water and mainly occurs in a plant as a sugar derivative known as glycoside. Pigments in flowers, fruit and seeds are responsible for flavonoids (Maria de Lourdes, 2013). In this study, flavonoid content in the leaf extract was higher than in other parts of the neem plant; it was highest in the seed extract, followed by the bark (Table 3), which is consistent with the results of Ghimeray et al. (2009) and Kiranmai et al. (2012) but slightly different from those of Naseer et al. (2014), concluded that the highest flavonoid content was found in the leaf extract followed by the bark and then the seed extract. Nevertheless, Abdulkadir et al. (2015a) recently reported high flavonoid content in *Moringa oleifera* leaves compared with that of bark and seed extract of the plant. This may be related to the higher cellular activities in the leaf. These activities are related to providing protection for the leaves from environmental stress. Total flavonoid content observed in this study was found to be high in the leaf extract but

low in the seed extract (Kiranmai et al., 2012). These differences may be attributed to solvent polarity, geographical location and concentration used in the experiment.

DPPH Assay

Free radical scavenging assay is the most rapid, reliable and sensitive method for measuring antioxidant activity in plant samples, measuring the absorbance of DPPH stable radicals spectrophotometrically (Waghulde et al., 2011). This study showed a decrease in percentage (%) inhibition in the following order: seed > leaf > bark > control (Table 4). This figures recorded in this study were higher than those recorded in previous studies such as those by Naseer et al. (2014), Nahak and Sahu (2010) and Abdulkadir et al. (2016a) but consistent with those recorded by Waghulde et al. (2011). However, the result stated by Ramamurthy et al. (2012) using a different solvent of *Solanum torvum* fruit at 500 µg/ml showed higher scavenging activity than was found in this study. Quercetin had a lower IC_{50} value 15.64 (µg/ml) than did the bark and leaf extract. Nevertheless, the seeds showed a significantly higher amount of IC_{50} than the control, probably due to the higher antioxidant potential but low antioxidant activity (Table 4). This result is consistent with the findings of Kiranmai et al. (2012) of low IC_{50} in leaf extract compared to seed extract of the same plant. However Pandey et al. (2014) reported a higher IC_{50} for the leaves of *Azadirachta indica* than was found in this current research. This difference may be attributed to the differences in solvent polarity, concentration and environmental factors.

FRAP Assay

The ferric-reducing power (FRAP) assay encompasses transfer of an electron, through which Fe^{3+} / ferricyanide complex is reduced to the ferrous (Fe^{2+}) form. The reducing compounds are an indicator of the substance's electron-donation potential and antioxidant activity (Cheynier, 2012). The FRAP value of bark extract was found to be higher than that of the leaf to seed extract (Table 5), indicating higher electron mobilisation in the bark due to direct contact with environmental conditions. This result is consistent with the result obtained by Kiranmai et al. (2012) using ethanolic extract of *A. indica* and by Abdulkadir et al. (2015b) using methanolic extract of *Moringa oleifera.* This difference may be related to the differences in solvent polarity, concentration and environmental factors.

CONCLUSION

Many studies have focussed on natural antioxidants from fruit, vegetables and medicinal plants. The results obtained may be related to high prevalence of oxidative diseases as well as the lethal effect of some synthetic antioxidants. Medicinal plants play a vital role in the treatment of many oxidative-related problems. The results obtained in this study from the DPPH, FRAP assay and total phenolic

and flavonoid content tests showed the potentiality of *Azadirachta indica* as a source of natural antioxidants. Bark and leaf extract of the plant revealed that these parts of the plant held the most potent antioxidant activity. Other species of neem under different environmental conditions can be examined in future studies for a more complete understanding of the antioxidant potential of this plant.

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REFERENCES

Abdulkadir, A. R. Zawawi, D. D., & Jahan, M. S. (2015a). DPPH antioxidant activity, total phenolic and total flavonoid content of different part of Drumstic tree (*Moringa oleifera* Lam.). *Journal of Chemical and Pharmaceutical Research 7*(4), 1423–1428.

Abdulkadir, A. R., Zawawi, D. D., & Jahan, M. S. (2016a) Proximate and phytochemical screening of different parts of *Moringa oleifera. Russian Agricultural Sciences, 42*(1), 24–26.

Abdulkadir, A. R., Jahan, M. S., & Zawawi, D. D. (2015b). Effect of chlorophyll content and maturity on total phenolic, total flavonoid contents and antioxidant activity of *Moringa oleifera* leaf (Miracle tree). *Journal of Chemical and Pharmaceutical Research, 7*(5), 1147–1152.

Abdulkadir, A. R., Zawawi, D. D., Yunusa, A. K., & Jahan, M. S. (2015c). *In Vitro* Anti-Oxidant Potential, Total Phenolic Content and Total Flavonoid Content of Methanolic Flower and Seed Extract of Miracle Tree (*Moringa Oleifera* Lam.). *Australian Journal of Basic and Applied Sciences, 9*(20), 27–31.

Abdulkadir, A. R., Nashriyah, M., Hasan, M. M., & Jahan, M. S. (2016b). *In Vitro* antioxidant activity of the eyhanolic extract from fruit, stem, and leaf of *Solanum torvum. Science Asia, 42*(3), 184–189.

Ainsworth, A. E., & Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using folin-ciocalteu reagent. *Nature Protocols, 2*(4), 875–877.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Analytical Biochemistry, 239*(1), 70–76.

Biswas, K., Chattopadhyay, I., Benerjee, R. K., & Bandyopadhyay, U. (2002). Biological activities and medicinal properties of neem (*Azadirachta indica*). *Current Science*, *82*(11), 1336-1345.

Cheynier, V. (2012). Phenolic compounds: from plants to foods. *Phytochemistry Review, 11*(2-3), 153–177.

Clarke, G., Ting, K., Wiart, C., & Fry, J. (2013). High correlation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing activity potential and total phenolics content indicates redundancy in use of all three assays to screen for antioxidant activity of extracts of plants from the Malaysian Rainforest. *Antioxidants, 2*(1), 1–10.

Ghimeray, A. K., Jin, C. W., Ghimire, B. K., & Cho, D. H. (2009). Antioxidant activity quantitative estimation of azadirachtin and nimbin in *Azadirachta indica* A. juss grown in foothills of Nepal. *African Journal of Biotechnology, 8*(13), 3084–91.

Giada, M. D. L. R. (2013). Food Phenolic Compounds: Main Classes, Sources and Their Antioxidant Power. In J. A. Morales-González (Ed.), *Oxidative Stress and Chronic Degenerative Diseases - A Role for Antioxidant*s (pp. 87-112). Rijeka: Croatia InTech.

Gupta, V. K., & Sharma, S. K. (2003). Plant as natural antioxidants. *Natural Product Radiance, 5*(4), 326–334.

Kalita, P., Barman, T. K., Pal, T. K., & Kalita, R. (2013). Estimation of total flavonoids content (TFC) and antioxidant activities of methanolic whole plant extract of *Biophytum sensitivum* linn. *Journal of Drug Delivery and Therapeutics,* 3(4), 33–37.

Kaushik, N., Singh, G. B., Tomar, U. K., Naik, S. N., Satya, V., Bisla, S. S., Sharma, S. K., & Thakkar, P. (2007). Regional and habitat variability in azadirachtin content of Indian neem (*Azadirachta indica* A Juss.). *Current Science, 92*(10), 1400–1406.

Kiranmai, M., Sri, U., Ibrahim, M., & Kumar, C.B.M. (2012). Antioxidant activity and total flavonoids content of different parts of *Azadirachta indica* A. Juss. *Journal of Medicinal Plants Research, 6*(46), 5737–5742.

Maria de Lourdes, R. G. (2013). Food Phenolic Compounds: Main Classes, Sources and Their Antioxidant Power. In J. A. Morales-Gonzalez (Ed.), *Oxidative Stress and Chronic Degenerative Diseases - A Role for Antioxidants*. InTech. doi: 10.5772/51687. Retrieved from: http:// www.intechopen.com/books/ oxidative-stress-and-chronic-degenerative-diseases -a-role-for-antioxidants/food-phenolic-compounds-main -classes-sources-and-their-antioxidant-power

Nahak, G., & Sahu, R. K. (2010). *In vitro* antioxidative acitivity of *Azadirachta indica* and *Melia azedarach* Leaves by DPPH scavenging assay. *Nature Science, 8*(4), 22–28.

Naseer, R., Sultana, B., Anwar, F., Mehmood, Z., & Mushtaq, M. (2014). Variations in phenolics, antioxidant and antifungal activities among different parts of selected medicinal plants. *Pakistan Journal of Botany, 42*(2), 705–712.

Pandey, G., Verma, K. K., & Singh, M. (2014). Evaluation of phytochemical, antibacterial and free radical scavenging properties of *Azadirachta indica (neem)* leaves. *International Journal of Pharmaceutical Sciences, 6*(2), 444–447.

Ramamurthy, C. H., Kumar, M. S., Suyavaran, V. S. A., Mareeswaran, R., & Thirunavukkarasu, C. (2012). evaluation of antioxidant, radical scavenging activity and polyphenolics profile in *Solanum torvum* L. fruits. *Journal of Food Science, 77*(8), 907–913.

Waghulde H., Kamble, S., Patankar, P., Jaiswal, B., Pattanayak, S., Bhagat, C., & Mohan, M. (2011). Antioxidant activity, phenol and flavonoid contents of seeds of *Punica granatum* (Punicaceae) and *Solanum torvum* (Solanaceae). *Pharmacology Online, 1*, 193–202.

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Effects of Elemental Sulphur Application Rates on Soil Al^{3+} Solubility and Its Concentration in Maize Plants (*Zea mays L.*)

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ABSTRACT

A greenhouse experiment was conducted to elucidate the influence of soil acidification due to application of different doses of elemental sulphur (0, 0.5, 1.0 and 2.0 g S kg^{-1} soil) on Al^{3+} solubility at 0, 20 and 40 days after incubation. Maize plants were grown after soil treatment with the elemental S and were allowed to grow for 45 days. The results showed that addition of elemental sulphur significantly increased the soil acidity; each g S decreased soil pH for 1.52 units. The Al^{3+} concentration in soil remained rather unchanged from the pH value of 7.3 to around 5 and experienced a 22000-time increase at the pH value of 4. Soil acidification from the background of 7.03 to 6.29 resulted in 41.83% increase in root Al^{3+} concentration and it was not significantly affected with further soil acidification. Soil acidification progressively decreased Al^{3+} concentration in the stem but it failed to affect Al^{3+} concentration in the leaves. The optimum rate of sulphur for maize without the risk of Al^{3+} toxicity under greenhouse conditions was 0.82 g S kg^{-1} soil. Further evaluation under field conditions is required.

Keywords: Soil acidification, Al^{3+} toxicity, maize growth

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INTRODUCTION

The high pH soils that are mainly located in arid regions amount to about 50 mil km^2 or more than 30% of world soils (Manahan, 2004; Shenker et al., 2005). In addition, these soils can be found in several isolated areas, especially in regions near limestone hills, which occur widely in Malaysia (Tan, 2002). Plant growth is usually restricted

in high pH soils because of the limited availability of essential nutrients (Lindsay, 1979; Shenker et al., 2005; Wang et al., 2006). Therefore, acidification of high pH soils is recommended to enhance nutrient availability and to improve plant growth (Ye et al., 2011).

Elemental sulphur is of special interest in increasing soil nutrient mobility (Jankowski et al., 2015; Sienkiewicz-Cholewa & Kieloc, 2015) as it is slow-release acidifying and is commonly available (Chien et al., 2011). The acidifying function of S originates from its microbial oxidation to sulphuric acid over time (Vidyalakshmi et al., 2009). However, according to some authors, application of elemental sulphur in soil amendment in their studies did not show significant change in soil chemical properties such as acidity and nutrient availability (Sameni et al., 2004; Shenker & Chen, 2005; Skwierawska et al., 2012). This might be due to both unsuccessful oxidation of applied sulphur as well as high carbonate content of the soil. However, successful oxidation of elemental sulphur and a significant change in soil chemical properties and nutrient availability are well documented for some soils (Ye et al., 2010; Khalid et al., 2012). Nonetheless, the response of nutrients to soil acidification is difficult to predict. In addition, their interaction affects their availability to crops as an over-abundance of one nutrient may result in a deficiency of another. For instance, soil acidification may increase Al solubility in soil; Al is not considered an essential nutrient (Meriño-Gergichevich et al., 2010). Among all available Al species trivalent aluminum (Al^{3+}) is the most toxic to plants and its concentration enhances in acidic conditions. Al-toxicity results in alterations of the physiological and biochemical processes of plants and consequently their productivity. The decrease in root growth is one of the initial and most evident symptoms of Al-toxicity at micro-molar concentrations in plants (Meriño-Gergichevich et al., 2010).

The present study was conducted to elucidate the effect of elemental S on Al^{3+} concentrations in the Bintang Series of soil and its effect on maize growth. In addition, the effect of elemental S on Al^{3+} accumulation in the root and the above-ground parts of maize plants in greenhouse conditions was also studied.

MATERIALS AND METHOD

Experimental Site

Soil samples were collected from the A horizon (0-20 cm) of the Bintang Series of soil located in Perlis, Malaysia (6° 31′ 01.61″ N and 100° 10′ 12.43″ E). The area, Bukit Bintang, is affected by limestone parent material and is host to natural vegetation (Karimizarchi et al., 2014). The experiment was conducted in greenhouse conditions at University Putra Malaysia (UPM).

Planting and Cultural Practices

Sweet maize (*Zea mays* L.) seeds, Masmadu, were collected from the

Malaysian Agricultural and Development Research Institute (MARDI). The seeds were germinated in laboratory conditions and transplanted to plastic pots (30 cm diameter and 50 cm height). Each pot contained 10 kg soil and held three plants, which were thinned to one within one week. The seedlings were grown for 45 days in greenhouse conditions. The pots were weighed and irrigated daily to maintain 90% soil field capacity moisture content. Fertiliser was applied according to MARDI's recommendation: 120 kg N/ha in the form of urea, 60 kg/ha P_2O_5 in the form of triple superphosphate and 40 kg/ha K_2O in the form of muriate of potash (Karimizarchi et al., 2014).

Experimantal Design and Treatment

The experiment was conducted in a completely randomised design (CRD) with four replications. There were 12 treatments including four levels of sulphur (0, 0.5, 1 and 2 g S per kg of soil) and three sampling times (0, 20 and 40 days) before the planting of maize.

Plant and Soil Sampling and Analysis

Soil samples were collected before the maize planting and after harvest. The soil samples were air dried and ground (<2 mm) before use. Soil electrical conductivity and pH were measured in a soil-water suspension (10 g soil to 25 ml deionised water) 24 hours after shaking for 30 min on a reciprocal shaker. Total carbon, nitrogen and sulphur was determined using a CHNS LECO analyser. Soil mechanical analysis was done using the pipette method (Gee et al., 1986) and textural class was determined by United States Department of Agriculture (USDA) soil textural triangle. Available Al^{3+} concentration in the soil was extracted using the un-buffered and neutral extracting solution of $CaCl_2$ (Jones, 2001; Ye et al., 2011).

The maize plants were harvested after 45 days. Plant leaf, shoot and root tissue was separately washed in deionised water, then dried at 65°C and weighed. After grinding, weighed plant tissue was ashed in a muffle furnace at 480°C for about 10 h and dissolved in a diluted acid mixture (Jones, 2001). Al concentration was determined by ICP-OES (Perkin Elmer, Optima 8300).

Statistical Analysis

To model the relationship between plant and soil properties, data collected were subjected to different regression models at the probability level of 0.05 with the help of the Sigmaplot software. The analysis of variance for different parameters was done following ANOVA technique. When F was significant at $p \leq 0.05$ level, treatment means were separated using DMRT. Data were analysed following standard procedure using SAS software (version 9.1).

RESULTS AND DISCUSSION

Physicochemical Properties of Bintang Series Soil

The physicochemical characteristics of the Bintang Series soil are presented in

Table 1. Being silt loam in texture, the soil was found to be slightly alkaline in nature (pH=7.3) as it was affected by limestone parent material from the nearby hills. Base saturation was high, (56%); however, the calcium carbonate content of the soil was not detected. Low calcium carbonate content that could be attributed to the high precipitation of the area, implied that the soil buffering capacity was low and did not need a high amount of acidic soil amendment such as elemental sulphur to reduce soil pH.

Table 1
Soil physico-chemical properties of Bintang series soil

Soil property	Unit	Value or Concentration	Soil property	Unit	Value or Concentration
pH	-	7.30	Sand	%	9.00
$CaCO_3$	%	Trace	Silt	%	66.40
C	%	1.75	Clay	%	24.60
N	%	0.12	Texture	-	Silt loam
S	%	0.004	FC	%	20.00
C/N	-	14.58	CEC	$Cmol_+ kg^{-1}$ soil	11.50
C/S	-	437.50	BS	%	56.00

Effect of Elemental Sulphur on Soil Acidity

Significant negative linear regression (P<1%) was found between soil pH and sulphur application rate (Figure 1), while increasing S rate soil pH decreased from the initial value of around 7.03 to 6.29, 5.26 and 3.94 at sulphur application rates of 0.5, 1 and 2 g kg^{-1}, respectively. The regression line slopes downwards with a slope of -1.52, which is consistent with the negative relationship anticipated between S rate and soil pH (Shenker & Chen 2005; Cui et al., 2004; Vidyalakshmi et al., 2009). This result reflected the successful oxidation of elemental sulphur in the Bintang Series soil and may have affected the availability of nutrients in the soil.

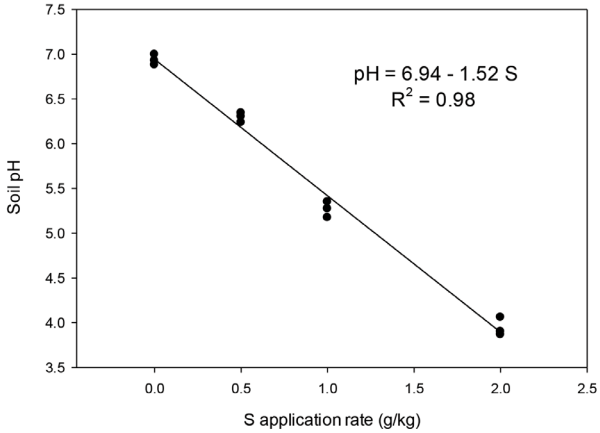

Figure 1. Effect of elemental sulphur application rates on Bintang Series soil pH

Effect of Elemental Sulphur Application Rate on Soil Extractable Al^{3+}

Sulphur application rate had a significant effect on Al^{3+} solubility in the Bintang Series soil (Figure 2). Application of 2 g S $kg^{-1}$of soil increased extractable Al^{3+} from the background of 0.001 mg kg^{-1} prior to S application to 21.78 mg kg^{-1} after 40 days and tended to level off thereafter. Application of elemental sulphur up to 1 g S kg^{-1} soil did not affect Al^{3+} solubility. The low concentration of Al^{3+} at the first three sulphur application rates was in line with the findings of Meriño-Gergichevich et al. (2010) i.e. most of the Al was bound to insoluble forms such as aluminosilicates or precipitated as Al hydroxide. They reported that Al was solubilised from silicates and oxides to Al^{3+} under low pH conditions. They also reported that there were various Al forms in soil and that their concentration depended on the degree and duration of Al compound hydrolysis. In addition, they found a significant correlation between low pH and high concentrations of phytotoxic Al species, which is related to the reduction of exchangeable bases in the soil solution. Below is the explanation of the relationship between soil Al^{3+} and soil pH in conditions of our experiment as we discovered.

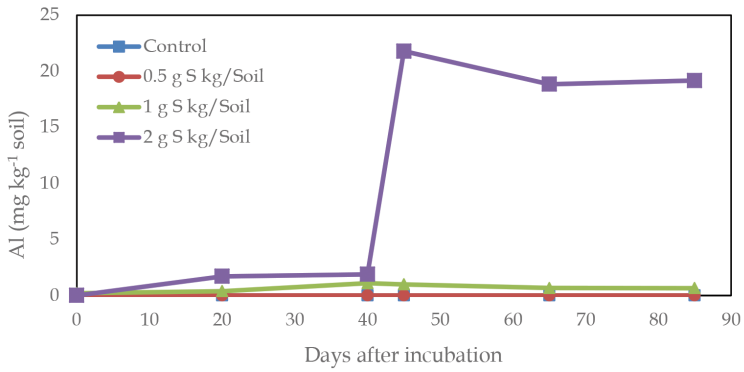

Figure 2. Effect of elemental sulphur on Al^{3+} concentration at different incubation times

Relationship Between Soil Al^{3+} Concentration and Soil pH

Bintang Series soil acidification significantly increased soil Al^{3+} solubility (Figure 3). The relationship between soil pH and Al^{3+} concentration was nonlinear, $Al = \frac{22.0}{\left(1+\left(\frac{pH}{4.3}\right)^{16.94}\right)}$, $R^2 = 0.94^{**}$ and soil Al^{3+} concentration failed to change from a pH value of 7 to 5, after which a sharp increase in Al^{3+} concentration was observed. This was in line with the data presented by Franz et al. (2007), Hesterbeg et al. (1993) and Ward et al. (2011). They reported the release of plant-available Al at pH ≤5.5. In addition, McBride (1994) revealed that once soil pH is lowered to below 5.5, aluminosilicate clay and Al hydroxide minerals begin to dissolve, releasing Al-hydroxyl cations. Al^{3+} will then exchange

with other cations from soil colloids, resulting in a build-up of Al^{3+} concentration in soil solution. As stated by Lambers et al. (2008) and Viani et al. (2014), the increase in weathering rate, the change in oxidation state of some nutrients and the displacement of cations from exchangeable sites due to high concentration of hydrogen ions accounted for the increases in soil nutrient mobility.

It was noted that the nonlinear relationship between soil pH and Al^{3+} concentration can be linearised if the relationship between the log of Al^{3+} concentration as a function of soil pH is considered, log Al=7.5-1.56 pH, R^2=0.92**. Using the solubility equation of Gibbsite (pAl=3pH-8.5), with 1 unit decrease in soil pH, Al solubility increases 10^3 times; in the conditions of our experiment, it increased $10^{1.56}$ times.

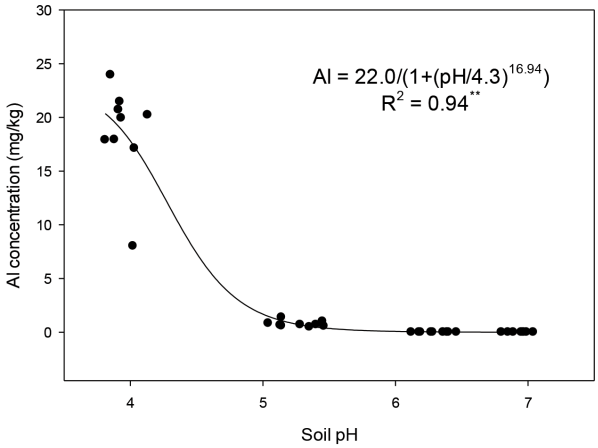

Figure 3. Effect of soil pH on Al^{3+} concentration in Bintang Series soil

Effect of Elemental Sulphur on Maize Growth

The relationship between sulphur application rates and maize dry weight was quadratic in nature (Figure 4). Maize dry weight increased as elemental sulphur rate increased up to 0.82 g kg^{-1} soil, after which there was a sharp decrease in maize dry weight. This was mainly due to the significant increase in soil Mn and Zn availability (Karimizarchi & Aminuddin, 2015) and uptake by maize as demonstrated by Karimizarchi et al. (2014b). In addition, our results showed that an addition of 2 g S kg^{-1} significantly decreased total dry weight of maize by 38.34% compared to the control (Figure 4). Although this reduction in maize biomass was related to the Mn and Zn toxicity (Karimizarchi et al., 2014b), the contribution of Al^{3+} toxicity remained unknown. Therefore, the effect of elemental sulphur on Al^{3+} concentration in different parts of maize became clear. At the same time, we also studied the relationship between Al^{3+} concentration in maize and growth.

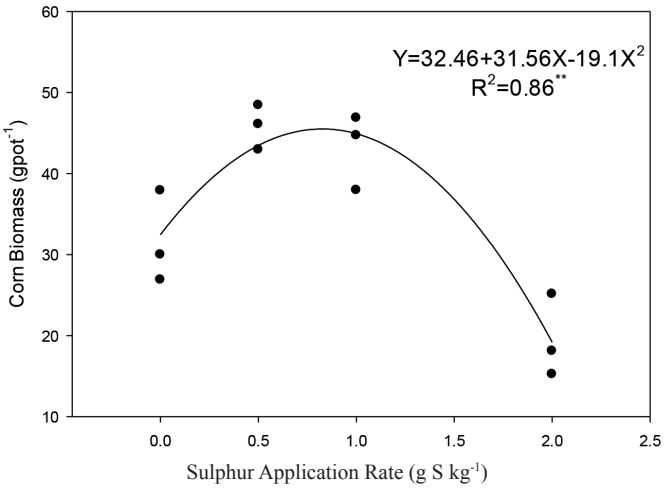

Figure 4. Effect of elemental sulphur application rate on maize dry weight

Relationship between Leaf Al^{3+} Concentration and Leaf Dry Weight

There was a linear relationship between leaf biomass and leaf Al^{3+} concentration (Figure 5). With one unit increase in leaf Al^{3+} concentration, leaf dry matter decreased by 8.49 g per pot. Although this was in line with the generally accepted theory that Al^{3+} is not considered an essential nutrient (Meriño-Gergichevich et al., 2010), the coefficient of determination for this relationship was low i.e. 0.5. In other words, Al^{3+} concentration in the leaves explained only 50% of the variation in leaf performance; other nutrients may have had a crucial effect on the maize growth. This conclusion was supported by the fact that Al^{3+} concentration in the leaves was far below the phytotoxicity level of Al^{3+}, 13 µg g^{-1} (Lidon et al., 2002). Karimizarchi et al. (2014b) also reported the toxicity of Mn and Zn in maize plants treated with 2 g S kg^{-1} soil.

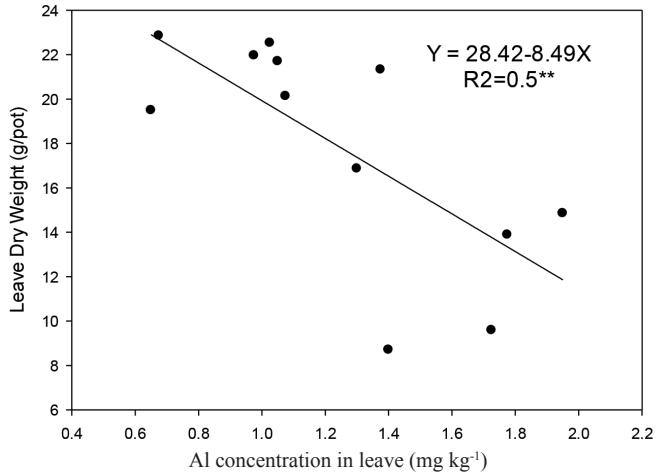

Figure 5. Relationship between Al^{3+} concentration and dry weight of maize leaf

Relationship between Root Al^{3+} Concentration and Root Dry Weight

The relationship between Al^{3+} concentration in root and root dry weight was quadratic in nature (Figure 6). Root dry weight increased as root Al^{3+} concentration increased up to 187.5 mg kg^{-1}, after which there was a slight decrease in root dry weight. This observation may signify the beneficial role of Al^{3+} on maize root growth. In line with our finding, Lee (2013) and Barker et al. (2007) also found a beneficial effect of low levels of aluminum on root and shoot growth of non-accumulator plants such as soybean and maize. However, their findings are contrary with the general belief that the specific biological functions of Al^{3+} for plants are unknown and this mineral is not regarded as an essential plant nutrient (Meriño-Gergichevich et al., 2010; Fernandes et al., 2013). The nontoxic high concentration of Al^{3+} in maize root in the conditions of our experiment can be related to the detoxifying role of sulphate (Meriño-Gergichevich et al., 2010; Robson, 2012; Karimizarchi et al., 2015), phosphate (Bennet et al., 1986; Robson, 2012), organic acid release by plants (Feng et al., 2001) and phosphorous deficiency (Bennet et al., 1986; Ward et al., 2011 & Karimizarchi et al., 2016). The relationship between sulphur application rate and Al^{3+} concentration in the roots is another confirmation of the nontoxic effect of Al^{3+} in maize in conditions of our experiment (Figure 7).

The maximum Al^{3+} concentration in root was found to be 0.5 mg kg^{-1} S, where maximum yield was obtained (Figure 4) but not in maximum S rate where the minimum plant growth (Figure 4) and maximum Al^{3+} concentration in soil (Figure 2) was achieved.

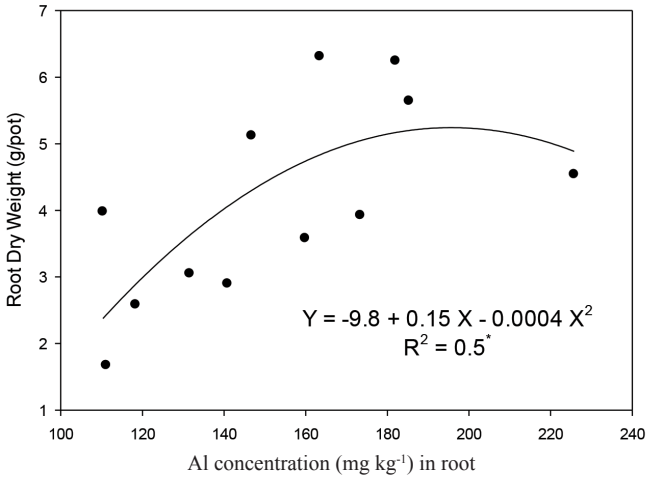

Figure 6. Relationship between Al^{3+} concentration and dry weight of maize

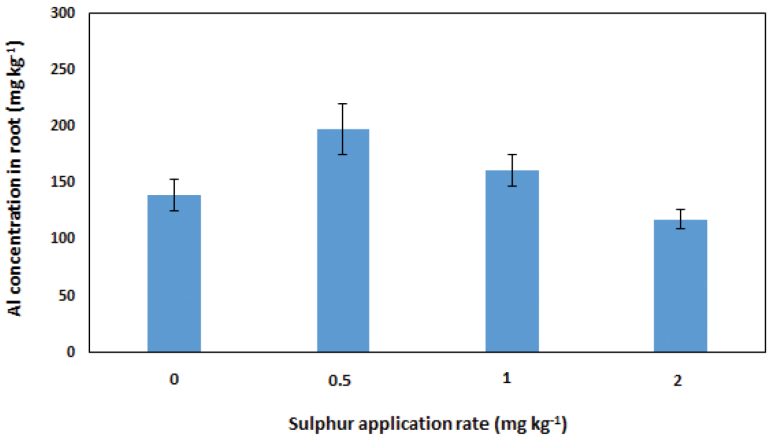

Figure 7. Effect of elemental sulphur rate on root Al^{3+} concentration (mg kg^{-1}). Bars show standard errors

Relationship between Stem Al^{3+} Concentration and Stem Dry Weight

The insignificant relationship between Al^{3+} in maize stem and stem performance (data not shown) is another confirmation of the nontoxic effect of Al^{3+} in maize in the conditions of our experiment. This conclusion is further supported by the decreasing trend in Al^{3+} concentration in the stem as a function of elemental sulphur application rate (Figure 8) and signifies the Al^{3+} exclusion ability of maize as stated by Taylor (1988) and Kochian (1995).

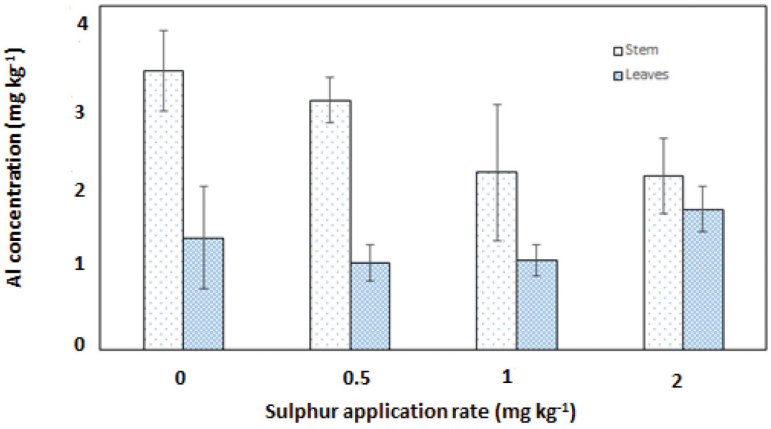

Figure 8. Effect of elemental sulphur application rate on Al^{3+} concentration in maize leaf and stem. Bars show standard errors

Al^{3+} is Immobile Inside the Maize Plant

The distribution of Al^{3+} in different parts of the maize plants grown in the conditions of our experiment signified the ability of maize to prevent Al^{3+} transfer from root to stem and leaves. Al^{3+} concentration in stem ranged from 2.02 to 3.24 mg kg^{-1} (Figure 8) and was considered very low compared with the concentration in the root, where it ranged from 110 to 225 mg kg^{-1} (Figure 7). Our data also demonstrated the lower Al^{3+} concentration in maize leaves (varied from 1 to 1.63 mg kg^{-1}) than in the stem. In addition, the low ratio of Al^{3+} concentration in maize leaves and stem to root, 0.008 and 0.016 respectively, demonstrated that Al^{3+} is an immobile nutrient in maize. This is in line with Barker and Pilbeam's (2007) finding. They reported a greater value of Al^{3+} concentration in the roots than in the young leaves of maize.

CONCLUSION

In this study, application of elemental sulphur decreased pH of the Bintang Series soil in linear trend. However, soil Al^{3+} solubility was not significantly affected unless soil acidity dropped to a pH of around 4, the pH of hydrous oxide precipitation, where application of 2 g S kg^{-1} soil increased the $CaCl_2$ extractable Al^{3+}. In addition, the 22000-time increase in soil Al^{3+} solubility due to application of 2 g S kg^{-1} soil failed to increase Al^{3+} concentration in maize tissue. The optimum rate of sulphur for maize production in greenhouse conditions was 0.82 g S kg^{-1} soil. Al^{3+} toxicity was not implicated in the significant decrease in maize growth at sulphur rate of 2 g S kg^{-1} soil.

REFERENCES

Barker, A. V., & Pilbeam, D. J. (2007). *Handbook of plant nutrition.* New York: CRC Press.

Bennet, R. J., Breen C. M., & Fey, M. V. (1986) Aluminium toxicity and induced nutrient disorders involving the uptake and transport of P, K, Ca and Mg in *Zea mays* L. *South African Journal of Plant and Soil, 3*(1), 11–17.

Carver, B. F., Inskeep, W. P., Wilson, N. P., & Westerman, R. L. (1988). Seedling tolerance to aluminum toxicity in hard red winter wheat germplasm. *Crop Science, 28*(3), 463–467.

Chien, S. H., Gearhart, M. M., & Villagarcía, S. (2011). Comparison of ammonium sulfate with other nitrogen and sulfur fertilizers in increasing crop production and minimizing environmental impact: A review. *Soil Science, 176*(7), 327–335.

Franz, B., Lichtenberg H., Hormes J., Modrow, H., Dahl, C., & Prange, A. (2007). Utilization of solid 'elemental'sulfur by the phototrophic purple sulfur bacterium Allochromatium vinosum: A sulfur K-edge X-ray absorption spectroscopy study. *Microbiology, 153*(4), 1268–1274.

Feng, J. M., Peter, R. R., & Delhaize, E. (2001). Aluminium tolerance in plants and the complexing role of organic acids. *Trends in Plant Science, 6*(6), 373–278.

Fernandes, M. B., Sebastião, I. M., Moacir, C. B., Augusto, M. B., José, O. G. A., & Takeyoshi, T. I. (2013). Aluminum in corn plants: Influence on growth and morpho-anatomy of root and leaf. *Revista Brasileira de Ciência do Solo, 37*(1), 177–187.

Gee, G. W., Bauder, J. W., & Klute, A. (1986). *Particle-size analysis. Methods of soil analysis. Part 1. Physical and mineralogical methods.* Madison, Wisconsin: American Society of Agronomy Inc.

Gigliotti, G., Businelli, D., & Giusquiani, P. L. (1996). Trace metals uptake and distribution in corn plants grown on a 6-year urban waste compost amended soil. *Agriculture, Ecosystems and Environment, 58*(2), 199–206.

Hesterberg, D., Bril, J., & Del Castilho, P. (1993). Thermodynamic modeling of zinc, cadmium, and copper solubilities in a manured, acidic loamy-sand topsoil. *Journal of Environmental Quality, 22*(4), 681–688.

Jankowski, K. J., Kijewski, Ł., Krzebietke, S., & Budzyński, S. (2015). The effect of sulphur fertilization on macronutrient concentrations in the post-harvest biomass of mustard. *Plant, Soil and Environment, 61*(6), 266–272.

Jones, J. B. (2001). *Laboratory guide for conducting soil tests and plant analysis.* Washington, D.C.: CRC Press.

Karimizarchi, M., Aminuddin, H., Khanif, M. Y., & Radziah, O. (2014a). Incorporation and transformations of elemental sulphur in high pH soils of Malaysia. *International Journal of Soil Science, 9*(3), 133–141.

Karimizarchi, M., Aminuddin, H., Khanif, M. Y., & Radziah, O. (2014b). Elemeantal sulphur application effects on nutrient availability and sweet Maize (*Zea mays* L.) response in a high pH soil of Malaysia. *Malaysian Journal of Soil Science, 18*, 75–86.

Karimizarchi, M., Aminuddin, H., Khanif, M. Y., & Radziah, O. (2016). Effect of elemental sulphur timing and application rates on soil P release and concentration in maize. *Pertanika Journal of Tropical Agricultural Science, 39*(2), 235–248.

Karimizarchi, M., & Aminuddin, H. (2015). Effect of elemental sulphur on soil micronutrients mobility. *Journal of Agricultural Science and Food Technology, 1*(3), 34–42.

Khalid, R., Khan, K. S., Islam, M., Yousaf, M., & Shabbir, G. (2012). Effect of different sulfur levels from various sources on *Brassica napus* growth and soil sulfur fractions. *Journal of the Chemical Society of Pakistan, 34*(4), 1023–1031.

Khan, T. A., & Mazid, M. (2011). Nutritional significance of sulphur in pulse cropping system. *Biology and Medicine, 3*(2), 114–133.

Kochian, L. V. (1995). Cellular mechanisms of aluminum toxicity and resistance in plants. *Annual Review of Plant Biology, 46*(1), 237–260.

Lambers, H., Chapin, F. S., & Pons, T. L. (2008). *Plant physiological ecology.* New York: Edward Arnold Ltd.

Lee, Y. P., Goh, K. J., & Teh, C. B. S. (2013). Effects of various acidity levels on the growth of oil palm seedlings and their nutrients uptake. In J. Hamdan & J. Shamshudin (Eds.), *Advances in tropical soil science* (Vol. 2). University Putra Malaysia, Malaysia.

Lidon, F. C., & Barreiro, M. G. (2002). An overview into aluminum toxicity in maize. *Brazilian Journal of Plant Physiology, 28*(3–4), 96–112.

Lindsay, W. L. (1979). *Chemical equilibria in soils.* New York: John Wiley and Sons Ltd.

Manahan S. E. (2004). *Environmental chemistry*. United States of America, USA: CRC press.

MARDI. (2008). *Jagung improved masmadu.* Kuala Lumpur: Malaysian Agricultural Research and Development Institute, Malaysia.

McBride, M. B. (1994). *Environmental chemistry of soils.* United States of America, USA: Oxford University Press.

Meriño-Gergichevich, C., Alberdi, M., Ivanov, A. G., & Reyes-Díaz, M. (2010). Al $^{3+}$-Ca^{2+} interaction in plants growing in acid soils: Al phytotoxicity response to calcareous amendments. *Journal of Soil Science and Plant Nutrition, 10*(3), 217–243.

Owen, K. M., Marrs, R. H., Snow, C. S. R., & Evans, C. E. (1999). Soil acidification – The use of sulphur and acidic plant materials to acidify arable soils for the recreation of heathland and acidic grassland at Minsmere, UK. *Biological Conservation*, *87*(1), 105–121.

Pineros, M. A., Shaff, J. E., Manslank, H. S., Alves, V. M. C., & Kochian, L. V. (2005). Aluminum resistance in maize cannot be solely explained by root organic acid exudation. *Plant Physiology,137*(1), 231–241.

Reddy, M. R., Lameck, D., & Rezania, M. E. (1989). Uptake and distribution of copper and zinc by soybean and corn from soil treated with sewage sludge. *Plant and Soil, 113*(2), 271–274.

Robson, A. (2012). *Soil acidity and plant growth.* Australia: Elsevier.

Sameni, A. M., & Kasraian, A. (2004). Effect of agricultural sulfur on characteristics of different calcareous soils from dry regions of Iran. I. Disintegration rate of agricultural sulfur and its effects on chemical properties of the soils. *Communications in Soil Science and Plant Analysis, 35*(9–10), 1219–1234.

Shamshuddin, J., Fauziah, C. I., Anda, M., Kapok, J., & Shazana, M. A. R. S. (2011). Using ground basalt and/or organic fertilizer to enhance productivity of acid soils in Malaysia for crop production. *Malaysian Society of Soil Science, 15*, 127–146.

Shenker, M., & Chen, Y. (2005). Increasing iron availability to crops: fertilizers, organo-fertilizers, and biological approaches. *Soil Science and Plant Nutrition, 51*(1), 1–17.

Sienkiewicz-Cholewa, U., & Kieloch, R. (2015). Effect of sulphur and micronutrients fertilization on yield and fat content in winter rape seeds (*Brassica napus* L.). *Plant Soil and Environment*, *61*(4), 164–170.

Skwierawska, M., Zawartka, L., Skwierawski, A., & Nogalska, A. (2012). The effect of different sulfur doses and forms on changes of soil heavy metals. *Plant, Soil and Environment-UZEI, 58*(3), 135–140.

Tan, B. K. (2002). *Environmental geology of limestone in Malaysia.* Geology Programme, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Malaysia. Retrieved March 10, 2015, from http://www. karst. edu. cn/ igcp/igcp448/2002/3-2-7.pdf

Taylor, G. J. (1988). The physiology of aluminum phytotoxicity. In H. Sigel & A. Sigel (Eds.), *Metal ions in biological systems: Aluminum and its rule in biology* (pp. 123-163). New York: Marcel Dekker.

Valencia, R., & Ligarreto, M. (2012). Differential response of plants to aluminum: a review. *Agronomía Colombiana, 30*(1), 71–77.

Viani, R. A. G., Rodriguesb, R. R., Dawsonc, T. E., Lambers, H., & Oliveira, R. S. (2014). Soil pH accounts for differences in species distribution and leaf nutrient concentrations of Brazilian woodland savannah and seasonally dry forest species. *Perspectives in Plant Ecology, Evolution and Systematics, 16*(2), 64–74.

Vidyalakshmi, R., Paranthaman, R., & Bhakyaraj, R. (2009). Sulphur oxidizing bacteria and pulse nutrition – a review. *World Journal of Agricultural Sciences, 5*(3), 270–278.

Wang, A. S., Angle, J. S., Chaney, R. L., Delorme, T. A., & Reeves, R. D. (2006). Soil pH effects on uptake of Cd and Zn by Thlaspi caerulescens. *Plant and Soil, 281*(1–2), 325–337.

Ward, C. L., Kleinert, A., Scortecci, K. C., Benedito, V. A., & Valentine, A. J. (2011). Phosphorus-deficiency reduces aluminium toxicity by altering uptake and metabolism of root zone carbon dioxide. *Journal of Plant Physiology, 168*(5), 459–465.

Ye, R., Wright, A. L., & McCray, J. M. (2010). Sulfur distribution and transformations in Everglades agricultural area soil as influenced by sulfur amendment. *Soil Science, 175*(6), 263–269.

Ye, R., Wright, A. L., & McCray, J. M. (2011). Seasonal changes in nutrient availability for sulfur – amended everglades soils under sugarcane. *Journal of Plant Nutrition, 34*(14), 2095–2113.

Zeng, F., Shafaqat, A., Zhang, H., Ouyang, Y., Qiu, B., Wu, F., & Zhang, G. (2011). The influence of pH and organic matter content in paddy soil on heavy metal availability and their uptake by rice plants. *Environmental Pollution, 159*(1), 84–91.

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Addition of L-Tyrosine to Improve Betalain Production in Red Pitaya Callus

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ABSTRACT

The aim of this study is to enhance betalain production in red pitaya (*Hylocereus polyrhizus*) callus through supplementation of the precursor L-tyrosine. Red-pigmented calli were produced on Murashige and Skoog (MS) basal salt medium supplemented with 2 mg/L naphthaleneacetic acid (NAA), 4 mg/L thidiazuron (TDZ) and different concentrations of L-tyrosine (20, 40, 60, 80, 100 mg/L). The addition of 20 mg/L L-tyrosine was able to increase betacyanin production by 1.5-fold compared to calli grown on Murashige and Skoog (MS) basal salt medium (MSO). The results also revealed that betalain production (calli cultured with 20 mg/L L-tyrosine) was higher than MS medium with 2 mg/L NAA and 4 mg/L TDZ (without L-tyrosine) up to 1.7-fold. Four compounds, namely betacyanin, betaxanthin and two additional compounds, phenolic and flavonoid compounds, were detected in the red-pigmented calli treated with 20 mg/L L-tyrosine that were not found in the fresh fruit sample. This indicates that the callus system has potential to produce betalain pigment and that red-pigmented calli could be an important antioxidant source.

Keywords: *Hylocereus polyrhizus*, betalains, precursor, L-tyrosine, pitaya callus, antioxidant

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INTRODUCTION

Colour additives refer to any dyes, pigments or substances that can impart colour to foods, cosmetics, medicines and other products. They can be classed into natural, synthetic, inorganic and natural-identical depending on their origin.

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Advancements in food technologies have made it feasible to chemically synthesise many synthetic colourants to impart colours to food, making it more appetising and attractive, and most importantly, not easily degraded. However, some synthetic colourants such as tartrazine, carmoisine, brilliant blue and erythrosine are harmful to humans due to their carcinogenic and toxic effects. Based on research carried out by El-Wahab and El-Deen Moram (2012), rats fed with synthetic colourants resulted in liver cells destruction as well as acute kidney dysfunction. They concluded that the administration of synthetic colours resulted in various alterations of the antioxidant system and the use of them in food needs to be limited. Recent studies also showed that tartrazine and carmoisine altered bioelements, especially iron and zinc, in the liver, kidney and brain of rats (Cemek et al., 2014). Consequently, there is growing interest in natural colourants such as plant pigments, namely anthocyanin, chlorophyll, carotenoid and betalain as alternatives to synthetic colourant substitutes. Betalain has gained interest among food scientists as it was found to have a broad pH range, from 3 to 7, a broad range of colours and health-benefit properties (Georgiev et al., 2010; Gandía-Herrero, Escribano, & García-Carmona, 2014).

Betalain is a class of water-soluble nitrogen-containing compounds that can be found in all families belonging to the order of Caryophyllales except the families of Molluginaceae and Caryophyllaceae (Brockington, Walker, Glover, Soltis, & Soltis, 2011). There are two main types of betalain, namely red-violet betacyanin and yellow-orange betaxanthin. Betalain is a secondary metabolite derived from L-tyrosine (Gandía-Herrero & García-Carmona, 2013), one of the amino acids synthesised in the Shikimate pathway. L-tyrosines will undergo hydroxylation and form an intermediate compound called L-dihydroxyphenylalanine (L-DOPA). From L-DOPA, betalamic acid and *cyclo*-dihydroxyphenylalanine (*cyclo*-DOPA) will be formed. Betacyanin is formed through the condensation of betalamic acid with *cyclo*-DOPA (Sakuta, 2013). Betalamic acid also condenses with amines or amino acids to give betaxanthin (Tanaka, Sasaki, & Ohmiya, 2008). Betacyanin and betaxanthis can be further divided into subclasses depending on the substitution pattern on the betalamic acid.

Beetroot has been the frequently used model of an important betalain source. However, beetroot contains a high nitrate concentration and an unfavourable earthy smell (Lu, Edwards, Fellman, Mattinson, & Navazio, 2003), which led to the search for new betalain sources. Red pitaya is the new focus as an alternative betalain source (Aberoumand, 2011). Red pitaya (*Hylocereus polyhizus*), also known as red dragon fruit, is a species in the family of Cacteceae. Dragon fruit has a thin scaly red peel and red-violet flesh that is interspersed with small digestible black seeds (Lim, 2012). Applications of betalain extracted from red pitaya in food have been

extensively studied. Yogurt added with red pitaya fruit enhanced the rate of milk fermentation, phenolic content, antioxidant activity and produced a high content of lactic acid compared to plain yogurt (Zainoldin & Baba, 2009). In milk, addition of betacyanin from red pitaya improved the colour acceptability and produced better thermal tolerance compared to that produced by red beetroot colourant, called E-162 (Gengatharan, Dykes, & Choo, 2016).

Natural colourants are limited to type and/or part of the plant; the production may be affected by seasonal changes and be susceptible to infection. In order to meet consumer needs and expectations, production of secondary metabolites, particularly natural colourants *in vitro* has been extensively studied. Plant tissue culture is an alternative tool that offers several advantages over field cultivation such as continuous production of desired metabolites in controlled environments. There are many reports on *in vitro* betalain production; however, betalain has been reported to be easily affected by abiotic stresses such as light, temperature and pH (Herbach, Stintzing, & Carle, 2006). Therefore, many strategies have been developed to improve yield such as by precursor feeding and supplementation of biosynthetic precursors in culture medium to increase the production of desired metabolites (Mulabagal & Tsay, 2004). The idea of supplying precursors or intermediate compounds of secondary metabolite routes has been effective in several studies. For example, the addition of L-phenylalanine was able to increase the production of tocopherol and carotene levels in *Rosa damascene* Mill. petal callus culture (Olgunsoy, Ulusoy, & Celikkol-Akcay, 2017). In another study by Mobin, Wu, Tewari and Paek (2015), feeding of L-tryptophan enhanced growth and accumulation of phenolic compounds, flavonoids and caffeic acid in adventitious root cultures of *Echinacea purpurea*. Similarly, phenolic acid concentration in shoot cultures of *Exacum affine* was increased when L-phenylalanine was added (Skrzypczak-Pietraszek, Słota, & Pietraszek, 2014). This study was carried out to enhance betalain production on established red pitaya callus through supplementation of L-tyrosine as a precursor.

MATERIALS AND METHODS

Sample Preparation

Red pitaya (*Hylocereus polyrhizus*) was used throughout this study. The fruit was purchased from a local farm located in Sepang Selangor, Malaysia (Longitude-101.74197; Latitude-2.68803). The fruit was washed in running tap water and detergent (GLO, Malaysia) to remove dirt from the peel and surface and sterilised using 70% (v/v) ethanol for 1 min in a laminar air flow hood. The fruit was halved and the flesh was excised for use as explants. The flesh was cut approximately into 1 cm^3 pieces and all the seeds were removed using forceps prior to culturing.

Callus and Pigment Production

The callus was induced based on the method described by Rogayah et al. (2014). Callus induction was carried out on Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) supplemented with 2 mg/L naphthaleneacetic acid (NAA), 4 mg/L thidiazuron (TDZ), 3% (w/v) sucrose and 0.3% (w/v) phytagel. The results showed that phytohormones at 2 mg/L NAA and 4 mg/L TDZ were found to produce a high amount of calli (bigger in size). This study also used 2 mg/L NAA and 4 mg/L TDZ supplementation to the MS basal medium with the addition of five concentrations of L-tyrosine (20, 40, 60, 80 and 100 mg/L). L-tyrosine was added separately to each sample to examine its effects on callus and pigment (betalain) productions. The pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. All the experiments were performed with five explants in each replicate. The cultures were kept in a dark condition at 25 ± 2ºC.

Analysis of Pigment Production

After one month, the calli produced were harvested for pigment content analysis. The betalain content was extracted by suspending the calli in 10 mL distilled water and filtered (cellulose acetate membrane, 0.45 µM pore size). Betalain content was quantified using a spectrophotometer (Biotek µQuant Microplate Spectrophotometer, Biotek Instrument Inc. Vermont, USA). Two pigments, betacyanin, indicated by red-purple pigmentation, and betaxanthin, indicated by yellow-orange pigmentation, were detected at the wavelength absorbance of 540 nm and 480 nm, respectively. The betalain content was then calculated from the absorbance reading obtained. The betalain content (BC) was calculated as BC (mg/L)=[(A x DF x MW x 1000) / (ε x 1)], where A was absorption, DF was the dilution factor and 1, the width of the spectrophotometer cell (1 cm). For betacyanin, the extinction coefficient (ε) was 60 000 L/(mol cm) and the molecular weight (MW) was 550 g/mol. For betaxanthin, the extinction coefficient (ε) was 48 000 L/(mol cm) and the molecular weight (MW) was 308 g/mol (Stintzing, Schieber, & Carle, 2003; Ravichandran et al., 2013). Betalain was extracted from calli cultured in an MS medium without plant growth regulators (PGRs) and a precursor (MSO), and calli culture in MS medium with 2 mg/L NAA and 4 mg/L TDZ was used as the main control in this study. All analyses were performed in five replicates with the data analysed by Duncan's test using SAS (SAS Institute Inc., Cary, NC) at $p<0.05$ defined as a significant difference. The data were reported as means ± standard deviation.

The betalain content of the calli was then further analysed using high-performance liquid chromatography (Biswas, Das, & Dey, 2013). Calli from each petri dish (10-15 plates) were ground using a mortar and pestle and freeze-dried with liquid nitrogen. The extraction method was adapted from Poh-Hwa, Yoke-Kqueen, Indu Bala and Son (2011), with

slight modification. HPLC Agilent 1200 Series (Agilent Technologies Inc., CA, US) was used for phytochemical profiling. Separation of samples was obtained at room temperature on reverse phase Thermo Hypersil C18 gold (150 x 4.1 mm ID) with guard column, and 20 µL of sample was injected into the column (flow rate at 0.75 ml/min). The mobile phase consisted of 0.5% formic acid (solvent A) and 0.5% formic acid in acetonitrile (solvent B). The following gradient procedure was used: 0-40 min: 5% to 95% B; 40-45 min: 95% B; and 45-50 min: 5% to 95% B. Monitoring was performed at 280 nm and 360 nm for phenolic compounds and flavonoids, respectively (Francisco & Resurreccion, 2009) at 480 nm for betaxanthin and at 540 nm for betacyanin.

RESULTS AND DISCUSSION

A previous study (unpublished data) was conducted to observe the effects of different combinations and concentrations of plant growth regulators (PGRs) used for callus induction in *H. polyrhizus*. It was found that the best treatment for callus induction was the combination of 2 mg/L naphthaleneacetic acid (NAA) and 4 mg/L thidiazuron (TDZ). Morphological appearance revealed the pigmented calli produced were red with a compact texture and bigger diameter in size (2.5 ± 0.02 cm) when compared to other PGR combinations and concentrations. Absorbance readings showed that a higher content of betalain, about 1.5-fold, was obtained compared to calli cultured on MS basal salt medium (control). Therefore, this study also used 2 mg/L NAA and 4 mg/L TDZ with addition of L-tyrosine, as a precursor, to increase betalain production in the red-pigmented calli.

Two main controls were used in this study to investigate the effects of plant growth regulators (PGRs) and precursors on callus production and betalain production. The first was calli cultured in MS medium without PGRs and a precursor (MSO) and the second control was calli cultured in MS medium with PGRs (2 mg/L NAA and 4 mg/L TDZ), without L-tyrosine. Betalain extracted from fresh fruit was used to compare with betalain extracted from the calli to study the potential of callus culture as a tool for betalain production. The explant, flesh of red pitaya, was cut into approximately 1 cm^3 pieces and cultured in the desired medium (five explants per plate) as shown in Figure 1(i). The results showed that L-tyrosine at 20, 40, 60, 80 and 100 mg/L produced calli of different colour intensities and texture. In general, all the calli produced were reddish and compact as depicted in Figure 1(ii). Calli cultured in MSO were dull and watery. The red colour of calli denoted the presence of betalain, particularly betacyanin. The development of calli could be seen after five to eight days of incubation. The colour intensity of the red-pigmented calli treated with 20 mg/L L-tyrosine was higher compared to other concentrations, where the explant was completely covered with red calli. The size of the callus clumps were also bigger (2.58 ± 0.06 cm) compared to calli grown with PGRs alone.

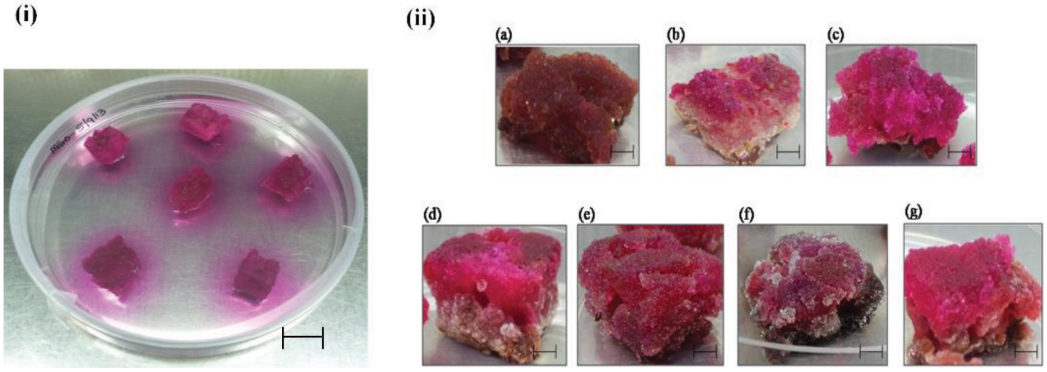

Figure 1. Red-pigmented calli from red pitaya. Five explants of seedless cubed pitaya were cultured per plate (i). After one month of incubation, different morphology appearances of calli were observed (ii), (a) callus induced in MS basal medium without PGRs and precursor (control) was watery (soft) and a dull red. The calli induced in 2 mg/L NAA and 4 mg/L TDZ with (b) 20 mg/L, (c) 40 mg/L, (d) 60 mg/L, (e) 80 mg/L and (f) 100 mg/L L-tyrosine produced different intensities of the colour red and had a friable texture.

Scale: ⊢⊣ indicate 1 cm

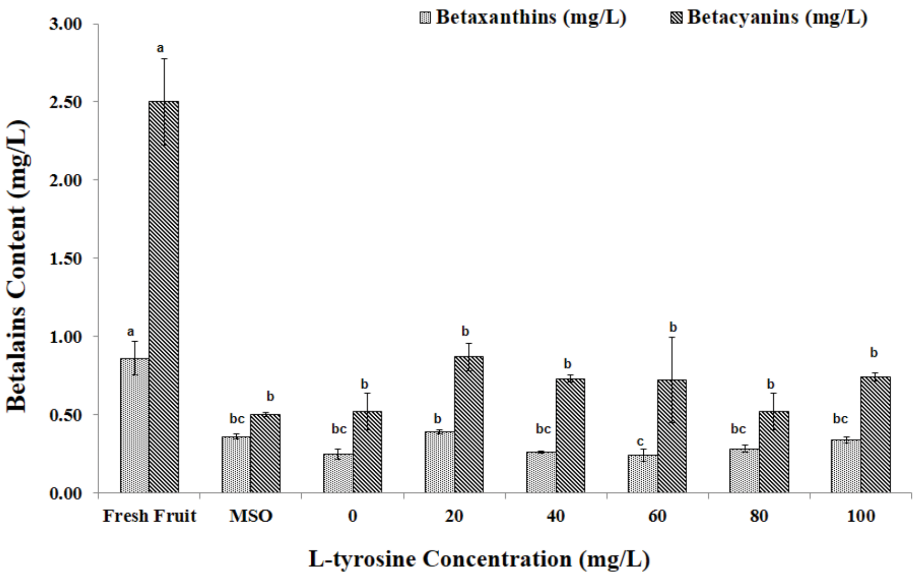

Figure 2. Effects of L-tyrosine concentration on betalain content in red-pigmented callus. Calli were induced in media containing 2 mg/L NAA, 4 mg/L TDZ and five different concentrations of L-tyrosine. Values shown are means of five replicates and error bars indicate standard deviations. Means with the same letter indicate no significant difference (Duncan's Test) at $p<0.05$

Figure 2 summarises the betalain content analysed in each respective red-pigmented callus grown in MS media supplemented with different L-tyrosine concentrations. High betalain content was observed in fresh fruit sample for both betaxanthin and betacyanin content, produced at 0.86 mg/L and 2.50 mg/L, respectively. *H. polyrhizus* is known as being rich in betacyanin content compared to betaxanthin (Wybraniec & Mizrahi, 2002). Betacyanin content in each sample was also higher than betaxanthin content, as predicted from looking at the colour of the calli. Betacyanin in red-pigmented calli showed no significant difference (p>0.05). Total betalain produced in calli cultured with addition of L-tyrosine was higher than in calli cultured in MSO and calli cultured with only PGRs. The highest betalain production was observed in calli cultured in MS medium supplemented with 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine, with 1.5-fold increment compared to MSO and 1.7-fold higher than calli cultured with only PGRs. Even though the amount produced in calli showed increment, the betalain content in fresh fruit was 2.7-fold higher. The size of the callus was observed to influence the betalain content as the biggest callus produced the highest betalain content.

The identification of compounds using reverse-phase high performance liquid chromatography (RP-HPLC) was based on absorbance of wavelength, where green represented betacyanin (540 nm), purple represented betaxanthin (480 nm), red represented flavonoid compounds (280 nm) and blue represented phenolic compounds, as shown in Figure 3. The HPLC analysis of betalain content in fresh fruit as shown in Figure 3(a) revealed the presence of two major compounds, betaxanthin (purple) and betacyanin (green), with the highest peaks being detected simultaneously at a retention time (Rt) of 11.83 min. Several peaks of betacyanin were also visible at different Rt, indicating detection of different compounds of betacyanin and betaxanthin. Betacyanin and betaxanthin were also detected in calli grown in MSO (Figure 3(b)) at Rt 8.67 min and 12.07 min. Similarly, HPLC analysis of calli grown in MS medium with 2 mg/L NAA and 4 mg/L TDZ (Figure 3(c)) and calli grown in MS medium with 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine (Figure 3(d)) showed the presence of betacyanin and betaxanthin at Rt 8.79 min and 12.04 min. The addition of 2 mg/L NAA and 4 mg/L TDZ did not produce any increment in betalain production compared to calli grown in MSO. However, when 20 mg/L L-tyrosine was added, absorbance of betacyanin in the calli increased by threefold and 1.42-fold compared to calli grown with PGRs only and MSO, respectively. The first *H. polyrhizus* pigments reported consisted of three main components i.e. betanin, hylocerenin and phyllocactin (Wybraniec et al., 2001). Thereafter, eight betacyanins were identified using positive ion electrospray mass spectrometry in *H. polyrhizus* (Stintzing, Schieber, & Carle, 2002). Naderi, Ghazali, Hussin,

Amid and Manap (2012) also reported that phyllocactin and betanin were the prominent betacyanins in the fruit pulp of *H. polyrhizus*.

Two additional compounds, identified as flavonoids (red) and phenolic compounds (blue) were detected in HPLC analysis of calli, which were not found in the fresh fruit sample. The addition of 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine produced several peaks of flavonoids and phenolic acids. Flavonoids and phenolic compounds are known as bioactive compounds, synthesised from the Shikimate pathway, a similar pathway used to synthesise tyrosine, phenylalanine and tryptophan. Phenolic compounds form a diverse group and are characterised by hydroxylated aromatic rings (Mandal, Chakraborty, & Dey, 2010) that include the widely distributed hydroxybenzoic and hydroxycinnamic acids. Flavonoids are synthesised by the phenylpropanoid pathway and the start-up component is phenylalanine. Flavonoids have been classified into six subgroups, namely flavones, flavols, flavanones, flavan-3-ols, isoflavones and anthocyanidin compounds (Ghasemzadeh & Ghasemzadeh, 2011). Phenolic compounds and flavonoids extracted from the treated red-pigmented calli may serve as an important source of antioxidants (Balasundram, Sundram, & Samman, 2006). Phenolic compounds and flavonoids have been extensively exploited for their multiple biological activities such as anti-scavenging, antioxidant, anti-inflammatory and anti-microbial effects (Cushnie & Lamb, 2011; González et al., 2011; Agati, Azzarello, Pollastri, & Tattini, 2012). The presence of phenolic compounds and flavonoids in the calli may be due to enzymatic reactions by the polyphenol oxidase (PPO) and as a response to stress conditions (Winkel-Shirley, 2002; Michalak, 2006). However, some of these compounds can contribute to the development of browning in plant callus cultures (Dong et al., 2016).

L-tyrosine was used in this study because it plays an important role as a precursor for betalain biosynthesis (Gandía-Herrero & García-Carmona, 2013). We postulated that addition of exogenous L-tyrosine in the callus induction medium might induce production of more betalain in the existing red-pigmented calli. A study on application of tyrosine as precursor was conducted by Kleinowski et al. (2014) to investigate its effect in pigment production and growth of Alternanthera plants. The application of 50 µM tyrosine increased betacyanin production to 36.5 mg/100g in the plant shoots. However, the mode of action of L-tyrosine in plant tissue culture is currently not well understood. The lower betalain content in the calli may be due to several factors such as exposure to light, unfavourable temperature or pH, all of which may cause betalain degradation during culturing and incubation (Woo, Ngou, Ngo, Soong, & Tang, 2011). Another possibility is that the precursor itself may not be effective for inducing betalain or the concentration used might not be optimal for betalain induction. Besides

that, the callus culture is only a partial system involving undifferentiated cells for production of secondary metabolites. Secondary metabolites are thought to be produced following long biosynthetic pathways that involve dozens of enzymes (Bourgaud, Gravot, Milesi, & Gontier, 2001). L-tyrosine may not be involved in the activation of specific genes for certain enzymes or it does not interact with membrane receptors that generate signalling compounds.

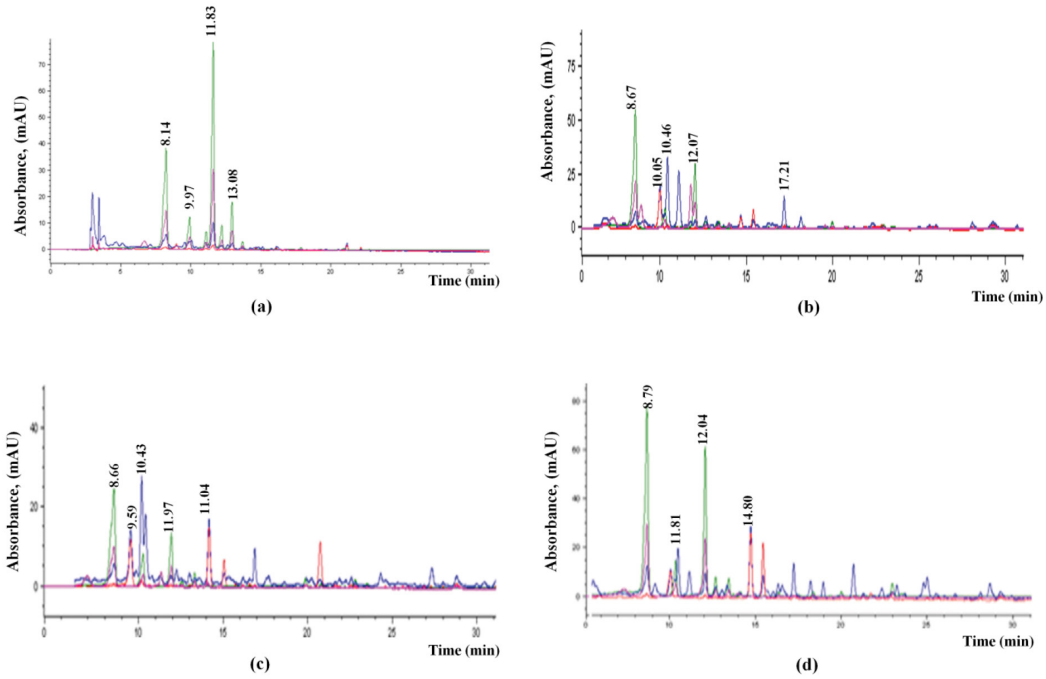

Figure 3. HPLC chromatographs. Fresh fruit sample (a) showed the presence of betacyanin and betaxanthin only, while in calli grown in MSO (b), MS medium with 2 mg/L NAA and 4 mg/L TDZ (c) and MS medium with 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine, two additional compounds were detected, phenolic compounds and flavonoids.
*Green represents betacyanins; purple represents betaxanthins; red represents flavonoids; blue represents phenolic compounds

CONCLUSION

Among the different concentrations of L-tyrosine tested as aprecursor, the highest intensity of pigmented calli produced was observed in MS medium containing 2 mg/L NAA, 4 mg/L TDZ and 20 mg/L L-tyrosine. Reverse-phase HPLC analysis of extracts obtained from the red-pigmented calli revealed the presence of four different compounds i.e. betacyanin, betaxanthin and phenolic and flavonoid compounds. Further analysis of enhancement of betalain production using other elicitors should be tested as this group of pigments has

beneficial values for the food industry. The callus system established is expected to be useful as a potential *in vitro* system for the production of secondary metabolites.

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REFERENCES

Aberoumand, A. (2011). A review article on edible pigments properties and sources as natural biocolorants in foodstuff and food industry. *World Journal of Dairy and Food Sciences, 6*(1), 71–78.

Agati, G., Azzarello, E., Pollastri, S., & Tattini, M. (2012). Flavonoids as antioxidant in plants: Location and functional significance. *Plant Science, 196*, 67–76.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: antioxidant activity, occurrence and potential uses. *Food Chemistry, 99*(1), 191–203.

Biswas, M., Das, S. S., & Dey, S. (2013). Establishment of a stable *Amaranthus tricolor* callus line for production of food colorant. *Food Science and Biotechnology, 22*(1), 1–8.

Bourgaud, F., Gravot, A., Milesi, S., & Gontier, E. (2001). Production of plant secondary metabolites: A historical perspective. *Plant Science, 161*(5), 839–851.

Brockington, S. F., Walker, R. H., Glover, B. J., Soltis, P. S., & Soltis, D. E. (2011). Complex pigment evolution in the Caryophyllales. *New Phytologist, 190*(4), 854–864.

Cemek, M., Büyükokuroğlu, M. E., Sertkaya, F., Alpdağtaş, S., Hazini, A., Önül, A., & Göneş, S. (2014). Effects of food color additives on antioxidant functions and bioelement contents of liver, kidney and brain tissues in rats. *Journal of Food and Nutrition Research, 2*(10), 686–691.

Cushnie, T. P. T., & Lamb, A. J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. *International Journal of Antimicrobial Agents, 38*(2), 99–107.

Dong, Y. S., Fu, C. H., Su, P., Xu, X. P., Yuan, J., Wang, S., … & Yu, L. J. (2016). Mechanisms and effective control of physiological browning phenomena in plant cell cultures. *Physiologia Plantarum, 156*(1), 13–18.

El-Wahab, H. M. F. A., & El-Deen Moram, G. S. (2012). Toxic effects of some synthetic food colorants and/or flavour additives on male rats. *Toxicology and Industrial Health, 29*(2), 224–232.

Francisco, M. L., & Resurreccion, A. V. A. (2009). Development of a reversed-phase high performance liquid chromatography (RP-HPLC) procedure for the simultaneous determination of phenolic compounds in peanut skin extracts. *Food Chemistry, 117*(2), 356–363.

Gandía-Herrero, F., Escribano, J., & García-Carmona, F. (2014). Biological activities of plant pigments betalains. *Critical Reviews in Food Science and Nutrition*, *56*(6), 937–945.

Gandía-Herrero, F., & García-Carmona, F. (2013). Biosynthesis of betalains: yellow and violet plant pigments. *Trends in Plant Science, 18*(6), 334-343.

Gengatharan, A., Dykes, G. A., & Choo, W. S. (2016). Stability of betacyanin from red pitahaya (*Hylocereus polyrhizus*) and its potential application as a natural colorant in milk. *International Journal Food Science and Technology, 51*(2), 427–434.

Georgiev, V. G., Weber, J., Eva-Maria, K., Denev, P. N., Bley, T., & Pavlov, A. I., (2010). Antioxidant activity and phenolic content of betalain extracts from intact plants and hairy root cultures of the red beetroot *Beta vulgaris* cv. Detroit dark red. *Plant Foods for Human Nutrition, 65*(2), 105–111.

Ghasemzadeh, A., & Ghasemzadeh, N. (2011). Flavonoids and phenolic acids: Role and biochemical activity in plants and human. *Journal of Medicinal Plants Research, 5*(31), 6697–6703.

González, R., Ballester, I., López-Posadas, R., Suárez, M. D., Zarzuelo, A., Martínez-Augustin, O., & Sánchez De Medina, F. (2011). Effects of flavonoids and other polyphenols on inflammation. *Critical Review in Food Science and Nutrition, 51*(4), 331–362.

Herbach, K. M., Stintzing, F. C., & Carle, R. (2006). Betalain stability and degradation-structural and chromatic aspects. *Journal of Food Science, 71*(4), R41–R50.

Kleinowski, A. M., Brandão, I. R., Einhardt, A. M., Márcia, V. R., Peters, J. A., & Braga, E. J. B. (2014). Pigment production and growth of *Alternanthera* plants cultured *in vitro* in thepresence of tyrosine. *Brazilian Archives of Biology and Technology, 57*(2), 253–260.

Lim, T. K. (2012). Hylocereus polyrhizus. In T. K. Lim (Ed.), *Edible Medicinal and Non-Medicinal Plants* (pp. 643–649). New York, NY: Springer Dordrecht Heidelberg.

Lu, G., Edwards, C. G., Fellman, J. K., Mattinson, D. S., & Navazio, J. (2003). Biosynthetic origin of geosmin in red beets (*Beta vulgaris* L.). *Journal of Agricultural and Food Chemistry, 51*(4), 1026–1029.

Mandal, S. M., Chakraborty, D., & Dey, S. (2010). Phenolic acids act as signalling molecules in plant-microbe symbioses. *Plant Signaling and Behavior, 5*(4), 359–368.

Michalak, A. (2006). Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. *Polish Journal of Environmental Studies*, *15*(4), 523–530.

Mobin, M., Wu, C. H., Tewari, R. K., & Paek, K. Y. (2015). Studies on the glyphosate-induced amino acid starvation and addition of precursors on caffeic acid accumulation and profiles in adventitious roots of *Echinacea purpurea* (L.) Moench. *Plant Cell, Tissue and Organ Culture, 120*(1), 291–301.

Mulabagal, V., & Tsay, H. S. (2004). Plant cell cultures – An alternative and efficient source for the production of biologically important secondary metabolites. *International Journal of Applied Science and Engineering, 2*(1), 29–48.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Plant Physiology, 15*(3), 473–497.

Naderi, N., Ghazali, H. M., Hussin, A. S. M., Amid, M., & Manap, M. Y. A. (2012). Characterization and quantification of dragon fruit (*Hylocereus polyrhizus*) betacyanin pigments extracted by two procedures. *Pertanika Journal of Tropical Agricultural Science, 35*(1), 33–40.

Olgunsoy, P., Ulusoy, S., & Celikkol-Akcay, U. (2017). Metabolite production and antimicrobial activities of callus cultures from Rosa damascene Mill. Petals. *Marmara Pharmaceutical Journal, 21*(3), 590–597.

Poh-Hwa, T., Yoke-Kqueen, C., Indu Bala, J., & Son, R. (2011). Bioprotective properties of three malaysia *Pyhllanthus* species: An investigation of the antioxidant and antimicrobial activities. *International Food Research Journal, 18*(3), 887–893.

Ravichandran, K., Saw, N. M. M. T., Mohdaly, A. A. A., Gabr, A. M. M., Kastell, A., Riedel, H., … & Smetanska, I. (2013). Impact of processing of red beet on betalain content and antioxidant activity. *Food Research International, 50*(2), 670–675.

Rogayah, S., Faizal, N. F. A., Hashim, Y., Wee, C. Y., Janna, O. A., Shaharuddin, N. A., & Yahya, S. F. (2017, August). *Effects of elicitors on betalain production in red dragon fruit*. Paper presented at Bio Johor 2014 Conference, Persada Johor Convention Centre, Johor Bahru, Johor, Malaysia.

Sakuta, M. (2013). Diversity in plant red pigment: anthocyanins and betacyanins. *Plant Biotechnology Reports, 8*(1), 37–48.

Skrzypczak-Pietraszek, E., Słota, J., & Pietraszek, J. (2014). The influence of L-phenylalanine, methyl jasmonate and sucrose concentration on the accumulation of phenolic acids in *Exacum affine* Balf. f. ex Regel shoot culture. *Acta Biochimica Polonica, 61*(1), 47–53.

Stintzing, F. C., Schieber, A., & Carle, R. (2002). Betacyanins in fruits from red-purple pitaya, *Hylocereus polyrhizus* (Weber) Britton and Rose. *Food Chemistry, 77*(1), 101–106.

Stintzing, F. C., Schieber, A., & Carle, R. (2003). Evaluation of colour properties and chemical quality parameters of cactus juices. *European Food Research and Technology, 216*(4), 303–311.

Tanaka, Y., Sasaki, N., & Ohmiya, A. (2008). Biosynthesis of plant pigments: Anthocyanins, betalains, and carotenoids. *The Plant Journal, 54*(4), 733–749.

Winkel-Sherley, B. (2016). Biosynthesis of flavonoids and effects of stress. *Current Opinion in Plant Biology, 5*(3), 218–223.

Woo, K. K., Ngou, F. H., Ngo, L. S., Soong, W. K., & Tang, P. Y. (2011). Stability of betalain pigment from red dragon fruit (*Hylocereus polyrhizus*). *American Journal of Food Technology, 6*(2), 140–148.

Wybraniec, S., & Mizrahi, Y. (2002). Fruit flesh betacyanin pigments in *Hylocereus* cacti. *Journal of Agricultural and Food Chemistry, 50*(21), 6086–6089.

Wybraniec, S., Platzner, I., Geresh, S., Gottlieb, H. E., Haimberg, M., Mogilnitzki, M., & Mizrahi, Y. (2001). Betacyanins from vine cactus *Hylocereus polyrhizus*. *Phytochemistry, 58*(8), 1209–1212.

Zainoldin, K. H., & Baba, A. S. (2009). The effect of *Hylocereus polyrhizus* and *Hylocereus undatus* on physiochemical, proteolysis, and antioxidant activity in yogurt. *World Academy of Science, Engineering and Technology, 60,* 361–366.

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Isolation and Characterisation of Ascomycetes Isolated from *Eurycoma longifolia* Jack and Malay Traditional Vegetables

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ABSTRACT

Plants are the most common host for fungal communities. However, vegetables and herbs traditionally consumed by the Malay community have not been thoroughly investigated for their association with fungi. The main objective of the present study is to identify the Ascomycetes fungi associated with *Eurycoma longifolia* Jack and vegetables traditionally consumed by the Malay community. In the present study, we isolated and identified 34 isolates of the Ascomycetes fungi obtained from five traditional vegetables (*Oenanthe javanica, Cosmos caudatus, Persicaria odorata, Psophocarpus tetragonolobus* and *Cantella asiatica*) and *Eurycoma longifolia* Jack. The isolates are identified as eight species, which are *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Fusarium oxysporum, Fusarium proliferatum, Fusarium solani, Penicillium paraherquei* and *Trichoderma asperellum*. *Aspergillus* and *Fusarium* are dominant among the isolated fungi. This report provides additional information on the diversity of fungi isolated from traditional vegetables and *Eurycoma longifolia* based on the Internal Transcribed Spacer (ITS) sequence analysis.

Keywords: Traditional vegetables, *Aspergillus* species, *Fusarium* species, *Penicillium* species and *Trichoderma* species

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INTRODUCTION

Numerous studies have been conducted on fungi and their ecological advantages, biomedical properties, natural products and taxonomic relationship (Radu & Kqueen, 2002; Guo, Wang, Sun, & Tang, 2008; Aly, Debbab, Kjer, & Proksch, 2010; Nath,

Raghunatha, & Joshi, 2012; Madhusudan, Barathi, & Prakash, 2015). Malaysia is rich in rainforest land teeming with thousands of plants with potential medicinal value such as traditional vegetables and *Eurycoma longifolia* Jack, which are the most consumed by Malaysians. Traditional vegetables (locally known as *ulam*) were reported in previous studies to contain many benefits in terms of their biomedical properties (Kumar & Sagar, 2007; Guo et al., 2008, p.136; Shukri, Alan, & Noorzuraini, 2011; Fatimah, Norazian, & Rashidi, 2012). *Eurycoma longifolia*, which belongs to the Simaroubaceae family, is a woody plant locally known as *Tongkat Ali*, that has pharmacological and medicinal value in treating various diseases.

Commonly, traditional vegetables can be eaten raw or boiled, usually as part of a side dish served with rice. It can be served as a salad and is very popular among Malaysians and visiting foreigners because of its flavour and savoury taste. Previous studies have recorded that there are more than 100 plant species from various plant families that are consumed as *ulam* in Malaysia (Hussain, Anwar, Sherazi, & Przybylski, 2008). *Ulam* is also applied in Malaysia as a medicinal remedy for controlling blood pressure and improving blood glucose concentrations among diabetes patients (Abas, Ozpinar, Kutay, Kahraman, & Eseceli, 2005). Five *ulam* were chosen in the present study, namely *Oenanthe javanica, Cosmos caudatus, Persicaria odorata, Psophocarpus tetragonolobus* and *Cantella asiatica* as the study samples. Selection of the *ulam* was due to availability, popularity as a local favourite and frequency of use in Malaysian cuisine.

There is very limited information on the diversity of fungi isolated from herbs and vegetables, especially in tropical area. Realising the importance of this research to the nation and the world, a study of the diversity of fungi associated with these plants was conducted. The objectives of this study were to isolate the Ascomycetes fungi from *Eurycoma longifolia* Jack and the traditional vegetables and to identify the fungal isolates based on the Internal Transcribed Spacer (ITS) region sequence analysis. The findings of this study provide basic information on fungal diversity that can be used as reference in aetiology, disease control studies and understanding the role of fungi in their hosts.

MATERIALS AND METHOD

Fungal Isolation, Purification and Preservation

Thirty-four fungal isolates were isolated from *Eurycoma longifolia* Jack and five traditional vegetables commoly consumed in Malaysia (*Oenanthe javanica, Cosmos caudatus, Persicaria odorata, Psophocarpus tetragonolobus* and *Cantella asiatica*). Three 5 × 5 mm^2 pieces from the margins of leaf tissue of each plant were taken and surface sterilised in 1% sodium hypochlorite solution by dipping for 3 min and then rinsed three times with sterilised distilled water. After the blot was dried, the pieces of tissue were placed on the surface

of a Potato Dextrose Agar (PDA) plate and incubated for 4 days. Single hyphal tip isolation was conducted to obtain pure fungal colonies. All contaminated isolates were sub-cultured on water agar (WA). The cultures were incubated for 24 hours, and then by using a dissecting microscope, a single hyphal tip of fungal growth was transferred to a PDA plate. The culture plates were incubated at 25°C for 5-7 days to allow the colony to grow. Conidial suspension of the fungi was preserved in 25% glycerol complete medium with xylose (CMX), which was then kept at -80°C for further use.

Morphological Characterisation

The fungal cultures were transferred to water agar (WA) for single conidial isolation using the conidial suspension method. All the purified isolates were tentatively identified based on morphological characteristics that were emphasised on growth rate, pigmentation, size and the shape of micro- and macroconidia, presence of chlamydospore and type of conidiophore (Raper & Fennell, 1965; Pitt, 1979; Leslie & Summerell, 2006).

DNA Extraction and Amplification of ITS Region

All the isolates were cultured on PDA and incubated for 7 days. DNA was extracted from the young fungal mycelia of the culture growing on the PDA agar plate using the UltraClean® Microbial DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the user's manual instructions. A standard Polymerase Chain Reaction (PCR) protocol was used to amplify the ITS gene region. The PCR reactions of the ITS region were carried out in a thermal cycler with the primer ITS1 (5'TCCGTAGGTGAACCTGCGG-3') and the primer ITS4 (5'TCCTCCGCTTATTGATATGC-3') (White, Bruns, Lee, & Taylor, 1990). Amplification reactions were performed in a 20 µl reaction volume containing 1x Green GoTaq Buffer, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.5 mM primer ITS1, 0.5 mM primer ITS4, 0.1U *Taq* Polymerase and a 20-ng DNA template. The ITS was amplified in a thermal cycler (Biometra TProfessional) with the following cycling protocol: initial denaturation at 95°C for 30 s; followed by 35 cycles of 95°C for 10 s, 59°C for 15 s and 72°C for 30 s, and a final extension at 72°C for 5 min.

The PCR products with an expected length of 600-650 bp were examined by gel electrophoresis in 2% agarose gel and stained with FloroSafe DNA (BIO-5170-1 ml) (Axil Scientific Pte Ltd, Singapore). The amplicons were viewed under an UV transluminator. All the PCR products were given good quality of amplicons; the DNA was amplified to 50 µl in volume for sequencing.

ITS Sequencing and Phylogenetic Analysis

The amplification product for the ITS of each isolate was purified with QIAquick® Gel Extraction Kit according to the

manufacturer's instructions. The ITS gene fragment was sent for DNA sequencing using the ABI3730XL sequencer completed by MyTACG Bioscience Company, Selangor, MY. These sequences were BLAST against GenBank (http://blast.ncbi.nlm.nih.gov/). BLASTn was used to run a search for maximum identity and the number of bases of aligned sequence. Sequence alignments and analysis were performed using the Mega 6.06 (Molecular Evolutionary Genetics Analysis 6.06; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The maximum-likelihood phylogenetic tree was constructed using the bootstrap (1000 replicates) method.

Seven ITS sequences of the Ascomycetes fungi, *Aspergillus flavus* (JX292092), *Aspergillus fumigatus* (AY214446.1), *Aspergillus niger* (JX535496.1), *Fusarium oxysporum* (KM246753), *Fusarium proliferatum* (AF291061.1), *Fusarium solani* (JN9830007) and *Penicillium paraherquei* (NR111052.1) obtained from the NCBI GenBank dataset were used. The ITS sequence of the Ustilaginomycetes fungi, *Ustilago maydis* (AJ235275.1), was used as the outgroup reference in the phylogenetic analysis.

RESULTS AND DISCUSSION

A total of 34 isolates of fungi from five traditional vegetables and one herb, namely *Oenanthe javanica* (4 isolates), *Cosmos caudatus* (8 isolates), *Persicaria odorata* (5 isolates), *Psophocarpus tetragonolobus* (4 isolates), *Cantella asiatica* (4 isolates) and *Eurycoma longifolia* (9 isolates), were evaluated in this study according to their morphological characteristics (Table 1) and ITS sequences. All the isolated fungi can be considered endophytic as all the samples collected in this study were free of disease symptoms. Endophytic fungi are microorganisms that live in the plant tissue of the host plant and build colonies without causing any harm to the host plant. They represent an abundant and dependable source of novel bioactive compounds with huge potential for exploitation in a wide variety of medicinal, agricultural and industrial uses (Radu & Kqueen, 2002; Guo et al., 2008, p. 136; Aly et al., 2010, p. 2; Nath et al., 2012, p. 8; Madhusudan et al., 2015, p. 111). However, they are a poorly investigated group of microorganisms. Metabolites emitted by endophytes are recognised as a versatile source of antimicrobial and antioxidant agents. Thus, the fungal endophytes of plants need to be explored and studied to describe their distribution, morphology and diversity.

Variations were observed in the cultures in colour, appearance, margins, texture and sectors of the colonies (Table 1; Figure 1). Based on morphological characteristics, the most important colony features of *Fusarium* species are a white to yellow or pale violet colour of abundant mycelia and white tinged with yellow or purple pigmentation in agar. The colonies of the *Aspergillus* species on the PDA were observed to have a cottony and powdery texture and they exhibited a dark green (*A. fumigatus* and *A. flavus*) or black (*A. niger*) pigmentation. The colonies of *Penicillium*

paraherquei were observed to be greyish green and have a cottony texture on the PDA, whereas *Trichoderma asperellum* displayed a cottony and powdery texture with white to light green pigmentation that became dark green with age.

Table 1
List of microfungi isolates used in this study and their morphological characteristics

Isolate no.	Host (Scientific name)	Location	Species	Pigmentation	Colony features
B2468 C2473	*Psophocarpus tetragonolobus*	Cameron Highland, Pahang	*A. fumigatus*	Grey with green to dark green, becomes black with age	Cottony and powdery
B2478	*Cosmos caudatus*	Puchong, Selangor			
B2479 B2480	*Oenanthe javanica*	Semenyih, Selangor			
C2483	*Cosmos caudatus*	Cameron Highland, Pahang			
C2471	*Psophocarpus tetragonolobus*	Cameron Highland, Pahang	*A. flavus*	Yellowish, becomes green with age	Powdery
B2474 B2475	*Cosmos caudatus*	Puchong, Selangor			
B2482	*Oenanthe javanica*	Semenyih, Selangor			
B2469 B2484	*Persicaria odorata*	Kajang, Selangor	*A. niger*	White, becomes black or dark brown with age	Cottony and powdery
C2470 C2472	*Psophocarpus tetragonolobus*	Cameron Highland, Pahang			
B2476 B2477	*Cosmos caudatus*	Puchong, Selangor			
B2481	*Oenanthe javanica*	Semenyih, Selangor			
A223, A225 A229, A242	*Eurycoma longifolia*	Grik, Perak	*F. solani*	White, becomes pale yellow with age	White sparse mycelia in concentric rings
B2486	*Centella asiatica*	Puchong, Selangor			
B475, B480 B481, B483 B484	*Eurycoma longifolia*	Ayer Hitam Forest Reserve, Selangor			
B2485	*Centella asiatica*	Puchong, Selangor	*F. oxysporum*	White, becomes yellow with age	Cottony and fluffy
B2487	*Centella asiatica*	Puchong, Selangor	*F. proliferatum*	White, becomes purple with age	Cottony
B2488	*Cosmos caudatus*	Puchong, Selangor	*Penicillium paraherquei*	Greyish green	Cottony and powdery
B2489	*Persicaria odorata*				
B2490	*Persicaria odorata*	Kajang, Selangor	*Trichoderma asperellum*	White to light green, becomes dark green with age	Cottony and powdery
B2491 B2492	*Centella asiatica*	Puchong, Selangor			

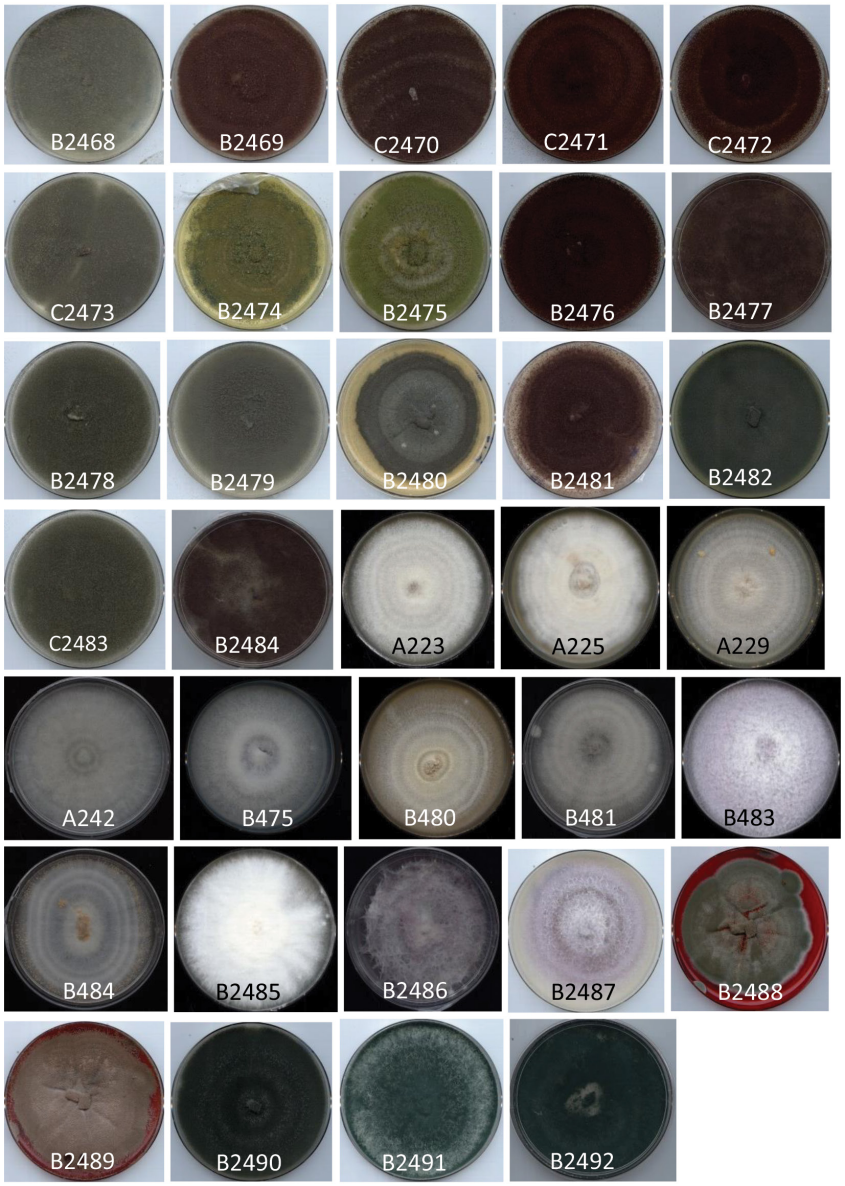

Figure 1. Colony features of *A. flavus (*isolates C2471, AB2474, B2475, B2482), *A. fumigatus* (isolates B2468, C2473, B2478, B2479, B2480, C2483), *A. niger* (isolates B2469, C2470, C2472, B2476, B2477, B2481, B2484), *F. solani* (isolates A223, A225, A229, A242, B475, B480, B481, B483, B484, B2486), *F. oxysporum* (isolate B2485), *F. proliferatum* (isolate B2487), *P. paraherquei* (isolates B2488 and B2489) and *T. asperellum* (isolates B2490, B2491 and B2492)

The *Aspergillus* and *Fusarium* species were observed to have the highest number of fungal isolates. According to the BLASTn analysis in the NCBI database, four isolates (C2471, B2474, B2475 and B2482) were successfully identified as *A. flavus,* six isolates (B2479, B2468, B2480, C2483, C2473 and B2478) as *A. fumigatus* and seven isolates (C2472, B2481, B2476, B2469, B2477, C2470 and B2484) as *A. niger* in a range of 97-99% maximum sequence identity. Twelve isolates were identified as the *Fusarium* species, mainly as *F. oxysporum* (B2485), *F. proliferatum* (B2487) and *F. solani* (A223, A225, A229, A242, B475, B480, B481, B483, B484 and B2486) with 98-99% maximum sequence identity. Isolates B2488 and B2489 were subjected to a BLAST analysis as *P. paraherquei* with 99% sequence similarity, while the remaining isolates (2490, B2491 and B2492) were identified as *T. asperellum* with 99% sequence similarity.

A study conducted by Radu and Kqueen (2002) reported on endophytes that were isolated from different types of medicinal plant including *Cantella asiatica* and *Eurycoma longifolia* Jack. In addition, Nur Ain Izzati and Wan Hasmida (2011) reported that a total number of 40 microfungi were isolated from traditional vegetables, namely *O. javanica, C. caudatus, P. odorata, P. tetragonolobus* and *C. asiatica*, that were only morphologically identified into four genera such as *Aspergillus, Fusarium, Penicillium* and *Trichoderma* species.

A phylogenetic tree was constructed using the neighbour-joining method of the MEGA Version 6.06 software with 1000 bootstrap replications. Nine additional sequences obtained from the NCBI GenBank databases were used as references including for the outgroup. Generally, the phylogenetic tree consisted of four genera, mainly *Aspergillus, Fusarium, Penicillium* and *Trichoderma.* Results from the phylogenetic tree (Figure 2) based on ITS region revealed that all the 34 endophytic fungi isolates belonging to the Ascomycete family could be divided into four terminal clades. *Aspergillus* sp. was the dominating group of fungi represented by four isolates of *A. flavus,* six isolates of *A. fumigatus* and seven isolates of *A. niger* in a 97-99% maximum sequence identity. Twelve isolates were identified as the *Fusarium* species, with *F. oxysporum*, *F. proliferatum* and *F. solani* showing a 98-99% maximum sequence identity. Isolates of *P. paraherquei* and the remaining three isolates identified as *T. asperellum* showed a 99% sequence identity.

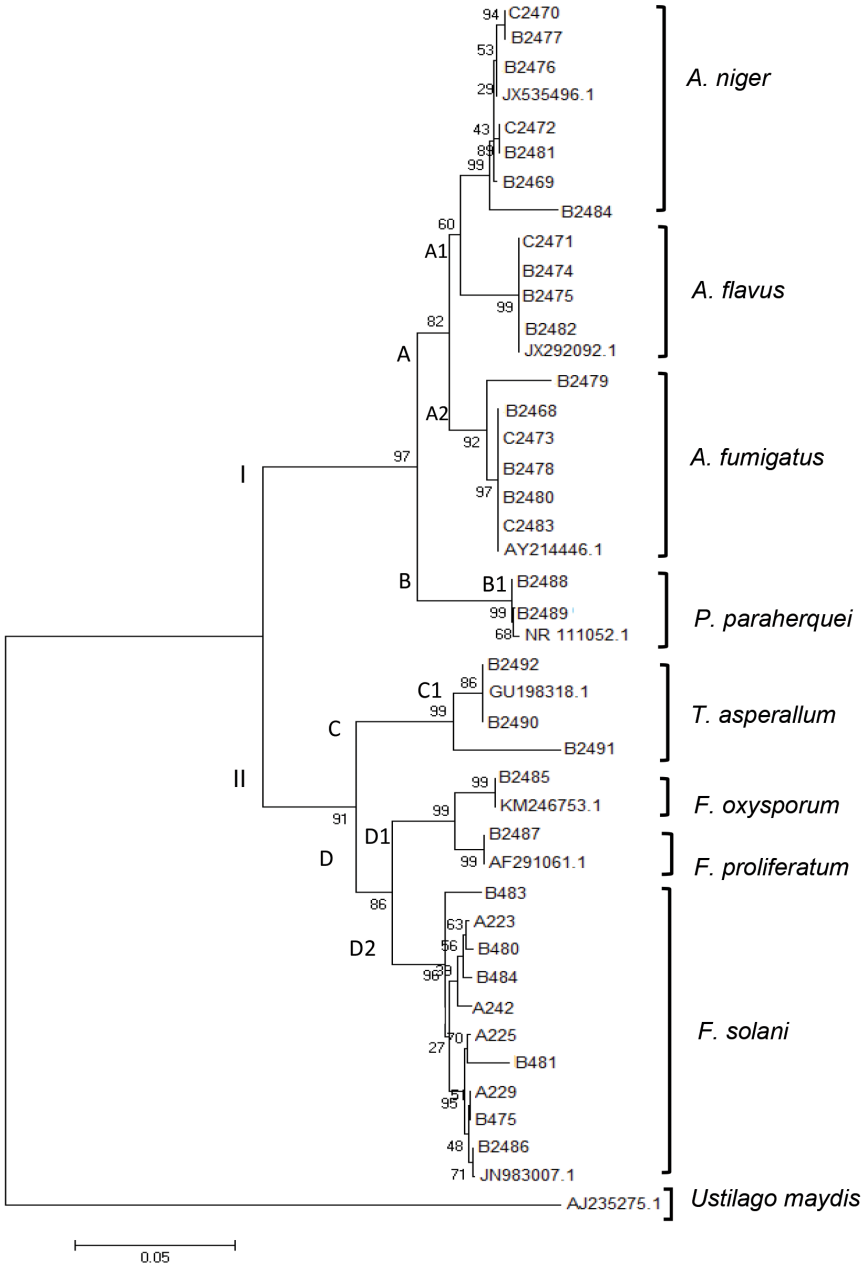

Figure 2. The evolution history was inferred using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 43 nucleotide sequences. *Ustilago maydis* (AJ235275) was taken as the outgroup.

The phylogenetic tree grouped the isolates into two major clades (Clade I and Clade II) with the outgroup (*U. maydis*) clade (Figure 2). Clade I was grouped into two clades (Clade A and Clade B) that consisted of 19 isolates, while Clade II was also grouped into two clades (Clade C and Clade D), which consisted of 15 isolates. Clade A1 consisted of 10 isolates of *A. niger* and *A. flavus*, which was the cluster formed supported by a 60% bootstrap value with two reference isolates identified as JX535496.1 (*A. niger)* and JX292092.1 (*A. flavus*). Clade A1 was branched into two small clusters that comprised 11 isolates classified into two species containing: i) *A. niger* (B2469, C2470, C2472, B2476, B2477, B2481 and B2484 isolated from *Psophocarpus tetragonolobus, Cosmos caudatus* and *Oenanthe javanica*) supported with a 99% bootstrap value; and ii) *A. flavus* (C2471, B2474, B2475 and B2482 isolated from *Psophocarpus tetragonolobus, Cosmos caudatus* and *Oenanthe javanica*) with a 99% bootstrap value. On the other hand, isolates of B2468, C2473, B2478, B2479, B2480 and C2483 (isolated from *Persicaria odorata, Psophocarpus tetragonolobus, Cosmos caudatus* and *Oenanthe javanica*) were closely related to *A. fumigatus* (AY214446.1) with a 92% bootstrap value clustered in Clade A2. This proved that the ITS region of some strains was highly conserved. This is highly supported by previous studies stating that ITS region is a highly conserved region (Sumida et al., 2004; Maryam & Ehsan, 2015). Based on the phylogenetic tree, all the species were clearly placed into separate clades.

CONCLUSION

In this study, 34 fungal isolates were grouped under eight different species, *Aspergillus flavus, A. fumigatus, A. niger, Fusarium oxysporum, F. proliferatum, F. solani, Penicillium paraherquei* and *Trichoderma asperellum*. All the isolates were identified based on morphological characteristics and ITS sequence analyses. *Aspergillus* sp. was the dominant genus with 17 isolates, while two isolates of *P. paraherquei* and three isolates of *T. asperellum* were also successfully identified. These findings can be used as reference for future study on the diversity of microfungi isolated from herbs as well as the role of fungi in herbs.

REFERENCES

Abaş, İ., Özpinar, H., Kutay, H. C., Kahraman, R., & Eseceli, H. (2005). Determination of the metabolizable energy (ME) and net energy lactation (NEl) contents of some feeds in the Marmara region by *in vitro* gas technique. *Turkish Journal of Veterinary and Animal Sciences, 29*(3), 751–757.

Aly, A. H., Debbab, A., Kjer, J., & Proksch, P. (2010). Fungal endophytes from higher plants: A profilic source of phytochemicals and other bioactive natural products. *Fungal Diversity*, *41*(1), 1–16.

Fatimah, A., Norazian, M., & Rashidi, O. (2012). Identification of carotenoid composition in selected *'ulam'* or traditional vegetables in Malaysia. *International Food Research Journal*, *19*(2), 527–530.

Guo, B., Wang, Y., Sun, X., & Tang, K. (2008). Bioactive natural products from endophytes: A review. *Applied Biochemistry and Microbiology*, *44*(2), 136–142.

Hussain, A. I., Anwar, F., Sherazi, S. T. H., & Przybylski, R. (2008). Chemical composition, antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. *Food Chemistry, 108*(3), 986–995.

Kumar, S., & Sagar, A. (2007). Microbial associates of *Hippophae rhamnoides* (seabuckthorn). *Plant Pathology Journal*, *6*(4), 299–305.

Leslie, J. F., & Summerell, B. A. (2006). *The Fusarium laboratory manual*. Iowa, USA: Blackwell Publishing Professional.

Madhusudan, M. C., Barathi, T. R., & Prakash, H. S. (2015). Isolation and purification of bioactive metabolites from fungal endophytes – a review. *Current Biochemical Engineering*, *2*(2), 111–117.

Maryam, T., & Ehsan, H. (2015). Molecular detection of endophytic *Myrothecium* spp. by ITS-sequencing technique. *International Journal of Research Studies in Biosciences, 3*(1), 60–66.

Nath, A., Raghunatha, P., & Joshi, S. R. (2012). Diversity and biological activities of endophytic fungi of *Emblica officinalis* and ethnomedicianl plant of India. *Mycobiology*, *40*(1), 8–13.

Nur Ain Izzati, M. Z., & Wan Hasmida, W. I. (2011). Isolation of microfungi from Malay traditional vegetables and secondary metabolites produced by *Fusarium* species. *Sains Malaysiana*, *40*(5), 437–444.

Pitt, J. I. (1979). *The Genus Penicillium and Teleomorphic States Eupenicillium and Talaromyces*. London: Academic Press Inc.

Radu, S., & Kqueen, C. Y. (2002). Preliminary screening of endophytic fungi from medicinal plants in Malaysia for antimicrobial and antitumor activity. *The Malaysian Journal of Medical Sciences, 9*(2), 23–33.

Raper, K. B., & Fennell, D. I. (1965). *The Genus Aspergillus*. Baltimore, USA: The Williams and Wilkins Company.

Shukri, M. M., Alan, C., & Noorzuraini, A. S. (2011). Polyphenols and antioxidant activities of selected traditional vegetables. *Journal Tropical Agricultural and Foundation Science*, 39(1), 1–7.

Sumida, M., Kato, Y., & Kurabayashi, A. (2004). Sequencing and analysis of the internal transcribed spacers (ITSs) and coding regions in the EcoR I fragment of the ribosomal DNA of the Japanese pond frog *Rana nigromaculata*. *Genes and Genetic Systems,* 79(2), 105–118.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*(12), 2725–2729.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications, 18*(6), 315–322.

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Interaction between the Long-Tailed Macaque and the Dung Beetle in Langkawi

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ABSTRACT

The interaction between dung beetles and primates was studied at Telaga Tujuh, Langkawi Island, Malaysia using such as observation and sampling method. The dung beetles were caught, and their dung balls collected. The beetles were identified as *Paragymnopleurus maurus*, and their dung balls were identified as originating from *Macaca fascicularis*, using a molecular approach involving the cytochrome *b* (Cytb) marker. This is the first record of *Paragymnopleurus maurus* from the study site in the Langkawi Islands and from this part of Malaysia. *Paragymnopleurus maurus* is attracted to the omnivorous dung of *M. fascicularis*, because it is a preferred food source for the beetle. Daytime is the active period for *P. maurus* and this study shows that the foraging area of *P. maurus* is restricted to the forest, even though the beetle's food source (*M. fascicularis*'s dung) can be found outside the forest.

Keywords: Primate, new record, dung beetle, species interactions, Langkawi Island

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INTRODUCTION

Dung beetles (Coleoptera: Scarabaeidae: Scarabaeinae) perform many crucial roles in the ecosystem, including soil enrichment, nutrient cycling, seed dispersal (Willson et al., 1990; Jordano, 1992), fly

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control (Haufe, 1989; Guglielmone et al., 1999; Nichols et al., 2008). The most important characteristic of dung beetles is their preference for animal faces as a food source and breeding medium. In addition to faces, dung beetles also use decaying matter as food during their adult and larval stages (Halffter & Matthews, 1966; Halffter & Edmonds, 1982).

The survival of dung beetles are highly dependent on other animals, especially mammals Some species of dung beetles are specific with regard to habitat selection (Hanski & Camberfort, 1991) and may not be able to survive in open vegetation where the number of small- or medium-sized mammals producing faces is lower. A study conducted by Davis (2000) in Borneo reported that the dung beetle diversity was lower in logged forests than in undisturbed forest areas.

The relationship between dung beetles and mammals are likely to be more specific than previously expected; for example, as documented by Hanski and Camberfort (1991), herbivorous, omnivorous, and carnivorous mammals will attract different species of dung beetles.

Three primate families are found on Langkawi Island: Hylobatidae, Lorisidae, and Cercopithecoidea. Likewise, the superfamily Cercopithecoidea can be divided into two subfamilies: Cercopithecinae and Colobinae. The genus *Macaca*, a representative of the omnivorous Cercopithecinae subfamily, has three species that are found in Malaysia: *M. fascicularis* (long-tailed macaques), *M. nemestrina* (pig-tailed macaques), and *M. arctoides* (stump-tailed macaques). Of the Cercopithecinae, only *M. fascicularis* and *M. nemestrina* are found on Langkawi. The genera *Trachypithecus, Presbytis,* and *Nasalis* represent colobines, or the leaf-eating group (Bennett, 1991); *Presbytis* and *Trachypithecus* are specific to the Langkawi Islands. Different primate species tend to have different lifestyles (i.e., arboreal or terrestrial) and different diets (Fam & Nijman, 2011). Therefore, different species of dung beetles are associated with primate species that have different diets. The objective of this study was to record the interactions between the dung beetles and the primates inhabiting this island ecosystem.

METHOD

Study Site

The sampling site selected for this study is the Telaga Tujuh Waterfalls, near the Mat Chinchang forest, a tropical mixed dipterocarp rainforest. The forest is on the island of Langkawi, located on the west coast of Peninsular Malaysia (6° 22.162′ N, 99° 40.827′ E).

Sampling of Dung Beetles and Faeces

Field observations were conducted to search for any interactions between primates and dung beetles that specialise in rolling dung. The observation of macaque troops was conducted from 21–23 August 2015, from 10am to 1pm each day, with 45-minute observation period and 15-minute interval.

The conditions were bright and sunny throughout the sampling period. Only diurnal species was observed because it coincided with the specific active period of the dung beetles under study (Davis, 1999; Niino et al., 2014). No passive trapping or bait trapping was used to collect the dung beetles. However, when any rolling dung balls were observed, both the dung beetle and the dung ball were collected. The collected dung beetles were preserved and identified in Universiti Kebangsaan Malaysia (UKM) laboratory using available taxonomic keys (Ochi et al., 1996; Ek-Amnuay, 2008); the diagnostic characters were elongated legs and two frontal lobes on the clypeus. Photographs of the dung beetles were taken using a Canon EOS 6D camera attached to a stereomicroscope (Zeiss Stemi SV11). Faeces from *M. fascicularis* and from beetles' dung balls were taken to the UKM laboratory to identify the associated macaque species using a molecular approach. To avoid cross-contamination, only a single faecal sample was collected per session and was carefully stored in a 15-ml vial with 99% ethanol, to preserve the traces of DNA in the faces.

The faecal samples were visually examined to identify the target species; usually, the genus *Macaca* has brown-coloured faces, while that of *Trachypithecus* is greenish. The colours are different because of the omnivorous and frugivorous diets of the macaques and the strictly herbivorous and folivorous diets of the langurs (called "*lutung*" locally). Fresh samples of the faces were easily found by looking for flies or by observing the dung beetles rolling the faces into dung balls.

DNA Extraction

The DNA was extracted from 0.2–0.4-g samples of the faces (2 replicates) using the innuPREP Stool DNA Kit (Analytik, Jena, Germany), following the manufacturer's protocol.

Polymerase Chain Reaction (PCR)

To confirm the identity of the macaque species, PCR was conducted using the mitochondrial DNA Cytochrome *b* (Cytb) as a primer (Table 1). This primer was designed specifically to avoid species cross-contamination. A sequence of *M. fascicularis* was retrieved from GenBank to be used as template in designing primer. The Primer-BLAST program was used to generate species-specific primer (Ye et al., 2012). The Needleman-Wunsch global alignment algorithm (Needleman & Wunsch, 1970) is used by Primer-BLAST to check the specificity of the primer pairs to the template sequences. To choose the best primer pair, the physical characteristics such as length (mer), guanine-cytosine (GC) content (%), molecular weight (g/mole), extinction coefficient at $260_{nm,}$ dimer and complementary sequences of the primer pairs were analysed using Oligo Analyzer 3.1. By using the Phusion Flash-High-Fidelity PCR Master Mix (Thermofisher scientific) which contains Phusion Flash II DNA Polymerase, 2x

reaction buffer, dNTPs and $MgCl_2$ as PCR reagent, a three-step PCR process was employed, including an initial denaturation at 98°C held for 10 seconds, followed by 30 cycles of denaturation at 98°C for 1 second, 50°C of annealing for 30 seconds, 72°C for 15 seconds of extension, and a final extension at 72°C for 1 minute (see Table 2).

Table 1
The designed primer sequences for PCR

Primer name	Sequence (5'-3')	Locus	Annealing Temperature (°C)	Species
Latiff1018_F	CAATACACTACTCACCAGAC	Cyt *b*	50.0	*M. fascicularis*
Latiff1069_R	TAGGTTGTTTTCGATTAGGG			

Table 2
PCR components in DNA amplification

Chemical	Volume (x1) (µl)
Phusion Flash High Fedelity (Mastermix)	10.0
100 µM Primer Forward	1.0
100 µM Primer Reverse	1.0
Template DNA	1.0
dd	7.0
TOTAL	20.0

RESULTS

Dung Beetle Species

Field observations showed that only a single species of dung beetle had a strong preference for primate faces. Dung-rolling beetles of the species *Paragymnopleurus maurus* (Figure 1 & Figure 2) consumed primate faces as their main food source from this area.

Figure 1. Dung beetle *Paragymnopleurus maurus* making a dung ball and ready to roll it to other places

Figure 2. *Paragymnopleurus maurus*

Primate Species

Faeces rolled by *P. maurus* were collected to determine their host DNA because, aside from hair follicles, faces are the best non-invasive source of DNA from primates (Inoue et al., 2007; Marangi et al., 2015). The entire mitochondrial DNA (mtDNA) sample from the faeces were successfully extracted using the standardised method described in the manufacturer's protocol. The PCR products with the most visible bands on the agarose gel (Figure 3) were sequenced to analyse the effectiveness of the primer and the PCR optimisation.

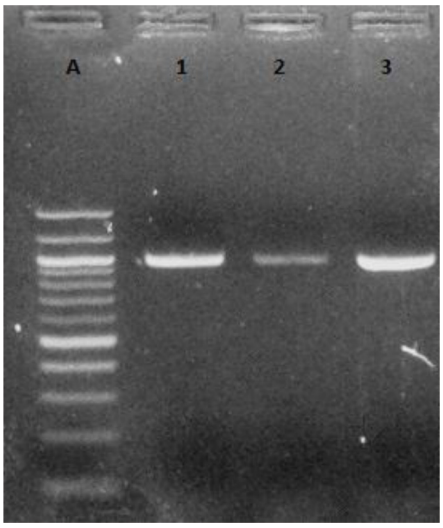

Figure 3. Result of PCR process on 1.5% agarose gel. Well A=100bp molecular marker

The sequences obtained were analysed using the GenBank BLAST to ensure that the targeted loci of the selected species were amplified. The parameters used to ensure the specificity of the DNA sequences included the percentage of queries covered by alignment to the database sequence, the best expected value (E-Value) of the alignments from the database sequence, and the highest percent identity (Max Identity) of all the query-subject alignments. The sequence obtained from the PCR product had 98% specificity to the database sequence of *M. fascicularis,* agreeing with the Max Identity and an E-Value ≤0. The specifically designed primer successfully amplified the targeted loci, and the overall molecular approach confirmed that the faces rolled by *P. maurus* on Langkawi Island belonged to a single species, *M. fascicularis* (Figure 4).

Figure 4. *Macaca fascicularis* (Long-tailed macaque) enjoying food in Langkawi Island, Kedah, Malaysia

DISCUSSION

Three genera of dung-rolling beetles have been recorded in Malaysia: *Paragymnopleurus*, *Ochicanthon*, and *Sisyphus*. Among these, *Paragymnopleurus* is the largest (2–4 cm in length) and was the only species found in this study. *P. maurus*, a large-bodied species, is associated with the long-tailed macaque, *M. fascicularis*. The existence of the interaction has been documented based on molecular data and live observations in the field. Though *P. maurus* is classified as large dung beetle, it is not the largest dung beetle that can be found in Malaysia (Muhaimin et al., 2015). Species such as *Heliocopris tyrannus* (Goh et al., 2014), *Catharsius renaudpauliani*, and *C. molossus* are much larger than *P. maurus* and have been previously recorded in Singapore (Ong et al., 2013). However,

in this study, which was conducted during the daytime, we focused only on diurnal species of dung beetles. Niino et al. (2014) reported that *P. maurus* is active during the day, while a closely related species, *P. striatus*, is active at night. It is likely that if we had conducted our sampling activities at night, the presence of *P. striatus* might have been detected.

The interactions between a primate species and a dung beetle species were recorded for the first time at the Telaga Tujuh Waterfalls, a site adjacent to the Mat Chinchang forest on the main island of Langkawi. This dung beetle species is common, due to its wide distribution in Peninsular Malaysia (Doll et al., 2014; Niino et al., 2014), Borneo (Davis, 2000; Davis et al., 2001), and the Oriental region (Davis et al., 2002). Unlike the genus *Ochicanthon*, which prefers to consume carrion (Krikken & Huijbergts, 2007), *Paragymnopleurus* is similar to the genus *Sisyphus* in terms of food preference.

According to Lee et al. (2009), large-bodied dung beetle species are more sensitive to forest disturbances than smaller species. This is because large dung beetles require big forest areas inhabited by large population of mammals. As the mammals provide food and a breeding medium for the dung beetles, declining population of mammals directly affect the survival of dung beetles. This relationship was documented by Scheffler (2005) and Gardner et al. (2008), who observed the same direct correlations between the mammal and dung beetle populations in the tropical forests of Brazil. However, a different circumstance was observed for *P. maurus,* which seems to be more resistant to habitat disturbance and can thrive in smaller forest patches. According to Qie et al. (2011), *P. maurus* can also be found in the small fragmented and isolated recreational forest patches of Kenyir Lake, Terengganu. Lee et al. (2009) discovered the same dung beetle species in small forest patches in Singapore, a case similar to that of Langkawi Island, in which island effects and similar abiotic factors prevail.

Regarding the dung preference of *P. maurus*, Hanski and Camberfort (1991) claimed that *P. maurus* is closely associated with the dung of omnivorous animals. However, this species is also found on carrion (Sakai & Inoue, 1999), human dung (Davis et al., 2000), cattle dung (Muhaimin et al., 2015), and pig dung (Slade et al., 2007). *P. maurus* specimens have also been collected from elephant dung (Doll et al., 2014; Goh et al., 2014), which is their food source and breeding medium. In this study, *P. maurus* preferred the dung of the omnivorous primate *M. fascicularis*. This indicates that either *P. maurus* is a generalist or that it has expanded its food preferences due to the scarcity of its preferred food source. Further research should be conducted to show the actual diet preference of this species.

The long-tailed macaque *M. fascicularis* is a mutualist–edge species, resilient and well-adapted to interaction with humans (Abdul-Latiff et al., 2014a, 2014b). Garbage dump sites provide

the macaques with ample food sources. However, the dung beetle *P. maurus* is incapable of venturing far out into human areas because its flying ability and mobility is limited (Niino et al., 2014) and specific requirements such as the forest floor and vegetation types that only the forest can provide (Doube, 1983; Andresen, 2005). Understanding the interactions and preferences of dung beetles, both tunnellers and rollers, with regard to primate dung is crucial for understanding the dynamics of the surrounding ecosystems (Nichols et al., 2007). This study has reported the interactions between the dung beetle *P. maurus* and the macaque *M. fascicularis*. Future works should include the identification of the components that attract this dung beetle to the faces of the long-tailed macaque, as well as the mode of seed dispersal involved in this interaction. Further and more specific studies on the rich biodiversity of the Langkawi Islands should be conducted for developing community-level conservation strategies for this invaluable UNESCO Geopark site.

CONCLUSION

This study has shown *Paragymnopleurus maurus* has direct interactions with the long-tailed macaque (*M. fascicularis*); this dung beetle evidently prefers omnivorous faces, a main diet of this primate. This study can contribute to knowledge by showing interactions among species, utilising practical methodologies and field samples such as the dung or faces of primates and other vertebrate hosts.

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REFERENCES

Andresen, E. (2005). Effects of season and vegetation type on community organization of dung beetles in a tropical dry forest. *Biotropica*, *37*(2), 291-300.

Bennett, E. L. (1991). Diurnal primates. In R. Kiew (Ed.), *The State of Nature Conservation in Malaysia* (pp. 150-172). Kuala Lumpur: Malaysia Nature Society.

Davis, A. J. (1999). Species packing in tropical forests: diel flight activity of rainforest dung-feeding beetles (Coleoptera: Aphodiidae, Scarabaeidae, Hybosoridae) in Borneo. *The Raffles Bulletin of Zoology*, *47*(2), 473-486.

Davis, A. J. (2000). Does reduced-impact logging help preserve biodiversity in tropical rainforests? A case study from Borneo using dung beetles (Coleoptera: Scarabaeoidea) as indicators. *Community and Ecosystem Ecology*, *29*(3), 467-475.

Davis, A. J., Holloway, J. D., Huijbregts, H., Krikken, J., Kirk-Springgs, A. H., & Sutton, S. L. (2001). Dung beetles as indicators of change in the forest of northern Borneo. *Journal of Applied Ecology*, *38*(3), 593-616.

Davis, A. J., Huijbregts, H., & Krikken, J. (2000). The role of local and regional processes in shaping dung beetle communities in tropical forest plantations in Borneo. *Global Ecology and Biogeography*, *9*(4), 281–292.

Davis, A. L., Scholtz, C. H., & Philips, T. K. (2002). Historical biogeography of scarabaeine dung beetles. *Journal of Biogeography*, *29*(9), 1217-1256.

Doll, H. M., Butod, E., Harrison, R. D., Fletcher, C., Kassim, A. R., Ibrahim, S., & Potts, M. D. (2014). Environmental and geographic factors driving dung beetle (Coleoptera: Scarabaeidae: Scarabaeinae) diversity in the dipterocarp forests of Peninsular Malaysia. *Raffles Bulletin of Zoology*, *62*, 549-560.

Doube, B. M. (1983). The habitat preference of some bovine dung beetles (Coleoptera: Scarabaeinae) in Hluhluwe Game Reserve, South Africa. *Bulletin of Entomological Research*, *73*(3), 357–371.

Ek-Amnuay, P. (2008). *Beetles of Thailand.* Bangkok: Amarin Printing and Publishing Co., Ltd.

Fam, S. D., & Nijman, V. (2011). Spizaetus hawk-eagles as predators of arboreal colobines. *Primates*, *52*(2), 105-110.

Gardner, T. A., Barlow, J., Araujo, I. S., Avila-Pires, T. C., Bonaldo, A. B., Costa, J. E., … & Mestre, L. A. M. (2008). The cost-effectiveness of biodiversity surveys in tropical forest. *Ecology Letters*, *11*(2), 139–150.

Goh, T. G., Huijbregts, J., Ning, H., & Campos-Arceiz, A. (2014). Bettles recorded to visit elephant dung in Temenggor Forest, Malaysia. *Journal of Wildlife and Parks*, *29*, 45-48.

Guglielmone, A. A., Gimeno, E., Idiart, J., Fisher, W. F., Volpogni, M. M., Quaino, O., … & Warnke, O. (1999). Skin lesions and cattle hide damage from Haematobia irritans infestations in cattle. *Medical and Veterinary Entomology*, *13*(3), 323–328.

Halffter, G., & Edmonds, W. (1982). *The nesting behavior of dung beetles* (*Scarabaeinae): an ecological and evolutive approach.* Mexico, D. F.: Instituto de Ecologıa.

Halffter, G., & Matthews, E. (1966). The natural history of dung beetles of the subfamily Scarabaeinae (Coleoptera, Scarabaeidae). *Folia Entomologica Mexicana*, *12-14*, 1–312.

Hanski, I., & Camberfort, Y. (1991). *Dung Beetle Ecology.* Princeton: Princeton University Press.

Haufe, W. (1989). Host–parasite interaction of blood feeding dipterans in health and productivity of mammals. *International Journal of Parasitology*, *17*(2), 607–614.

Inoue, T., Hiratsuka, M., Osaki, M., & Oshimura, M. (2007). The molecular biology of mammalian SIRT proteins: SIRT2 in cell cecle regulation. *Cell Cycle*, *6(9)*, 1011-1018.

Jordano, P. (1992). Fruits and frugivory. In M. Fenner (Ed.), *Seeds: the ecology of regeneration in plant communities* (p. 127). Wallingford, UK: CAB International.

Krikken, J., & Huijbregts, J. (2007). Taxonomic diversity of the genus *Ochicanthon* in Sundaland (Coleoptera: Scarabaeidae: Scarabaeinae). *Tijdschrift voor Entomologie*, *150*(2), 421-479.

Lee, J. S., Lee, I. Q., Lim, S. L., Huijbregts, J., & Sodhi, N. S. (2009). Changes in dung beetle communities along a gradient of tropical forest disturbance in South-East Asia. *Journal of Tropical Ecology*, *25*(6), 677–680.

Marangi, M., Koehler, V. A., Zanzani, S. A., Manfredi, M. T., Brianti, E., Giangaspero, A., & Gasser, R. B. (2015). Detection of Cyclospora in captive chimpanzees and macaques by a quantitative PCR-based mutation scanning approach. *Parasites and Vectors*, *8*(1), 274-278.

Muhaimin, A. M. D., Hazmi, I. R., & Yaakop, S. (2015). Colonization of dung beetles (Coleoptera: Scarabaeidae) of smaller body in the Bangi Forest Reserve, Selangor, Malaysia: A model sampling site for a secondary forest area. *Pertanika Journal of Tropical and Agricultural Sciences*, *38(4)*, 519 - 532.

Needleman, S. B., & Wunsch, C. D. (1970). A general method applicable to the search for similarities in the amino acis sequence of two proteins. *Journal of Molecular Biology, 48*(3), 443-453.

Nichols, E., Larsen, T., Spector, S., Davis, A. L., Escobar, F., Favila, M., & Vulinec, K. (2007). Global dung beetle response to tropical forest modification and fragmentation: A quantitative literature review and meta-analysis. *Biological Conservation*, *137*(1), 1-19.

Nichols, E., Spector, S., Louzada, J., Larsen, T., Amezquita, S., Favila, M. E., & Vulinec, K. (2008). Ecological functions and ecosystem services provided by Scarabaeinae dung beetles. *Biological Conservation*, *141*(6), 1461-1474.

Niino, M., Hosaka, T., Kon, M., Ochi, T., Yamada, T., & Okuda, T. (2014). Diel flight activity and habitat preference of dung beetles (Coleoptera: Scarabaeidae) in Peninsular Malaysia. *Raffles Bulletin of Zoology*, *62*, 795–804.

Ochi, T., Kon, M., & Kikuta, T. (1996). Studies on the family Scarabaeidae (Coleoptera) from Borneo. I. Identification keys to subfamilies, tribes and genera. *Giornale Italia Entomologia*, *8*, 37-54.

Ong, X. R., Chua, S. C., & Potts, M. D. (2013). Recent records of the dung beetle *Catharsius molossus* (Coleoptera: Scarabaeidae) in Singapore. *Nature in Singapore*, *6*, 1-6.

Owczarzy, R., Tataurov, A. V., & Wu, Y. (2008). IDT SciTools: a suite for analysis and design of nucleic acid oligomers. *Nucleic Acids Research, 36*(suppl_2), 163-169.

Qie, L., Lee, T. M., Sodhi, N. S., & Lim, S. L. (2011). Dung beetle assemblages on tropical land-bridge islands: small island effect and vulnarable species. *Journal of Biogeography*, *38*(4), 792-804.

Sakai, S., & Inoue, T. (1999). A new pollination system: Dung-beetle pollination discovered in *Orchindantha inouei* (Lowiaceae, Zingiberales) in Sarawak, Malaysia. *American Journalof Botany*, *86*(1), 56-61.

Scheffler, P. (2005). Dung beetle (Coleoptera: Scarabaeidae) diversity and community structure across three disturbance regimes in eastern Amazonia. *Journal of Tropical Ecology*, *21*(1), 9-19.

Slade, E. M., Mann, D. J., Villanueva, J. F., & Lewis, O. T. (2007). Experimental evidence for the effects of dung beetle functional group richness and composition on ecosystem function in a tropical forest. *Journal of Animal Ecology*, *76*(6), 1094–1104.

Willson, M., Rice, B., & Westoby, M. (1990). Seed dispersal spectra - a comparison of temperate plant communities. *Journal of Vegetation Science*, *1*(4), 547–562.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*(1), *134*.

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Enhancement of Performance of Farmed Buffaloes Pasture Management and Feed Supplementation in Sabah, Malaysia

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ABSTRACT

A buffalo-breeding farm run on an extensive grazing system without feed supplementation was selected for this study. Farm records between 2004 and 2011 were analysed for selected parameters, particularly calving rate, calving interval, average daily gain of calves and calf mortality. Following the analysis, interventions were implemented in January 2012, which included increase in pasture area from 399 to 441 acres followed by application of organic fertiliser. The selected breeder buffaloes were prepared for breeding by supplementing palm kernel cake-based feed at the rate of 1.5 kg/animal/day for two weeks before breeder males were introduced at the rate of one male to 20 females. Weaning age was reduced from six to three months. Prior to the intervention, proximate analysis of pasture revealed 7.6% crude protein content; approximately 79% of breeder buffaloes were found with a body score of ≥3; the average annual calving rate was 22%; the calving interval was 24±11.2 months; average daily gain of calves was 0.89±0.21 kg; the average birth weight was 28.31±3.26 kg; and calf mortality was 26.8±7.0%. Following intervention, proximate analysis of grass revealed 12% crude protein content. With feed supplementation, the percentage of breeder females with a body score of ≥3 increased to 95%, leading to an average annual calving rate of 50%. Average birth weight was significantly ($p<0.05$) improved to 35.4±5.39 kg, while the average daily gain was 0.95±0.32 kg. Subsequently, the average calving interval

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was reduced to 15.2±9.2 months. Similarly, calf mortality rate was significantly ($p<0.05$) reduced to 17.6±4.7%. In conclusion, improved management, particularly of pasture and feeding, significantly enhanced the performance of farmed buffaloes.

Keywords: Farmed buffaloes, feed supplementation, pasture, performance, body weight

INTRODUCTION

Asian buffalo or water buffalo is classified under the genus Bubalus, species bubalis. The domestic water buffalo, commonly found in Malaysia, has been classified into two sub-species known as the river and swamp types, both of which are different genetically and morphologically as well as in purpose (FAO, 2005). The river buffalo, which also known as murrah, has 50 chromosomes, of which five pairs are submetacentric, while 20 are acrocentric while the swamp buffalo has 48 chromosomes, of which 19 pairs are metacentric. Swamp buffalo is largely concentrated in Southeast Asia (e.g. Thailand, the Philippines, Indonesia, Vietnam, Myanmar, Laos, Sri Lanka, Malaysia) and Southern China, whereas river buffalo is mainly found in South Asia (e.g. India). Indeed, these Asian buffalo remains of high economic importance for farmers in many developing countries in Asia (Cruz, 2010). Besides being utilised as draught power for smallholders, they also provide high quality milk and meat and other by-products such as hide and skin (Wanapat & Wachirapakon, 1990). They are commonly kept under traditional management, which is mostly concentrated in the rice-growing states of Kelantan, Terengganu, Kedah and Pahang. They are usually provided with relatively poor digestible feeds (Yindee, 2011). Indeed, buffaloes are known to be better at converting poor-quality roughage (e.g. crop by-products and poor quality grasses) into milk and meat. They are reported to have a 5% higher digestibility of crude fibre and 4-5% higher efficiency of utilisation of metabolic energy than high-yielding cows (Mudgal, 1988). They graze in harvested paddy fields, along roadsides and on the edges of cultivated plots during the day and are kept within the village, usually under the house, at night (Khajarern & Khajarern, 1989). Nowadays, the buffalo has lost its prominence due mainly to farm mechanisation and urbanisation. Both breeds, murrah and swamp buffalo, are farmed mainly for meat production under an extensive production system. Some oil palm estates use swamp buffalo as draught animals for pulling carts carrying oil palm bunches (FAO, 2005). In addition, crossbreeding between murrah and swamp buffalo was also initiated as a programme at the Buffalo Breeding and Research Centre Farm in Telupid, Sabah. In general, murrah and swamp buffalo have a small body size, with body weight ranging between 350 and 450 kg, and a slow growth rate that leads to poor reproductive performance (e.g. late maturity and a long calving interval), but are very well adapted to local ecological conditions and display good

disease resistance (Nanda et al., 2003). Nevertheless, the body size and body weight in crossbreds are greater than in the pure breed (FAO, 2005). This was also reported in other studies conducted in Asia and Southeast Asia that concluded that the murrah crossbred buffaloes were heavier and grew faster compared to indigenous swamp buffaloes (Momongan et al., 1990; Kamonpatana et al., 1991; Situmorang & Sitepu, 1991; Parker et al., 1991; Salas et al., 2000). Indeed, there is a need to enhance scientific and technological development in buffalo nutrition, production, reproduction, biotechnologies and genetic improvement (Sethi, 2003). Thus, this paper describes the attempts to enhance the selected performance parameters of murrah crossed buffaloes that are kept at a breeding farm in Sabah, Malaysia through improved pasture and feeding management.

MATERIALS AND METHOD

The Farm

This study was conducted at the Buffalo Breeding and Research Centre Farm located at Telupid, Sabah, Malaysia (5^{o} 30' N, 117^{o} 7' E). The average daily temperature and annual rainfall in the farm recorded were between 29˚C and 30˚C and 200 and 400 mm/year, respectively. At the start of the study, the farm had a total of 335 buffaloes. Of these, 180 were breeder females, 78 were breeder heifers, seven were breeder males and 70 were calves, of which 51 were females and 19 were males (Othman et al., 2014). The 399 acres of pasture land were divided into paddocks with established pasture (*Brachiaria decumbens)* and wallowing sites. Besides *Brachiaria decumbens,* the paddocks were also covered with other vegetation, which included herbage, legumes, weeds and ferns, most of which are palatable to buffalo. However, the identification of each vegetation species was not done in this study. In addition, the farm did not apply a proper pasture management system that practised fertilisation and soil analysis as a routine. Although the pasture was generally poorly maintained, the farm practised an extensive 30-day rotational grazing system without feed supplementation (Othman et al., 2014).

At the start of the study, eight-year farm records between 2004 and 2011 were selected and analysed retrospectively for selected parameters associated with feed and feeding. Among the parameters analysed were calving rate, calving interval, birth weight, average daily gain of calf and calf mortality. Pasture samples were collected randomly in quadruplicate from four representative paddocks. A pair of scissors was used to cut off the mixed herbage sample on each paddock at six sites of 1m^{2} area. The samples were then dried and used for proximate analysis of the nutrient content (Galyean, 2010). Proximate analysis was performed to determine the nutrient content, which was found to include the following: dry matter, crude protein, crude fat, crude fibre and metabolisable energy. All the analyses were carried out according to certified procedures

outlined by the Manual of Laboratory Techniques, Universiti Putra Malaysia, and developed according to procedures of AOAC (1990). The proximate analysis of the samples was done in replicates of four.

Intervention

Intervention was implemented in January 2012. This included the use of organic fertiliser on pastureland at the rate of 20 tonnes of organic fertiliser supplemented with 200 kg of urea/ha/year to improve the pasture. After six months, pasture samples were collected from the four paddocks for re-analysis of the proximate nutrient content. After six months, the organic fertiliser as supplemented again, at the end of June 2012, as the farm was scheduled for pasture management. However, the results presented here are only for the first six months (January-June 2012) after supplementation with organic fertiliser.

A total of 150 breeder females of more than 350 kg body weight were selected as breeders. They were re-grouped into 20 heads per group according to body weight and were allowed to graze in the paddocks. They were prepared for breeding by being fed palm kernel cake-based supplemented feed at the rate of 1.5 kg/animal/day for 14 days before the breeder males were introduced in January 2012 at the rate of one male to 20 females for a period of three months. Pregnant buffaloes were re-grouped into 20 heads per group according to body weight and allowed to graze on supplemented feed of 1 kg/animal/day. The non-pregnant females were prepared again for breeding by being fed supplemented feed at 1.5 kg/animal/day for 14 days before the breeder males were re-introduced. The procedure was repeated one more time before the remaining non-pregnant breeder females were culled.

Data Analysis

The pre-intervention calves that were produced between 2004 and 2011 were weaned at six months of age, while those post-intervention calves that were produced between 2012 and 2014 were weaned at three months of age. All dead calves were recorded. Birth weight of the calves was measured and recorded within three days of birth. The body weight of calves was measured and recorded every three months and the average daily gain was calculated based on the body weight of the first three months according to this formula:

$$\text{Average daily gain (ADG)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{90 days}}$$

In this study, birth and body weight of calves between 2009 and 2011 were used to calculate the average birth and daily weight gain during pre-intervention, while those between 2012 and 2014 were used for post-intervention calculations. The body condition score was measured using a scale of 1 to 5 as described by Anitha et al. (2011). Briefly, after each check point was observed thoroughly by sight and palpation, the scores were recorded and an average body condition score was assigned to the buffaloes. The percentage

of calving rate was calculated according to this formula:

$$\text{Calving rate (\%)} = \frac{\text{Total no of calf born}}{\text{Total no of cows calving}} \times 100$$

whereas the percentage of calf mortality was calculated according to this formula:

$$\text{Mortality rate (\%)} = \frac{\text{Total no of calf died}}{\text{Total no of calf born}} \times 100$$

The calving interval was calculated based on the number of days between the birth of a calf and the birth of a subsequent calf, both from the same cow (Mellado et al., 2004).

Statistical Analysis

All data were analysed using the Statistical Package for the Social Sciences (SPSS) version 20 software. Means were tested using the T-test based on analysis of variance (ANOVA), with $p<0.05$ considered a significant difference.

RESULTS

Pre-Intervention

Prior to the intervention, proximate analysis of the pasture revealed 7.6% crude protein content (Table 1), while 79% of the breeder buffaloes showed a body score of $\geq$3 and were ready for breeding. The annual calving rate ranged between 15% and 31%, with an average of 22.1±6.4%, and the calving interval ranged between nine and 56 months with an average of 24±11.2 months. During the eight-year period, 73 (29%) breeders calved twice and 35 (14%) calved three times. The average birth weight was 28.31±3.26 kg, the average daily gain was 0.89±0.21 kg (Table 2) and calf mortality was 26.8±7.0% (Table 3).

Table 1
The nutrient composition (% DM) of pasture and palm kernel cake

	Pasture	Palm kernel cake
Dry matter	95.3	89.1
Ash	10.1	3.5
Crude protein	7.6	16.9
Crude fat	2.3	5.5
Crude fibre	36.4	13.0
Acid detergent fibre	44.7	34.3
Neutral detergent fibre	81.2	66.8
Acid detergent lignin	8.2	6.7
Metabolisable energy (MJ/kg DM)	7.7	11.9

Table 2
Average birth weight and daily weight gain of buffalo calves before and after intervention

Year	Birth weight (kg) (Mean±SD)	Daily weight gain (kg) (Mean±SD)
Pre-intervention		
2009	28.5±1.36	1.0±0.16
2010	27.2±1.04	1.0±0.16
2011	28.7±2.08	0.7±0.21
Average	28.3±3.26	0.9±0.21
Post-intervention		
2012	30.5±3.43	0.9±0.24
2013	32.5±5.27	0.9±0.25
2014	37.1±4.76	1.2±0.35
Average	35.4±5.39*	1.0±0.32

*$p<0.05$ significant difference between pre-intervention and post-intervention

Table 3
Calf mortality before (2004 to 2011) and after intervention (2012 to 2014)

Year	No. of calves		% Calving	% Mortality
	Birth	Death		
Pre-intervention				
2004	60	13	24.0	21.6
2005	73	12	29.2	16.4
2006	43	15	17.2	34.8
2007	78	29	31.2	37.2
2008	42	13	16.8	31.0
2009	37	9	14.8	24.3
2010	66	17	26.4	25.8
2011	43	10	17.2	23.3
Average	55.3±15.9	14.8±6.3	22.1±6.4	26.8±7.0
Post-intervention				
2012	53	8	35.3	15.9
2013	101	14	67.3	13.8
2014	72	9	48.0	12.5
Average	73.3±24.2	10.3±3.2	50.2±16.1	14.1±1.7

Post-Intervention

Following intervention, proximate analysis of the pasture revealed a significantly higher content (12%) for crude protein compared to the crude protein content before intervention (7.6% crude protein). With feed supplementation, the percentage of breeder females with a body score of ≥ 3 increased significantly ($p<0.05$) to 95%, leading to a significant ($p<0.05$) increase

in the average annual calving rate to 50.2±16.1%. A total of 69 (46%) had calved twice, which was significantly ($p<0.05$) more than in the pre-intervention period and eight (5%) calved three times in the three-year study period, significantly ($p<0.05$) less than in the pre-intervention period. The average birth weight had significantly ($p<0.05$) improved to 35.4±5.39 kg and the average daily gain was improved to 1.0±0.32 kg (Table 2). Subsequently, the calving interval was significantly ($p<0.05$) reduced to 15.2±9.2 months. Similarly, the calf mortality rate was significantly ($p<0.05$) reduced to 17.6±4.7% (Table 3).

DISCUSSION

Diet is the main factor that affects body weight and the body condition of livestock (Zerbini & Wold, 1999). Nevertheless, Mahmoudzadeh and Fazaeli (2009) indicated that manipulation of diet could sometimes exert a profound effect on the weight gain of buffalo calves. This explained the slight increase in the post-intervention average daily weight gain of the buffalo calves compared to in the pre-intervention period of this study. These results were in agreement with those reported in previous studies such as the work of Situmorang and Sitepu (1991) in Indonesia, Kamonpatana et al. (1991) in Thailand and Momongan et al. (1990), Parker et al. (1991) and Salas et al. (2000) in the Philippines, who concluded that the murrah crossbred buffaloes were heavier and grew faster after having been fed with improved feed. However, feeding and pasture management can also enhance other parameters such as birth weight, which can show significant increases in post-intervention (Khajarern & Khajarern, 1989). Indeed, birth weight in this study was higher compared to that recorded in a study by Nordin et al. (2004) in Malaysia and Thevamonaharan et al. (2001) in Thailand. Similarly, the average birth weight of murrah crossbred buffaloes reported by Charlini and Sinniah (2015) in Sri Lanka was also lower compared that recorded in the present study. In addition, Mahmoudzadeh and Fazaeli (2009) reported that improvement of growing and fattening performance of buffalo calves could be achieved through nutritional and management manipulation. Indeed, the average daily gain was shown to reach the maximum figure recorded when the animals received 10% crude protein (CP) in the diet through improved pasture (CP, 12%) as observed in this study (Tatsapong et al., 2010). Similarly, the buffaloes were shown to have gained weight with feed supplementation; this effect was less pronounced when the basal diet was only grass (Van Thu & Preston, 1999). This was due to the high CP and total digestible nitrogen (TDN) levels in the supplemented diet that increased the feed intake, while ruminal pH was reduced and the ammonia nitrogen and blood urea nitrogen concentration were increased (Chanthakhoun et al., 2014). Therefore, feed supplementation helps in maintaining the body condition of breeder buffaloes by reducing weight loss (Sanh, 2005; Jabbar et al., 2013).

Body condition score (Anitha et al., 2011) has been used as an indicator of energy status resulting from feed supplementation (Qureshi, 2009). Animals receiving metabolisable energy (ME) above the requirements during the pre-partum period are able to maintain a relatively good body condition score (BCS) (Qureshi, 2009), a phenomenon observed in this study when the percentage of breeder buffaloes with a good body score increased to 95% following feed supplementation. Furthermore, feed supplementation improved reproduction, as evident from the short post-partum ovulation interval and low incidence of silent ovulation (Qureshi, 2009). Therefore, high-energy supplemented feed such as the palm kernel cake-based diet implemented in this study helps to improve the reproductive performance of buffaloes.

Early weaning at 45 days of age has been recognised as a major cause of mortality among buffalo calves (Parera, 1999). Nevertheless, climatic features, such as rainy season (Othman et al., 2014), and pre-weaning nutrition level also play an important role in calf mortality. Low supplies of CP and TDN for lactating mothers, such as during pasture-deficient period or poor quality pasture lowered milk yield (Hayashi et al., 2006), particularly among buffaloes in their second and more lactation cycle (Hayashi et al., 2005). Therefore, the average growth rate of buffalo calves is better when feed is supplemented (Lubis & Fletcher, 1987). In addition, the high average body weight and the rapid growth rate for buffalo calves may have been influenced by the feeding management of the dam. Indeed, good quality pasture together with high-energy concentrate feed provided to the dam have been shown to result in high production of milk (Donker et al., 1968; Sarwar et al., 2012) and better quality of milk (Slots et al., 2009; O'Donovan et al., 2011). A study on the nutrient composition of milk for lactating buffaloes reared on this farm revealed that the nutrient composition of the milk was higher in protein content and lower in fat content compared to that reported by Siregar et al. (2015) in Indonesia for swamp buffalo and by Chiangmai et al. (1987) in Thailand and Ren et al. (2015) in China for murrah crossbred buffalo. Apart from that, the better growth rate led to a better survival rate (Thevarnanoharan et al., 2001).

CONCLUSION

This study revealed that improved management of pasture through regular use of fertiliser and supplemented feed at 1.5 kg/animal/day for 14 days improved the body condition of breeder females. Maintaining pregnant females with supplemented feed at 1 kg/animal/day improved the reproductive performance of the breeders and enhanced the body weight gain of the calves.

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REFERENCES

Anitha, A., Rao, K. S., Suresh, J., Moorthy, P. S., & Reddy, Y. K. (2011). A body condition score (BCS) system in murrah buffaloes. *Buffalo Bulletin, 30*(1), 79–99.

Chanthakhoun, V., Wanapat, M., Berg, J., & Kang, S. (2014). Influence of crude protein and energy level on feed intake, ruminal ammonia nitrogen, and methylglyoxal production in swamp buffaloes (*Bubalus bubalis)*. *Journal of Animal and Plant Science, 24*(6), 1716–1723.

Charlini, B. C., & Sinniah, J. (2015). Performance of murrah, surti, nili-ravi buffaloes and their crosses in the intermediate zone of Sri Lanka. *Development, 27*(3), 1-17.

Cruz, L. C. (2010). Recent developments in the buffalo industry of Asia. *Proceedings of the 9th World Buffalo Congress* (pp. 7–19). Buenos Aires, Argentina.

FAO (2005). Buffalo production and research. *REU Technical Series 67,* 14–15.

Frisch, J. E., & Vercoe, J. E. (1984). Improving the swamp buffalo of South-east Asia. In Copland, J. W. (Ed.), *Evaluation of large ruminants for the tropics* (pp. 37-43). Australia: Australian Centre for International Agricultural Research.

Galyean, M. M. (2010). *Laboratory procedures in animal nutrition research*. Department of Animal and Food Science, Texas Tech University, Lubbock.

Hayashi, Y., Maharjan, K. L., & Kumagai, H. (2006). Feeding traits, nutritional status and milk production of dairy cattle and buffalo in small-scale farms in Terai, Nepal. *Asian Australasian Journal of Animal Science, 19*(2), 189–197.

Hayashi, Y., Shah, S., Shah, S. K., & Kumagai, H. (2005). Dairy production and nutritional status of lactating buffalo and cattle in small-scale farms in Terai, Nepal. *Livestock Research for Rural Development, 17(6), 65-74.* Retrieved February 3, 2015, from http://www.lrrd.org/lrrd17/6/haya17064.htm

Jabbar, M. A, Marghazani, I. B., Pasha, T. N., Khaliqueand, A., & Abdullah, M. (2013). Effect of protein supplements of varying ruminal degradability on nutrients intake, digestibility, nitrogen balance and body condition score in early lactating Nili-Ravi buffaloes. *Journal of Animal and Plant Science, 23*(Suppl.), 108–112.

Kamonpatana, M., Sophon, S., Jetana, T., Sravasi, S., Tongpan, R., & Thasipoo, K. (1991). Growth of female swamp and murrah x swamp buffalo under village conditions. In N. M. Tulloh (Ed.), *Buffalo and Goats in Asia: Genetics and Its Application,* (pp. 76–82). Australia: Australian Centre for International Agricultural Research.

Khajarern, S., & Khajarern, J. M. (1989). Feeding swamp buffalo for milk production. In A. Speedy & R. Sansoucy (Eds.), *Feeding dairy cows in the tropics* (pp. 115-126). Bangkok: FAO.

Lubis, A., & Fletcher, I. C. (1987). Post-partum reproduction in swamp buffalo cows at two feeding levels. *Animal Reproduction Science, 13*(3), 183–189.

Mahmoudzadeh, H., & Fazaeli, H. (2009). Growth response of yearling buffalo male calves to different dietary energy levels. *Turkish Journal of Veterinary and Animal Sciences, 33*(6), 447–454.

Mellado, M., Valdez, R., Lara, L. M., & Garcıa, J. E. (2004). Risk factors involved in conception, abortion, and kidding rates of goats under extensive conditions. *Small Ruminant Research, 55*(1), 191–198.

Momongan, V. G., Sarabia, A. S., Roxas, N. P., Palad, O. A., Obsioma, A. R., Nava, Z. M., & Del Barrio, A. N. (1990). Increasing the productive efficiency of carabaos under smallholder farming systems. Domestic buffalo production in Asia. *Proceedings of the final research co-ordination meeting on the use of nuclear techniques to improve domestic buffalo production in Asia-phase II,* Rockhampton, Australia, 1989, February 20-24 organised by the joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. (pp. 167-178). International Atomic Energy Agency.

Mudgal, V. D. (1988). Comparative efficiency for milk production of buffaloes and cattle in the tropics. *Proceedings of II World Buffalo Congress* (pp. 454–462). New Delhi, India.

Nanda, A. S., Barr, P. S., & Prabhakar, S. (2003). Enhancing reproductive performance in dairy buffalo: major constraints and achievements. *Reproduction (Cambridge, England) Supplement, 61,* 27–36.

Nordin, Y., Sani, R. A., Rosalan, A. M., & Ghadzi, M. S. (2004). Productivity of swamp buffaloes under three production system. *Journal of Tropical Agriculture and Food Science*, *32*(2), 229.

Othman, R., Bakar, M. Z. A., Kasim, A., & Zamri-Saad, M. (2014). Improving the reproductive performance of buffaloes in Sabah, Malaysia. *Journal of Animal Health and Production, 2*(1), 1–4.

Parera, O. (1999). Management of reproduction. In E. Zerbini, A. G. Wold, L. Falvey, & C. Chantalakhana (Eds.). *Smallholder dairying in the tropics* (pp. 241-264). ILRI (International Livestock Research Institute), Nairobi, Kenya.

Parker, B. A., Nava, Z. M., Lapitan, R. M., del Barrio, A. N., & Momongan, V. G. (1991). Comparative growth performance of carabao and carabao crosses under controlled (institutional) and smallholder farms. In N. M. Tulloh (Ed.), *Buffalo and Goats in Asia: Genetic Diversity and its Application* (pp. 83-92). Australia: Australian Centre for International Agricultural Research.

Qureshi, M. S. (2009). Nutritional and management support to reproduction in dairy buffaloes under tropical conditions. *Pakistan Journal of Zoology, 9*(Suppl.), 895–909.

Salas, R. C. D., van der Lende, T., Udo, H. M. J., Mamuad, F. V., Garillo, E. P., & Cruz, L. C. (2000). Comparison of growth, milk yield and draughtability of murrah-Philippine crossbred and Philippine native buffaloes. *Asian-Australasian Journal of Animal Sciences, 13*(5), 580–586.

Sanh, M. V. (2005). *Effects of supplementation on feed intake, working capacity and body weight change of swamp buffaloes in a grazing system*. Workshop-seminar "Making better use of local feed resources" (Editors: Reg Preston and Brian Ogle) MEKARN-CTU, Cantho, 23-25 May, 2005. Article #19. Retrieved February 2, 2016, from http://www.mekarn.org/proctu/sahn19.htm

Sethi, R. K. (2003). Improving riverine and swamp buffaloes through breeding. *Proceeding of the 4th Asian Buffalo Congress* (pp. 51-60). New Delhi, India.

Situmorang, P., & Sitepu, P. (1991). Comparative growth performance, semen quality and draught capacity of Indonesian swamp buffalo and its crosses. In N. M. Tulloh (Ed.), *Buffalo and Goats in Asia: genetic diversity and its application* (pp. 102–112). Australia: Australian Centre for International Agricultural Research.

Tatsapong, P., Peangkoum, P., Pimpa, O., & Hare, M. D. (2010). Effects of dietary protein on nitrogen metabolism and protein requirements for maintenance of growing Thai swamp buffalo (*Bubalus bubalis*) calves. *Journal of Animal and Veterinary Advances, 9*(8), 1216–1222.

Thevarnanoharan, K., Vandepitte, W., Mohiuddin, G., & Chantalakhana, C. (2001). Environmental factors affecting various growth traits of swamp buffalo calves. *Pakistan Journal of Agricultural Science, 38*(3-4), 5–10.

Van Thu, N., & Preston, T. R. (1999). Rumen environment and feed degradability in swamp buffaloes fed different supplements. *Livestock Research and Rural Development, 11*(3), 1-7. Retrieved from http://www.lrrd.org/lrrd11/3/thu113.htm

Wanapat, M., & Wachirapakorn, C. (1990). Utilization of roughage and concentrate by feedlot swamp buffaloes (*Bubalus bubalis*). *Asian-Australasian Journal of Animal Sciences, 3*(3), 195–203.

Yindee, M. (2011). *Reproduction and genetic diversity of the swamp buffalo*. (Doctoral dissertation) Utrecht University. Retrieved from www.vetscite.org/publish/articles/000096/index.html

Zerbini, E., & Wold, A. G. (1999). Feeding dairy cows for draught. In E. Zerbini, A. G. Wold, L. Falvey, & C. Chantalakhana (Eds.), *Smallholder dairying in the tropics* (pp. 133-156). Nairobi, Kenya: ILRI (International Livestock Research Institute).

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Weed Seed Bank of Parthenium Weed (*Parthenium hysterophorus* L.) in Batang Kali, Selangor, Malaysia

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ABSTRACT

The newly detected noxious weed (*Parthenium hysterophorus* L.) is an invasive alien species in Malaysia. The degree of seriousness of the invasion of parthenium weed can be predicted from the condition of weed seed bank in the soil. A study was conducted to investigate the soil seed bank of parthenium weed at different soil depths in five different locations of Ulu Yam Baru, Batang Kali, Selangor, Malaysia. The soil samples were collected from four depths, 0-5 cm, 5-10 cm, 10-15 cm and 15-20 cm; and five sampling areas were chosen, namely a waste disposal site, a vegetable farm, a cattle farm, a stretch of roadside and an undisturbed area of fallow land. The seeds were extracted using sieve shakers at the laboratory of Universiti Malaysia Kelantan, Jeli Campus. On average, 1321 seeds/m^2 (13.21 million seeds/ha) were found at the depth of 0-5 cm, 218 seeds/m^2 (2.18 million seeds/ha) at the depth of 5-10 cm and 121 seeds/m^2 (1.21 million seeds/ha) at the depth of 10-15 cm. No seed was found at the depth of 15-20 cm. The number of seeds varied in different locations as well. The highest number recorded, 1108 seeds/m^2 (11.08 million/ha), was from the sample taken from the waste disposal site, followed by the vegetable farm, with 514 seeds/m^2 (5.14 million seeds/ha). The maximum number of weed seeds (3547 seeds/m^2) was from the surface layer of the waste disposal site and the total number of seeds varied from 4432-396 seeds/m^2, and seeds remained within 15 cm soil depth. The collected seeds from the different soil depths showed 70.6% viability in a seed germination test. The total accumulation of 8300 seeds/m^2 (83.00 million seeds/ha) in soil of 15-cm depth in Batang Kali is a threat to the environment.

Keywords: Parthenium weed, weed seed bank, sieve shaking, soil depth

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INTRODUCTION

Parthenium weed (*Parthenium hysterophorus* L., locally called 'Rumpai Miang Mexico') is an invasive alien species in Malaysia. It is an environmental pollutant weed species that was accidentally introduced to many countries and later became a serious problem in the agriculture sector, affecting biodiversity and human and animal health (Karim, Norhafizah, Maszura, Fatimah, & Alam, 2016, pp.1006–1015). Weed is known as allergenic and lethal to cattle if consumed in large amounts. Human beings may also suffer allergies from long-time contact with this weed and from pollen intake during breathing (Karim, Norhafizah, & Maszura, 2017, pp. 175–182). There is no effective treatment for severe parthenium allergic diseases and the only thing to do would be to avoid contact with the weed and if possible, to leave the place (Anon., 2003). Cropton (2014) regarded parthenium weed as the worst weed of all on the planet.

Parthenium weed seeds are striped grey to black in colour, have a narrow diamond shape and are 1.5-2.5 mm long and flattened. The seeds are tightly grasped in a brown outer coat, which gives them a tufted triangle appearance (Lusweti, Wabuyele, Ssegawa, & Mauremootoo, 2017, p.1). The seeds of parthenium weed are very small and light with a wing-like structure that allows them to be carried easily to different places by the wind and other vectors (Navie et al., 1996). The biological characteristic contributing to the aggressiveness of parthenium weed is its special reproductive ability. Under stressed conditions, it can complete its life cycle within four to five weeks. The plant can produce four or more cohorts of seedlings in a single season and can add a lot of seeds to the soil in a year (Frew et al., 1996; Tamado, 2001). It was first detected in Batang Kali, Selangor in 2013 (Karim, 2013, p. 28) and has now spread to 10 states of Malaysia including Sabah, covering more than 70 hectares of land in Peninsular Malaysia (DOA, 2015). As it is spreading to other areas of the country, it is now necessary to implement proper measures for control of this weed (Maszura, Karim, & Norhafizah, 2016, p. 37). The Plant Biosecurity Division of the Department of Agriculture (DOA) is responsible for sustainable management of weed eradication. Currently, in Malaysia, it is a national agenda to control this invasive species (DOA, 2015).

A weed seed bank is a reserve of viable weed seeds that present on the soil surface and in soil profile that indicates the past history of weed vegetation. After viable seeds are produced, the dispersal of weed seeds takes place and is distributed over different distances via different agents e.g. birds, the wind, flood water etc. (Shabbir, 2013, p. 1). Finally, these seeds are accumulated in the soil in different layers depending on the time period and soil management activities. Seeds in the seed bank are subject to a different fate. Some seeds germinate, grow and produce more seeds and others germinate and then die, while some are attacked by predators and yet others remain dormant under certain

environmental conditions (Menalled, 2008). Whyte (1994, p. 1–15) confirmed that parthenium weed seeds remain viable in the soil seed bank for four to six years. Tamado et al. (2002) stated that parthenium seeds were buried in the soil for 26 months and observed that the viability of seeds was more than 50% and the 'half-life' of seeds in soil was three to four years. The weed seed bank in soil is an indication of the possibility of future infestation. Destroying weed seeds from the soil seed bank is an important aspect of weed management. Information on the weed seed bank in an area helps in predicting the degree to which crop-weed competition or environmental degradation may occur in that area. Data on the soil seed bank can also be used to calculate new plant recruitment in the area. Knowledge about seed banks is, therefore, important as it provides valuable data for developing weed management strategy (Golafshan & Yasari, 2012, pp. 1-9). The objective of this study was to examine weed seed density in four different soil depths in five locations in Batang Kali, Selangor, Malaysia.

MATERIALS AND METHOD

Collection of Soil Samples

Soil samples were collected using soil core of 7.5 cm diameter from four soil depths (0-5 cm, 5-10 cm, 10-15 cm and 15-20 cm) in five different locations (waste disposal place, vegetable farm, cattle farm, roadside and an undisturbed area of fallow land) in Batang Kali (03° 25.781' N, 101° 39.212' E) in the Hulu Selangor district (Figures 1 & 2). An area of 400 X 400 square metres in a parthenium-infested area was selected as the sampling area. Five locations, which were located more or less 200 metres away from each other within this area, were marked following a 'W' pattern. In every location, a 2 X 2 square metre area was selected during sample collection (Golafshan & Yasari, 2012). Within each sampling spot, five randomly selected sub-spots were marked for sample collection. Soil samples were collected from four soil depths in each sub-spot, and each core was separated with a differently marked plastic bag. The samples of five sub-spots from each area of each soil depth were then mixed together uniformly and from these composite samples, four working samples, which were regarded as four replications, were obtained. The experiment was laid out in randomised block design with four replications. Collected soil samples were taken to the UMK laboratory for seed analysis using a sieve shaker. The weed seeds were then extracted in the laboratory using the direct seed extraction technique (Mesgaran, Mashhadi, Zand, & Alizadeh, 2007, pp. 472–478).

Seed Extraction from Soil Samples

The collected soil samples were dried in an electric oven at 70ºC for one hour to improve dispersal of clay aggregates. The parthenium weed seeds were then extracted using a sieve shaker with sieves of different dimension e.g. 2.36 mm, 1.10 mm, 600 µm, 150 µm and 75 µm. The sieve

shaker with different sizes of mesh was used to remove all unwanted plant root, debris and different sizes of other particles from the weed seeds settled at the bottom of the sieve. When the fine particles had passed through the sieves, the remainder of the sample-like sand particles, organic debris, clay soil and seeds that had not fully dispersed from the soil remained on the top sieve. The parthenium seeds were separated from other seeds. They were identified through observation by noting their small size, triangular shape and black colour. The number of seeds on the sieves was counted and recorded.

The number of seeds was estimated using the following formula:

Number of seeds/m^2 = (Number of seeds/core) x (10000/Area of core in cm^2)

The area of core = πr^2

where π is a constant and is equal to 3.14, r = d/2, where d = diameter of the core (7.5 cm)

The collected seeds were tested for viability in the laboratory using paper towels and distilled water in Petri dishes and placing the Petri dishes in a seed germinator at 27^0C for seven days. The data then collected were tabulated for statistical analyses using the Statistical Package for the Social Sciences (SPSS) (Landau & Everitt, 2004, pp. 339). Analysis of variance (ANOVA) of the data was determined to discover the significant differences between the means. The Duncan New Multiple Test was used to indicate the significant difference between the treatment means.

Figure 1. Map showing Hulu Selangor, Malaysia

Figure 2. Sampling area at Batang Kali

(*Source*: The Internet)

RESULTS AND DISCUSSION

Weed Seed Bank in Different Locations

The number of weed seeds (mean values) in the different locations of the sampling area of Ulu Yam Baru, Batang Kali are presented below (Figure 3). Figure 3 makes it obvious that the size of the weed seed bank significantly differed between the different locations. Location 1, which was near a waste disposal area, recorded the highest number of weed seeds in the soil; on average, there were 1108 seeds/m^2 (11.08 million/ha) seeds in the soil. In an earlier survey, it was noted that the area was heavily infested with parthenium weed plants (Karim, 2013, p. 28). It is possible that local residents who had disposed of waste in the area had also thrown parthenium plants with seeds at the same time. When the seeds germinated, no one was concerned about the plant due to lack of knowledge about the dangers posed by the weed.

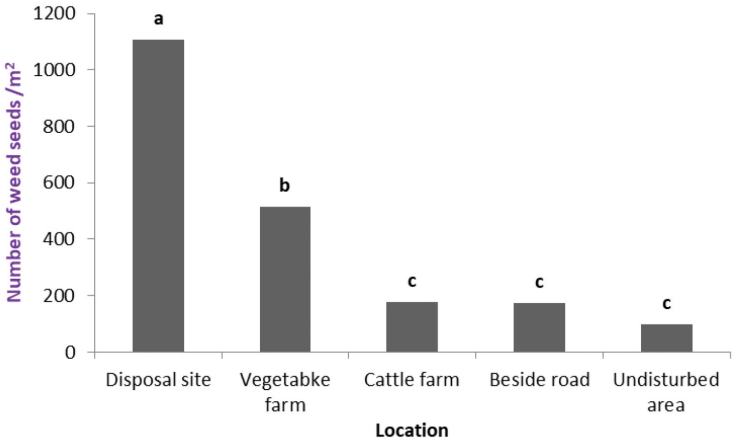

Figure 3. Number of weed seeds in different locations of the sampling area (bars bearing dissimilar letters indicate significant difference)

The plants were established slowly in the particular area, but over time, the plant community grew tremendously due to lack of disturbance. After the plant achieved maturity, it shed seeds, with each emerging as a new plant. The second location was a vegetable farm near the first location (the waste disposal site). On average, 514 seeds/m^2 (5.14 million/ha) were found in the location. This vegetable farm might have been the first source of parthenium infestation in Ulu Yam Baru. The owner of the farm might have imported the seeds from a parthenium-infested country either directly or via another seed trading company, with the parthenium seeds being carried to the farm in the form of vegetable seeds (Karim, 2013). Although the farm owner controlled the weed to some extent to reduce competition with the planted

vegetables, the parthenium weed was still present. However, the size of the weed seed bank was smaller than that of the waste disposal site. However, tillage practices on the farm had enhanced the growth of the weeds.

The other three locations had fewer seeds, between 179 and 99 seeds/m^2 (1.79 million to 0.99 million/ha), due probably to the greater distance from the main source of infestation in Ulu Yam Baru. These locations were probably infested through seed dispersal from the main source of parthenium infestation at Ulu Yam Baru. Seed dispersal might have been due to wind flow, vehicles or human activities. The seeds are easily dispersed via water, wind, vehicles, machinery and other vectors (Anon., 2003). According to Noroozi, Alizadeh and Mashhadi (2012, pp. 1–6), the factors that influence the rate of dispersal might be the habitat, environmental conditions, weed species, seed characteristics, seed density and also the seed distribution pattern.

Weed Seed Bank at Different Soil Depths

It is clear from Figure 4 that the number of seeds per unit area varied significantly among the different soil depths. The highest number of parthenium seeds, 1321 seeds/m^2 (13.21 million/ha), was noted to have come from the shallower depth, 0-5cm. This was the surface layer of the bed where all the seeds fell initially. Later on, the seeds were moved to different depths due to tillage activities, the action of insects or through rain water. There are many factors that can alter the location of seed dispersal such as animal movement, social behaviour, dispersal agents, the wind, relative humidity and pattern of rainfall (Willson & Traveset, 2000, pp. 85–110). Around 60% of the total number of weed seeds were found at the soil depth of 0-5 cm, with the number of weed seeds decreasing logarithmically with soil depth (Buhler, Kohler, & Thompson, 2009, pp. 70–76).

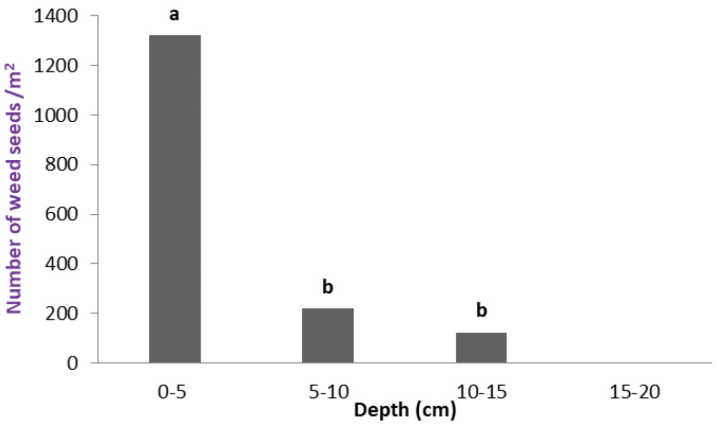

Figure 4. The number of weed seeds in different soil depths of Ulu Yam Baru, Batang Kali (bars bearing dissimilar letters indicate significant difference)

The second layer i.e. at the soil depth of 10-15 cm, contained about 17% of the total number of seeds in the first layer i.e. 5-10 cm. The number of parthenium seeds in the third layer, was around 55% of the number found in the second layer. In Ulu Yam Baru, the sampling spots, particularly the waste disposal area, was undisturbed, and only the land in the vegetable farm was disturbed to some extent. In a similar study in northern Sri Lanka, the number of parthenium seeds found in the soil depth of 5-10 cm was 5-9 seeds per square metre (Nishanthan, Sivachandiran, & Marambe, 2013, pp. 56–68). However, in this study, on average, 121 seeds/m^2 (1.21 million/ha) were found in the soil depth of 10-15 cm. It is not clear why the seeds were moved to the deeper layer of 10-15 cm depth. This might have been due to human activities on the soil like mulching, tillage and soil turning, which pushed the seeds at the top layer deep into the soil. According to Mulugeta and Stoltenberg (1997, pp. 706–715), the vertical distribution of weed seeds in the soil was influenced by tillage, with 43% to 74% of the total viable seeds found in the soil depth of 5-10 cm. The number of weed seeds found differed between the tillage systems used, decreasing with the soil depth (Auškalnienė & Auškalnis, 2009, pp. 156–161). Skuodienė, Karčauskienė, Čiuberkis, Repšienė and Ambrazaitienė (2013, pp. 25–32) stated that primary soil tillage significantly influenced soil weed seed banks. No seeds were recorded in the next soil depth of 15-20 cm, which indicated that no heavy force such as deep tillage or other activities had been exerted on the soil. This is supported by Caroca, Candia and Hinojosa (2011, pp. 40–47), who had found that non-tillage soil had a higher number of seeds in the soil surface. In Sri Lanka, no parthenium seeds were also found at this level of depth (Nishanthan et al., 2013).

The results of the viability test indicated that 70.6% of the collected seeds were viable. When the seeds were collected directly from the mature plants and then tested, after proper drying, for viability, almost 90% of the seeds had germinated. Mulugeta and Stoltenberg (1997) noted 43% to 74% viable seeds in the sample from the soil depth of 5-10 cm.

Interaction Between Location and Soil Depth

Interaction effects indicated that the number of weed seeds varied in the different soil depths from the five different locations of Ulu Yam Baru (Table 1 and 2).

Table 1
Analysis of variance showing mean effects and interaction effects of location and depth of soils on number of weed seeds extracted

Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9260.50*	19	487.395	36.669	0.000
Intercept	2420.00	1	2420.000	182.069	0.000
Location	1977.50	4	494.375	37.194	0.000
Depth	3924.40	3	1308.133	98.418	0.000
Location * Depth	3358.60	12	279.883	21.057	0.000
Error	797.50	60	13.292		
Total	12478.00	80			
Corrected Total	10058.00	79			

*R squared = 0.921 (Adjusted R squared = 0.896)

Table 2
Interaction effects of soil depth and location on the number of parthenium weed seeds (No./m^2) in Ulu Yam Baru, Batang Kali

Soil depth (cm)	Location						
	1	2	3	4	5	Total	Mean
0-5	3547	1698	453	548	358	6604	1321^{a}
5-10	527	208	188	131	38	1092	218^{b}
10-15	358	152	75	18	0	603	121^{b}
15-20	0	0	0	0	0	0	0
Total	4432	2058	716	697	396	8300	1660
Mean	1108^{A}	514^{B}	179^{C}	174^{C}	99^{C}		

Locations: 1 = Disposal site, 2 = Vegetable farm, 3 = Cattle farm, 4 = Beside road, 5 = Undisturbed area. (Dissimilar superscript small letters in the vertical column of means and superscript capital letters in the horizontal row of means indicate significant difference).

Based on Table 2, the number of weed seeds in the different depths of soil was significantly different in the five locations. The maximum number of weed seeds, 3547 seeds/m^2 (35.47 million/ha), was noted at the disposal site under the surface layer (Table 2). This might be due to different environmental conditions and human activities in the area. According to Kelton, Price, Van Santen, Balkcom, Arriaga and Shaw (2011, pp. 21–30), weed seed density is influenced by the level of tillage, manure application and depth range. The sample from the seed bank in the non-tillage area showed the highest number of seeds in the 0-5 cm depth i.e. in the top soil (Swanton, Shrestha, Knezevic, Roy, & Ball-Coelho, 2000, pp. 455–457).

Ezemvelo KZN wildlife ecologist, Ian Rushworth, stated that the parthenium weed could become the biggest natural disaster ever to befall communities and

their lands in KwaZulu-Natal (South Africa). Many livelihoods are threatened by this weed than by any other disaster ever experienced (Compton, 2014). Navie, McFadyen, Panetta and Adkins (1996, pp. 76–88) estimated that the soil seed bank of parthenium weed had invaded pasture fields of Australia and reported that the seed bank size of 3200 to 5100/m^2 was a dangerous level for the environment.

Navie et al. (1996) stated that the presence of 3200 to 5100 seeds/m^2 in Australia was considered a danger and that it posed a threat to the environment. Moreover, a single mature plant can produce more than 20,000 seeds (Belgeri, Sheldon, & Adkins, 2012, pp. 727–731). The weed can produce four or more cohorts of seedlings in a year. The seeds germinate in different flashes, with some remaining dormant for more than five years (Tamado et al., 2002). All these plant traits of parthenium make the problem of the quick spread of this weed more serious in Malaysia.

CONCLUSION

The discovery of 13.21 million seeds/ha of the dangerous weed, parthenium, in the surface layer and the presence of the seeds in the soil depth of 15 cm in Ulu Yam Baru, Batang Kali is alarming. The total number of seeds in the soil depth of 15 cm was 8300 seeds/m^2 (83.0 million/ha). Continuous control and monitoring over a long period of time is required to solve this problem and to make sure that all the seeds are removed from the soil. The highest number of seeds at a shallower depth, 0-5 cm, was found in the waste disposal site near a vegetable farm. The farm owner needs to be aware of this weed as it can take over cultivated land on his farm. Any parthenium found on the farm must be immediately uprooted and burnt.

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REFERENCES

Anonymous. (2003). *Parthenium biological control agents.* Department of Natural Resources and Mines, Queensland. Retrieved December 9, 2014, from http://www.weeds.org.au/WoNS/parthenium/docs/Parthenium_ Mgmnt-2.pdf

Auškalnienė, O., & Auškalnis, A. (2009). The influence of tillage system on diversities of soil weed seed bank. *Agronomy Research*, *7*(Special Issue I), 156–161.

Belgeri, A. M., Sheldon, C. N., & Adkins, S. W. (2012). Screening parthenium weed (*Parthenium hysterophorus* L.) seedlings for their allelopathic potential. *Pakistan Journal of Weed Science and Research, 18,* 727–731.

Blackmore, P., & Gray, P. (2012). *Parthenium weed.* Invasive Species Unit, Biosecurity NSW. Retrieved December 8, 2014, from http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0009/256590/ Parthenium -weed- Primefact-707-web.pdf

Buhler, D. D., Kohler, K. A., & Thompson, R. L. (2009). Weed seed bank dynamics during a five-year crop rotation 1. *Weed Technology 15*(1), 170–176.

Caroca, R. P., Candia, P. S., & Hinojosa, E. A. (2011). Characterization of the weed seed bank in zero and conventional tillage in central Chile. *Chilean Journal of Agricultural Research*, *71*(1), 140–147.

Compton, R. (2014, March 14). Invasive plant threatens health, livelihoods. *Daily News.* http//:www.iol.co.za/dailynews/news/invasive-plant-threatens-health-livelihoods-1.1661199#VBj-RPmSyCk

Cooksey, D., & Sheley, R. (2002). Montana noxious weed survey and mapping system. *MSU Extension Service MT199613 AG 7*.

DOA. (2015). *Report of a meeting on parthenium management in Malaysia*. Department of Agriculture (DOA), Wisma Tani, Jalan Sultan Salahuddin, Kuala Lumpur on 2015, February 17.

Frew, M., Solomon, K., & Mashilla, D. (1996). Prevalence and distribution of Parthenium hysterophorus L. in Eastern Ethiopia. In R. Fessehaie (Ed.), *Proceedings of the first annual conference, Ethiopian Weed Science Society, 24–25 Nov. 1993* (pp. 19-26). Addis Ababa, Ethiopia.

Golafshan, M. G., & Yasari, E. (2012). Comparison of sampling methods for estimating seed bank and weed population densities during the growing season. *Journal of Agricultural Science*, *4*(9), 1–9.

Karim, S. M. R. (2014, January 9). Malaysia invaded: Weed it out before it is too late. *International Parthenium News*. Retrieved from http://apwss.org/documents/newsletters/parthenium/Parthenium_News_January_2014.pdf

Karim, S. M. R., Norhafizah, M. Z., Maszura, C. M., Fatimah, K., & Alam, Z. A. (2016). Perception of local people about parthenium weed (Rumpai Miang Mexico) of Kedah, Malaysia. *International Journal of Biology. Pharmacy and Allied Sciences*, *5*(5), 1006–1015.

Karim, S. M. R., Norhafizah, M. Z., & Maszura, C. M. (2017). Incidence of parthenium allergy on human health in Kedah, Malaysia. *International Journal of Biology. Pharmacy and Allied Sciences*, *6*(2), 175–182.

Kelton, J. A., Price, A. J., Van Santen, E., Balkcom, K. S., Arriaga, F. J., & Shaw, J. N. (2011). Weed seed bank density and composition in a tillage and landscape variability study. *Communications in Biometry and Crop Science*, *6*(1), 21–30.

Khan, M. A., Hussain, Z., Khan, I., Khan, R., Waqas, M., Haroon, M., & Khan, A. I. (2012). Studies on weed seed bank of the new developmental farm, KPK Agricultural University Peshawar, Pakistan. *Pakistan Journal of Weed Science Research*, *18*(2), 183–189.

Landau, S., & Everitt, B. S. (2004). *A handbook of statistical analyses using SPSS.* Washington D. C.: Chapman & Hall/CRC Press LLC.

Lusweti, A., Wabuyele, E., Ssegawa, P., & Mauremootoo, J. (2017). *Parthenium hysterophorus* (Parthenium weed) (pp. 1-4). BioNET-INTERNATIONAL Secretariat – UK. Retrieved from http://keys.lucidcentral.org/keys/v3/eafrinet/weeds/key/weeds/Media/Html/Parthenium_hysterophorus_(Parthenium_Weed).htm

Maszura, C. M., Karim, S. M. R., & Norhafizah, M. Z. (2016). Parthenium weed survey at Batang Kali, Selangor, and Sungai Petani, Kedah, Malaysia. In *Proceeding of UMTAS 2016 Annual Symposium on Sustainability Science and Management* (p. 37). Universiti Malaysia Terengganu, Terengganu 2016, December 13-15.

Menalled, F. (2008). Weed seed bank dynamics and integrated management of agricultural weeds. In *Montana State University Extension guide*. Retrieved from: http://www.ipm.montana.edu/cropweeds/documents_cropweeds/extension/Weed%20Seed bank%20dynamics%20MT200808AG.pdf

Mulugeta, D., & Stoltenberg, D. E. (1997). Weed and seed bank management with integrated methods as influenced by tillage. *Weed Science, 45*(5), 706–715.

Navie, S. C., McFadyen, R. E., Panetta, F. D., & Adkins, S. W. (1996). The biology of Australian weeds, 27. *Parthenium hysterophorus* L. *Plant Protection Quarterly, 11*(2), 76–88.

Nishanthan, K., Sivachandiran, S., & Marambe, B. (2013). Control of *Parthenium hysterophorus* L. and its impact on yield performance of tomato (*Solanum lycopersicum* L.) in the northern province of Sri Lanka. *Tropical Agricultural Research, 25*(1), 56–68.

Noroozi, S., Alizadeh, M. H., & Mashhadi, R. H. (2012). Factors affecting post dispersal of weed seed predation in barely fields. *International Scholarly Research Network ISRN Agronomy, 2012,* 1-6.

Rezaul, K. (2013, October 25). Parthenium invasion: Weed it out before it is too late. *New Straits Times*. Retrieved from http://www.nst.com.my/opinion/letters-to-the-editor/parthenium-invasion-weed-it-out-before-it-s-too-late-1.384246

Shabbir, A. (2006). Parthenium weed: A threatening weed for agricultural lands. *Parthenium News*. University of the Punjab Lahore, Pakistan.

Skuodienė, R., Karčauskienė, D., Čiuberkis, S., Repšienė, R., & Ambrazaitienė, D. (2013). The influence of primary soil tillage on soil weed seed bank and weed incidence in a cereal-grass crop rotation. *Zemdirbyste-Agriculture*, *100*(1), 25–32.

Swanton, C. J., Shrestha, A., Knezevic, S. Z., Roy, R. C., & Ball-Coelho, B. R. (2000). Influence of tillage type on vertical weed seed bank distribution in a sandy soil. *Canadian Journal of Plant Science*, *80*(2), 455–457.

Tamado, T. (2001). *Biology and management of Parthenium hysterophorus L. in Eastern Ethiopia* (Ph.D. thesis). University of Agricultural Sciences Uppsala, Sweden.

Tamado, T., Ohlander, L., & Milberg, P. (2002). Interference by the weed *Parthenium hysterophorus* L. with the grain sorghum: Influence of weed density and duration of the competition. *International Journal of Pest Management, 48*(3), 183–188.

Whyte, G. G. (1994). *Parthenium weed.* Corporate Research Centre for Tropical Pest Management, Brisbane, Australia.

Willson, M. F., & Traveset, A. (2000). The ecology of seed dispersal. In M. Fenner (Ed.), *Seeds: The ecology of regeneration in plant communities* (pp. 85-110). United Kingdom, UK: CABI Publishing.

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Micropropagation of *Dendrobium signatum* Rchb.f.

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ABSTRACT

A study was conducted to elucidate the effects of using different media (MS, ½MS, VW) supplemented with different combinations of 15% coconut water, 10% potato extract and 5% mashed banana with an extra 0.2% activated charcoal on seed germination of *Dendrobium signatum* Rchb.f. in *in vitro* cultures. The results showed that the most suitable media, providing 100% seed germination, were MS added to 10% potato extract, ½MS supplemented with 10% potato extract and MS added to 5% mashed banana. Young plantlets were then transferred to MS and ½MS medium supplemented with various kinds of cytokinin including BA, Kinetin and TDZ at the concentrations of 0, 1 and 2 mg/l, and combined with 0 and 0.5 mg/l of NAA for plantlet development. The results showed that young shoots could be differentiated and regenerated into plantlets in all the treatments. The results revealed that the most suitable medium for shoot proliferation and root induction was ½MS medium with 2 mg/l BA added and combined with 0.5 mg/l NAA.

Keywords: *Dendrobium*, *in vitro* culture, cytokinin, seed germination, organic supplements

INTRODUCTION

Dendrobium signatum Rchb.f. belongs to the genus *Dendrobium*, which is the largest genus of orchid species in Thailand (Seidenfaden, 1985). Genus *Dendrobium* is also one of the most well attended orchids for trade production because of the enormous increase in demand for cut flowers and pot plants over the years (Peyachoknagul et al., 2014). It was reported that many *Dendrobium* species have antidiabetic, anti-cancer and anti-pyretic properties (Pant, 2013). Some phytochemical compounds found in *Dendrobium* are alkaloids, flavonoids, sesquiterpenoids as well as pigments (Singh et al., 2012; Attri, 2016). *D.*

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signatum Rchb.f. is a sympodial epiphytic wild orchid. Its local names are '*Ueang kham kew*' or '*Ueang tin ped*', and it has a geographical distribution in tropical forests ranging from elevations of 200 to 1200 metres and are mostly found in North and Northeast Thailand. The characteristics of the stem are a yellow color, succulence, a diameter of 1.5-2 cm and a height of 30-50 cm. The leaves are also succulent and lanceolate. The leaves grow alternately over the whole length of the stem. The *D. signatum* Rchb.f. blooms in the winter through early summer (approximately March to May) with a short inflorescence positioned on a mature leafless cane towards the apex with two flowers. They are fragrant and long-living (Thaitong, 2005). Since *Dendrobium* is popular in the ornamental market, this species has been collected from the forest with little regard for sustainability. This has initiated the extinction of the *Dendrobium* species (Zhang et al., 2013).

Other causes of the extinction of *Dendrobium* are seed germination and seedling development. In nature, orchid seeds do not have endosperm to support seed germination. Thus, orchid seed germination requires the presence of mycorrhizal fungi, classified as Ascomycota or Basidiomycota, which infect and supply nutrition essential for orchid seed germination (Behie & Bidochka, 2014). In the process of seedling development, the number of seedlings is limited because many protocorms and seedlings are destroyed by pests and aggressive fungi. The reduction of wild orchids has been subsequently increased and this can lead to the extinction of the species in the near future. Therefore, asymbiotic seed germination by tissue culture is essential.

In previous studies, the careful selection of media, cytokinins, auxins and natural supplements and their optimisation have been reported to be the most important factors in orchid propagation (Malabadi et al., 2005; Luo et al., 2009; Parthibhan et al., 2015). The aims of this study were to obtain *in vitro* seed germination and regeneration of *D. signatum* Rchb.f. through optimisation of their aseptic culture conditions. The objectives were: (1) to determine the suitable media for seed germination and regeneration of *D. signatum* Rchb.f., and (2) to determine the optimum concentrations of the organic form of cytokinin and NAA.

MATERIALS AND METHOD

Effect of Different Media and Organic Supplements on Seed Germination

The mature capsules of *D. signatum* Rchb.f. were collected six months after pollination. Capsules were then surface sterilised by dipping in 70% ethyl alcohol and flamed immediately four to five times in laminar air flow. The capsules were then cut longitudinally in a sterilised petri dish. Seeds were scraped from the capsule, mixed with sterile water and pipetted into 200 µl tubes and then cultured on the surface of the medium. Three different basal media were used in the whole experiment consisting of MS (Murashige & Skoog,

1962), ½MS (half strength of MS) and VW (Vacin & Went, 1949) supplemented with several organic ingredients including 15% coconut water, 10% potato extract and 5% mashed banana. *In vitro* cultured seeds were then kept at 25± 2°C under a photoperiod of 16 h light/8 h dark. The percentage of orchid seed germination was obtained by estimating the surface area of seed germination in the tissue culture bottle with a diameter of 4.5 cm. The total surface area of the tissue culture bottle was defined as 100%. After cultivation for eight weeks, the percentage of seed germination was recorded. Observations on the percentage germination of seeds, the number of leaves, length of leaves and number of roots were recorded 16 weeks after culture.

Effects of Different Cytokinin and NAA on Shoot Proliferation and Root Induction

The experiment was performed using young *in vitro* seedlings of *D. signatum* Rchb.f. of approximately 1 cm. height at age 16 weeks. Single plantlets were cultured on MS and ½MS media containing 0, 1 and 2 mg/l of benzyladenine (BA), 6-furfuryl aminopurine (Kinetin) and thidiazuron (TDZ) in combination with 0 and 0.5 mg/l of naphthaleneacetic acid (NAA). Culture conditions were the same as previously. After three months of culture, morphogenetic response to the treatments was evaluated in terms of percentage proliferation of shoot, height of shoot, number of leaves, number of roots and length of root.

All the experiments were set up in completely randomised design (CRD). Each treatment consisted of 10 replicates. The difference among the treatment means was compared based on Duncan's multiple range test (DMRT) analysis.

RESULTS AND DISCUSSION

Effect of Different Media and Organic Supplements on Seed Germination

The effect of different media and organic supplements on seed germination of *D. signatum* Rchb.f. was observed at 60 days after culture on a seed germination medium. It was observed that seeds taken from immature capsules had germinated and green protocorms had formed after culture for eight weeks. (Table 1). Figure 1 shows seed germination of *D. signatum* Rchb.f. cultured in various media supplemented with several organic ingredients after culture for 16 weeks. The most suitable media, providing 100% seed germination, were MS added to 10% potato, ½MS supplemented with 10% potato, and MS added to 5% banana. Naturally, orchid seeds have a poor germination rate because of the small size of the seeds and the lack of cotyledons and endosperm (Maneerattanarungroj, 2007) that contain food reserves in the form of starch grains, oil droplets and small amounts of proteins (Thomas & Michael, 2007). This study found that MS and ½MS were more suitable for seed germination than VW basal media. Miransari and Smith (2014) reported that seeds consume larger amounts of nitrogen

during seed germination. Therefore, the amount of nitrogen in MS and ½MS can influence seed germination of orchids.

Combinations of organic supplements in the medium enhanced seed germination. The best medium for seed germination in treatment was 10% potato extract. Potato extract consists of carbohydrates, amino acids, important vitamins (C, B1, B6) and mineral elements (potassium, iron, magnesium) (Molnár, Virág, & Ördög, 2011). In the *in vitro* culture, potato had useful effects on some orchid species such as *Phalaenopsis* and *Doritaenopsi*s (Thorpe, Stasolla, Yeung, de Klerk, Roberts, & George, 2008). A study of the effect of seedling media or nutrients added to coconut water for orchid growth found that the nutrients could activate low-level seed germination compared to other organic supplements. The composition of coconut water (CW) includes inorganic ions (e.g. phosphorus, potassium), nitrogenous compounds, amino acids, related substances (e.g. alanine, glutamic acid, lysine), enzymes, vitamins and sugar (Sandoval Prando et al., 2014). All these compounds may be the reason for seed germination in orchids (Arditti, 2008). Vijayakumar et al. (2012) reported that MS medium added with 3% sucrose 1.5 mg/l BA and 15% CW showed a higher rate of seed germination of *D. aggregatum*. Banana is frequently used to influence orchid *in vitro*. This study found that MS added to 5% mashed banana could increase the highest percentage for seed germination of *D. sigmnatum* Rchb.f. but 100% germination as a result of media added to mashed banana was observed only in full MS media. Many reports indicated that mashed banana combined with a cultured medium could improve the growth of orchid seedlings. The effect of mashed banana on seed germination in *D. sigmnatum* Rchb.f is similar to other plants such as in *D. wangliangii* and *D. strongylanthum* Rchb.f. (Zhao et al., 2013; Kong et al., 2007)

Table 1
Effect of different media and organic supplements on percentage of seed germination, average values of the number of leaves, leaf length and number of the roots of D. signatum *Rchb.f.*

Medium	**Seed Germination* (%)**	**Average Number of Leaves****	**Average Leaf Length ** (cm)**	**Average Number of the Roots****	**Average Root Length** (cm)**
MS	50 ± 0^{b}	3 ± 2^{bc}	0.3 ± 0.07^{ab}	1 ± 0^{ab}	0.5 ± 0.28^{de}
MS + 15% coconut water	25 ± 0^{a}	3 ± 1.73^{bc}	0.3 ± 0.23^{ab}	1 ± 0.71^{ab}	0.1 ± 0^{ab}
MS + 10% potato extract	100 ± 0^{d}	5 ± 1.22^{c}	0.9 ± 0.24^{d}	4 ± 2.00^{d}	0.3 ± 1^{bc}
MS + 5% mashed banana	100 ± 0^{d}	4 ± 1.22^{c}	0.5 ± 0.20^{bc}	4 ± 0^{d}	0.6 ± 0.12^{ef}

Table 1 (*continue*)

Medium	Seed Germination* (%)	Average Number of Leaves**	Average Leaf Length ** (cm)	Average Number of the Roots**	Average Root Length** (cm)
½MS	50 ±30.62^{b}	3 ± 1.87^{bc}	0.4 ± 0.20^{bc}	1 ± 0^{ab}	0.2 ± 0.14^{b}
½MS + 15% coconut water	25 ± 17.68^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
½MS + 10% potato extract	100 ± 0^{d}	4 ± 1.58^{c}	0.4 ± 0.20^{bc}	3 ± 1.41^{cd}	0.7 ± 0.14^{f}
½MS + 5% mashed banana	75 ± 30.62^{c}	5 ± 1^{c}	0.7 ± 0.37^{cd}	4 ± 0^{d}	0.5 ± 0.17^{de}
VW	50 ± 17.68^{b}	4 ± 1.58^{c}	0.3 ± 0.12^{ab}	1 ± 0^{ab}	0.3 ± 1^{bc}
VW + 15% coconut water	25 ± 0^{a}	2 ± 0^{b}	0.3 ± 0.21^{ab}	1 ± 0^{ab}	0.1 ± 0.07^{ab}
VW + 10% potato extract	75 ± 25.00^{c}	5 ± 1.00^{bc}	0.5 ± 0.23^{bc}	3 ± 0^{cd}	0.2 ± 0.14^{b}
VW + 5% mashed banana	75 ± 0^{c}	3 ± 1.00^{bc}	0.5 ± 0.16^{bc}	2 ± 0^{bc}	0.4 ± 0.16^{cb}

Means followed by the same letter within each column are not significantly different using Duncan's multiple range test at $p<0.05$

* Percentage of seed germination after culture for eight weeks.

** Data were recorded after culture for 16 weeks.

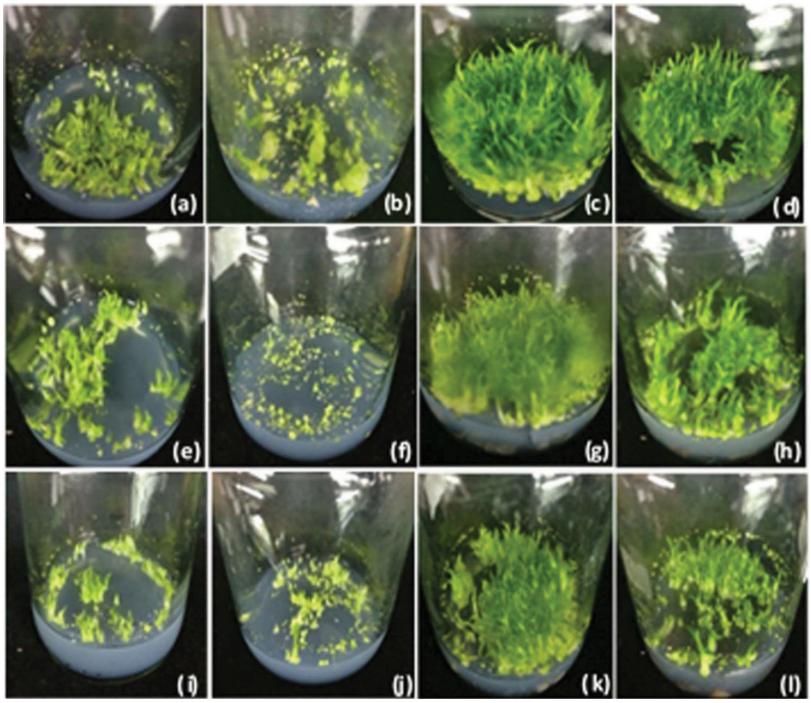

Figure 1. Seed germination of *D. signatum* Rchb.f. cultured in various media supplemented with several organic supplements, (a) MS, (b) MS + 15% coconut water, (c) MS + 10% potato extract, (d) MS + 5% mashed banana, (e) ½MS, (f) ½MS + 15% coconut water, (g) ½MS + 10% potato extract, (h) ½MS + 5% mashed banana, (i) VW, (j) VW + 15% coconut water, (k) VW + 10% potato extract, (l) VW + 5% mashed banana, after culture for 16 weeks

The Effect of Different Cytokinin and NAA on Shoot Proliferation and Root Induction

The results of the study on the effect of different cytokinin and NAA on shoot and root proliferation of the orchid are shown in Figure 2 and Table 2. The combination of cytokinin and auxin promoted the growth of shoots and roots and the vigorous growth of *D. signatum* Rchb.f. The results also showed that young shoots could be differentiated and regenerated into plantlets in all the treatments. The results revealed that the most suitable medium for plantlet development was ½MS medium supplemented with 2 mg/l BA combined with 0.5 mg/l NAA (Table 2). The synthetic kinetin (6-furfuryl aminopurine), benzyladenine (*N*6-benzylaminopurine, *N*6-benzyladenine, BA, BAP), dimethyl aminopurine (DMAP), thidiazuron (TDZ), and the naturally occurring zeatin are used most commonly in orchid culture media (Arditti, 2008). This study showed that the addition of 2 mg/l BA to 0.5 mg/l NAA could induce shoot proliferation and root induction of *D. signatum* Rchb.f. The beneficial effects of cytokinin in promoting the highest shoot proliferation in the *Dendrobium* hybrids, Sonia 17 and 28, which were cultured in half-strength Murashige and Skoog (MS) medium supplemented with 44.4 µM BA (Martin & Madassery, 2006) have been noted in previous studies. In *Dendrobium candidum,* the most suitable for callus induction was MS medium with half-strength macronutrients and full-strength micronutrients combined with 2 mg/l BA and 0.5 mg/L NAA (Zhao et al., 2007). In mass propagation of *Dendrobium* 'Zahra FR 62' half-strength MS medium containing 1 mg/l thidiazuron (TDZ) and 0.5 mg/l N6-benzyladenine (BA) were used, and this resulted in a high protocorm-like body (PLB) (Winarto et al., 2013). As a result of this study, the most commonly used auxins in orchid tissue culture media are the naturally occurring auxin, indoleacetic acid (IAA), synthetic naphthaleneacetic acid (NAA), indolebutyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Arditti, 2008). In the present study, 0.5 mg/l NAA combined with 2 mg/l BA applied to the medium induced the highest average of shoot height, number of leaves, number of roots and root length. Some reports explained that the application of a single regulator also influenced shoot proliferation. Sujjaritthurakarn and Kanchanpoom (2011) reported that the highest percentage for PLB induction and the highest number of PLBs per protocorm of dwarf *Dendrobium* were derived from using modified Murashige and Skoog (MS) liquid medium supplemented with 18 µM TDZ.

Table 2
Effect of cytokinin supplemented with naa for percentage of shoot proliferation, shoot regeneration, average of shoot height, number of leaves, number of roots and root length of D. signatum *Rchb.f. cultured for three months*

Number of Medium	Medium	Plant Growth Regulators (mg/l)				Shoot Proliferation (%)	Average of Shoot Height (cm.)	Average Number of Leaves	Average Number of Roots	Average Root Length (cm.)
		BA	**Kinetin**	**TDZ**	**NAA**					
M1	MS	-	-	-	-	33 ± 8.33^{abcd}	2.0 ± 0.15^{bcde}	2.3 ± 0.33^{abc}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M2	MS	1.0	-	-	-	25 ± 14.43^{abcd}	1.3 ± 0.23^{ab}	2.0 ± 0.58^{abc}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M3	MS	2.0	-	-	-	8 ± 8.33^{ab}	1.4 ± 0.12^{abc}	3.0 ± 1.00^{abcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M4	MS	-	1.0	-	-	33 ± 22.04^{abcd}	1.7 ± 0.28^{abcde}	4.0 ± 0.58^{abcde}	2.3 ± 2.33^{a}	0.1 ± 0.13^{a}
M5	MS	-	2.0	-	-	0 ± 0.00^{a}	2.0 ± 0.15^{bcde}	3.0 ± 0.58^{abcde}	0.7 ± 0.67^{a}	0.7 ± 0.67^{a}
M6	MS	-	-	1.0	-	17 ± 16.67^{abc}	1.8 ± 0.19^{abcde}	4.3 ± 0.33^{abcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M7	MS	-	-	2.0	-	42 ± 16.67^{abcde}	1.8 ± 0.32^{abcde}	4.3 ± 1.20^{bcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M8	MS	-	-	-	0.5	17 ± 16.67^{abc}	1.9 ± 0.09^{bcde}	4.3 ± 0.88^{bcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M9	MS	1.0	-	-	0.5	42 ± 16.67^{abcde}	1.8 ± 0.39^{abcde}	4.0 ± 2.00^{bcde}	0.3 ± 0.33^{a}	0.0 ± 0.03^{a}
M10	MS	2.0	-	-	0.5	58 ± 22.04^{cde}	1.6 ± 0.20^{abcde}	5.3 ± 1.20^{de}	0.3 ± 0.33^{a}	0.1 ± 0.07^{a}
M11	MS	-	1.0	-	0.5	25 ± 0.00^{abcd}	1.5 ± 0.09^{abcd}	3.7 ± 0.33^{abcde}	1.7 ± 0.88^{a}	0.1 ± 0.06^{a}
M12	MS	-	2.0	-	0.5	17 ± 8.33^{abc}	1.4 ± 0.15^{ab}	2.0 ± 0.00^{abc}	0.0 ± 0.00^{a}	0.0 ± 0.0^{a}
M13	MS	-	-	1.0	0.5	42 ± 8.33^{abcde}	2.1 ± 0.26^{cde}	3.3 ± 0.33^{abcde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M14	MS	-	-	2.0	0.5	58 ± 8.33^{cde}	2.1 ± 0.27^{bcde}	4.7 ± 0.33^{cde}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M15	½MS	-	-	-	-	67 ± 8.33^{de}	1.5 ± 0.15^{abcd}	2.0 ± 0.58^{abc}	1.7 ± 1.67^{a}	0.0 ± 0.01^{a}
M16	½MS	1.0	-	-	-	33 ± 22.04^{abcd}	1.9 ± 0.19^{bcde}	2.7 ± 0.33^{abcd}	1.0 ± 1.00^{a}	0.2 ± 0.20^{a}
M17	½MS	2.0	-	-	-	58 ± 16.67^{cde}	1.9 ± 0.25^{abcde}	2.7 ± 0.67^{abcd}	0.3 ± 0.33^{a}	0.2 ± 0.02^{a}
M18	½MS	-	1.0	-	-	25 ± 0.00^{abcd}	1.7 ± 0.12^{abcde}	3.7 ± 0.66^{abcde}	2.3 ± 1.20^{ab}	0.2 ± 0.12^{a}
M19	½MS	-	2.0	-	-	58 ± 22.04^{cde}	2.4 ± 0.31^{e}	4.0 ± 0.58^{abcde}	1.7 ± 0.33^{a}	0.3 ± 0.03^{ab}
M20	½MS	-	-	1.0	-	42 ± 16.67^{abcde}	1.9 ± 0.12^{abcde}	3.7 ± 0.33^{abcde}	1.0 ± 1.00^{a}	0.2 ± 0.20^{a}
M21	½MS	-	-	2.0	-	0 ± 0.00^{a}	1.7 ± 0.38^{abcde}	2.7 ± 1.20^{abcd}	1.3 ± 1.33^{a}	0.7 ± 0.07^{a}
M22	½MS	-	-	-	0.5	25 ± 14.43^{abcd}	1.2 ± 0.07^{a}	1.3 ± 0.33^{a}	0.3 ± 0.33^{a}	0.1 ± 0.10^{a}
M23	½MS	1.0	-	-	0.5	33 ± 8.33^{abcd}	1.4 ± 0.17^{abc}	1.7 ± 0.33^{ab}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M24	½MS	2.0	-	-	0.5	58 ± 22.05^{cde}	3.2 ± 0.17^{f}	5.7 ± 1.77^{e}	5.3 ± 2.02^{b}	0.7 ± 0.19^{b}
M25	½MS	-	1.0	-	0.5	42 ± 8.33^{abcde}	2.3 ± 0.32^{e}	4.3 ± 0.88^{bcde}	3.0 ± 2.08^{bc}	0.2 ± 0.10^{a}
M26	½MS	-	2.0	-	0.5	50 ± 0.00^{bcde}	1.4 ± 0.09^{ab}	3.0 ± 0.00^{abcde}	2.0 ± 0.00^{a}	0.0 ± 0.00^{a}
M27	½MS	-	-	1.0	0.5	83 ± 8.33^{e}	1.9 ± 0.12^{bcde}	5.3 ± 0.00^{de}	2.0 ± 1.15^{a}	0.2 ± 0.12^{a}
M28	½MS	-	-	2.0	0.5	67 ± 8.33^{de}	2.2 ± 0.12^{de}	5.3 ± 0.33^{abcd}	0.7 ± 0.67^{a}	0.2 ± 0.23^{a}

Means followed by the same letter within each column are not significantly different using Duncan's multiple range test at $p<0.05$

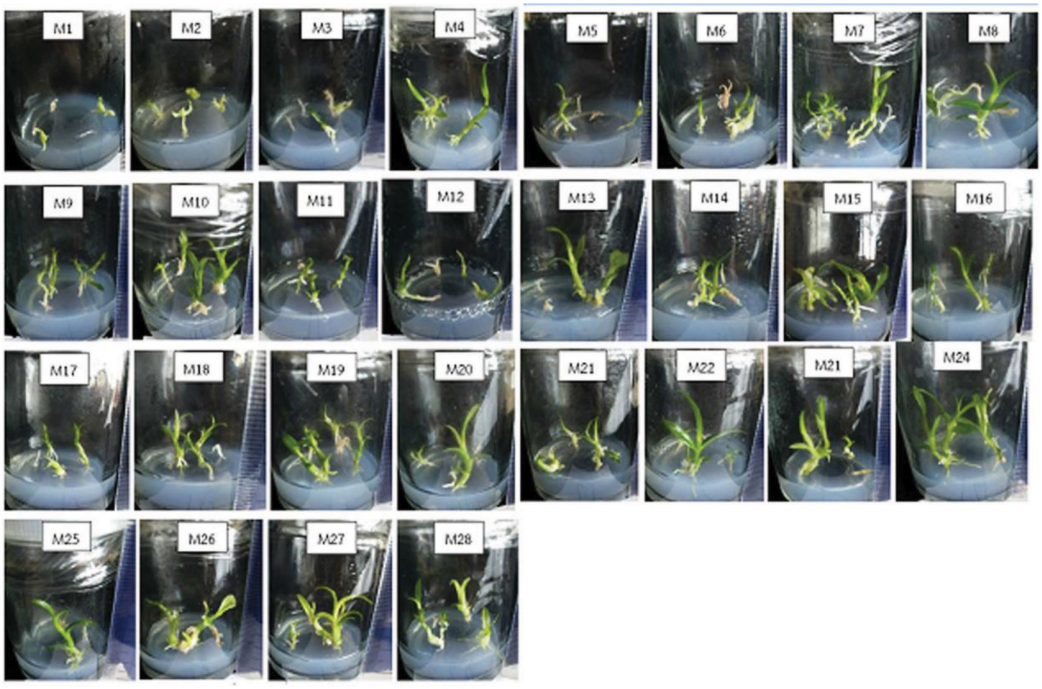

Figure 2. Shoot regeneration of *D. signatum* when cultured in MS and ½MS medium supplemented with BA, Kinetin and TDZ at 0, 1 and 2 mg/l, and 0 and 0.5 mg/l of NAA for three months

M1 = MS
M2 = MS + 1 mg/l BA
M3 = MS + 2 mg/l BA
M4 = MS + 1 mg/l Kinetin
M5 = MS + 2 mg/l Kinetin
M6 = MS + 1 mg/l TDZ
M7 = MS + 2 mg/l TDZ
M8 = MS + 0.5 mg/l NAA
M9 = MS + 1 mg/l BA + 0.5 mg/l NAA
M10 = MS + 2 mg/l BA + 0.5 mg/l NAA
M11 = MS + 1 mg/l Kinetin + 0.5 mg/l NAA
M12 = MS + 2 mg/l Kinetin + 0.5 mg/l NAA
M13 = MS + 1 mg/l TDZ + 0.5 mg/l NAA
M14 = MS + 2 mg/l TDZ + 0.5 mg/l NAA

M15 = ½MS
M16 = ½MS + 1 mg/l BA
M17 = ½MS + 2 mg/l BA
M18 = ½MS + 1 mg/l Kinetin
M19 = ½MS + 2 mg/l Kinetin
M20 = ½MS + 1 mg/l TDZ
M21 = ½MS + 2 mg/l TDZ
M22 = ½MS + 0.5 mg/l NAA
M23 = ½MS + 1 mg/l BA + 0.5 mg/l NAA
M24 = ½MS + 2 mg/l BA + 0.5 mg/l NAA
M25 = ½MS + 1 mg/l Kinetin + 0.5 mg/l NAA
M26 = ½MS + 2 mg/l Kinetin + 0.5 mg/l NAA
M27 = ½MS + 1 mg/l TDZ + 0.5 mg/l NAA
M28 = ½MS + 2 mg/l TDZ + 0.5 mg/l NAA

CONCLUSION

MS added to 10% potato extract, ½MS supplemented with 10% potato extract and MS added to 5% mashed banana can improve seed germination. In addition, the most suitable medium for improving shoot proliferation observed in this study was the ½MS medium added to 2 mg/l BA combined with 0.5 mg/l NAA. However, there was no significant difference in percentage of shoot proliferation and average number of leaves between media with and without plant growth regulators. Therefore, to save cost, some would choose to use MS media without plant growth regulators or use them at lower concentrations. As mentioned above, this experiment is simple and efficient and provides mass propagation in a short period of time as well as natural conservation of a rare orchid species.

REFERENCES

Arditti, J. (2008). *Micropropagation of orchids*. Oxford: Blackwell Publishing.

Attri, L. K. (2016). Therapeutic potential of orchids. *World Journal of Pharmacy and Pharmaceutical Sciences, 5*(2), 438–446.

Behie, S. W., & Bidochka, M. J. (2014). Nutrient transfer in plant-fungal symbioses. *Trends in Plant Science*, *19*(11), 734–740.

Kong, Q. L., Yuan, S. Y., & Végvári, G. (2007). Micropropagation of an orchid *Dendrobium strongylanthum* Rchb.f. *International Journal of Horticultural Science*, *13*(1), 61–64.

Luo, J. P., Wawrosch, C., & Kopp, B. (2009). Enhanced micropropagation of *Dendrobium huoshanense* C. Z. Tang et S. J. Cheng through protocorm-like bodies: The effects of cytokinins, carbohydrate sources and cold pretreatment. *Scientia Horticulturae*, *123*(2), 258–262.

Malabadi, R. B., Mulgund, S. G., & Kallappa, K. (2005). Micropropagation of *Dendrobium nobile* from shoot tip sections. *Journal of Plant Physiology*, *162*(4), 473–478.

Maneerattanarungroj, P., Bunnag, S., & Monthatong, M. (2007). In vitro conservation of *Cleisostoma areitium* (Rchb.f.) Garay, rare Thai orchid species by an encapsulation-dehydration method. *Asian Journal of Plant Sciences*, *6*(8), 1235–1240.

Martin, K. P., & Madassery, J. (2006). Rapid in vitro propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. *Scientia Horticulturae*, *108*(1), 95–99.

Miransari, M., & Smith, D. L. (2014). Plant hormones and seed germination. *Environmental and Experimental Botany, 99*, 110–121.

Molnár, Z., Virág, E., & Ördög, V. (2011). Natural substances in tissue culture media of higher plants. *Acta Biologica Szegediensis, 55*(1), 123–127.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiology Plant*, *15*(3), 473–497.

Pant, B. (2013). Medicinal orchids and their uses: Tissue culture a potential alternative for conservation Bijaya Pant. *African Journal of Plant Science*, *7*(10), 448–467.

Parthibhan, S., Venkateswara, M., & Kumar, T. S. (2015). *In vitro* regeneration from protocorms in *Dendrobium aqueum* Lindley – An imperiled orchid. *Journal of Genetic Engineering and Biotechnology, 13*(2), 227–233.

Peyachoknagul, S., Mongkolsiriwatana, C., Wanaapinpong, S., Huehne, P. S., & Srujyknath, K. (2014). Identification of native *Dendrobium* species in Thailand by PCR-RFLP of rDNA-ITS and chloroplast DNA. *ScienceAsia*, *40*(2), 113–120.

Sandoval Prando, M. A., Chiavazza, P., Faggio, A., & Contessa, C. (2014). Effect of coconut water and growth regulator supplements on in vitro propagation of *Corylus avellana* L. *Scientia Horticulturae*, *171*, 91–94.

Seidenfaden, G. (1985). *Orchid genera in Thailand XII*, *Dendrobium Sw. Opera Botanica* no. 83. Copenhagen: Council for Nordic Publications in Botany.

Singh, S., Singh, A. K., Kumar, S., Kumar, M., Pandey, P. K., & Singh, M. C. K. (2012). Medicinal properties and uses of orchids: A concise review. *Applied Botany*, *52*, 11627–11634.

Sujjaritthurakarn, P., & Kanchanpoom, K. (2011). Efficient direct protocorm-like bodies induction of dwarf *Dendrobium* using Thidiazuron. *Notulae Scientia Biologicae*, *3*(4), 88–92.

Thaitong, O. (2005). *The orchids of Thailand*. Thailand: Bangkok.

Thomas, D. T., & Michael, A. (2007). High-frequency plantlets regeneration and multiple shoot induction from cultured immature seeds of *Rhynchostylis retusa* Blume, an exquisite orchid. *Plant Biotechnology Reports*, *1*(4), 243–249.

Thorpe, T. A., Stasolla, C., Yeung, E. C., de Klerk, G. J., Roberts, A., & George, E. F. (2008). The components of plant tissue culture media II: Organic additions, osmotic and pH effects, and support systems. In E. F. George, M. A. Hall, & G. J. de Klerk (Eds.), *Plant propagation by tissue culture* (pp. 115–173). Verlag, Dordrecht: Springer.

Vacin, E., & Went, F. (1949). Some pH changes in nutrient solution. *Botanical Gazette*, *110*(4), 605–613.

Vijayakumar, S., Rajalkshmi, G., & Kalimuthul, K. (2012). Propagation of *Dendrobium aggregatum* by green capsule culture. *Lankesteriana*, *12*(2), 131–135.

Winarto, B., Rachmawati, F., Santi, A., & Teixeira da Silva, J. A. (2013). Mass propagation of *Dendrobium* 'Zahra FR 62', a new hybrid used for cut flowers, using bioreactor culture. *Scientia Horticulturae*, *161*, 170–180.

Zhang, Y., Lee, Y. I., Deng, L., & Zhao, S. (2013). Asymbiotic germination of immature seeds and the seedling development of *Cypripedium macranthos* Sw., an endangered lady's slipper orchid. *Scientia Horticulturae, 164*, 130–136.

Zhao, D., Hu, G., Chen, Z., Shi, Y., Zheng, L., Tang, A., & Long, C. (2013). Micropropagation and *in vitro* flowering of *Dendrobium wangliangii*. A critically endangered medicinal orchid. *Journal of Medicinal Plants Research*, *7*(28), 2098–2110.

Zhao, P., Wu, F., Feng, F. S., & Wang, W. J. (2007). Protocorm-like body (PLB) formation and plant regeneration from the callus culture of *Dendrobium candidum* Wall ex Lindl. *In Vitro Cellular Development Biology-Plant, 44*(3), 178–185.

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Morphological and Molecular Characterisation of *Campylocarpon fasciculare* and *Fusarium* spp., the Cause of Black Disease of Grapevine in Iran

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ABSTRACT

In 2014, disease symptoms of yellowing, foot rot and drying of leaves were observed in vineyards in Hormozgan province, Iran. The goal of the present study was to characterise fungal isolates associated with black foot of grapevines (*Vitis* spp.) using multi-gene DNA analysis (partial translation elongation factor-1 [*tef1*], internal transcribed spacers [ITS rDNA] and β-tubulin) and pathogenic characteristics of the isolates from the grapevines. Twenty-five isolates were obtained from diseased plants and identified as *Campylocarpon fasciculare* (14), *Fusarium solani* (7) and *F. decemcellulare* (4) through morphological characteristics. The three DNA regions analysed supported the morphological concept. All fungal isolates were evaluated for their pathogenicity on one-year-old rooted grapevine cultivar Askari in the planthouse. Typical root rot symptoms were observed within 90 days after inoculation. *Campylocarpon fasciculare* and an unnamed phylogenetic species of FSSC 20 were reported for the first time for Iranian mycoflora, indicating that grapevine vineyards have become the new host plants for *F. decemcellulare*.

Keywords: Grapevine, black disease, fungal species, multi-locus analysis, morphology

INTRODUCTION

Black disease, a disease affecting grapevines, is one of the most serious diseases to take note if in grape-producing plantations throughout the world. The disease is caused by different species of *Cylindrocarpon*, *Cylindrocladiella*, *Ilyonectria* and *Campylocarpon* (Alaniz et al., 2007; Auger et al., 2007). Black disease is characterised by root hairs, necrotic lesions on the outside of the foot and root reduction in root biomass. Mature grapevines presenting this disease are

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usually weak with small leaves, shortened internode, and uneven wood maturity (Halleen et al., 2006).

Black disease of grapevine was first described in 1961 (Grasso & Magnano, 1975). Grapevine black disease pathogens have been intensively investigated based on comprehensive morphological comparisons and molecular phylogeny analyses of multilocus DNA sequence (Halleen et al., 2004, 2006; Alaniz et al., 2007; Schroers et al., 2008; Lombard et al., 2012). According to previous studies, four distinct genera of *Cylindrocarpon*, *Cylindrocladiella*, *Ilyonectria* and *Campylocarpon* were reported as the causal agents of black disease of grapevine in most grapevine production areas of the world. Moreover, it is well known that these pathogens are distributed in soils and plant debris and usually act as decomposers. Therefore, control of these pathogens is difficult (Auger et al., 2007; Alaniz et al., 2007; Schroers et al., 2008; Lombard et al., 2012). Until now, no grapevine cultivar is sufficiently resistant to black disease (Casieri et al., 2009). Also, chemical control alone is not efficient to eradicate black disease pathogens in vineyards (Agustí-Brisach & Armengol, 2013). In addition, these pathogens have also been associated with different diseases of other economically important hosts, such as common olive (Úrbez-Torres et al., 2012), avocado (Vitale et al., 2012), and Scots pine (Menkis & Burokiene, 2012). Therefore, the correct identification of the causal agents of black disease of grapevine is necessary in order to develop proper management strategies to control pathogenic species (Agustí-Brisach & Armengol, 2013).

Mohammadi et al. (2009) introduced *Cylindrocarpon liriodendri* as the black disease of grapevine in Iran. But, until today, little attempt has been made to classify fungal isolates associated with black disease of grapevines in Iran. Therefore, the objectives of this study were (1) to identify fungal isolates associated with black disease of grapevines (Rishbaba, Askari and Black cultivars) in southern Iran by using morphological characteristics and sequencing of ITS regions, *tef1*and β-tubulin to determine genetic relationship among them, and (2) to determine their pathogenicity in grapevine cultivar, Askari.

MATERIALS AND METHOD

Fungal Cultures

A total of 25 fungal isolates were obtained from 21 diseased grapevines (Rishbaba, Askari and Black cultivars) in southern Iran that were showing severe symptoms of early decline (Table 1).

Table 1
Place of sample collection, Genbank Accession Numbers of fungal species isolated from black foot disease of grapevine vineyards collected from southern Iran

No.	Isolate Number	Species Identified	Location in Southern Iran (gps)			[a]*tef1*	[a]ITS	[a]*tub2*
1	CSC 1	*C. fasciculare*	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	KT935554	KT935546	KT935542
2	CSC 2	*C. fasciculare*	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	KT935555	KT935547	KT935543
3	CSC 3	*C. fasciculare*	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	-	-	-
4	CSC 4	*C. fasciculare*	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	-	-	-
5	CSC 5	*C. fasciculare*	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	-	-	-
6	CSC 6	*C. fasciculare*	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	-	-	-
7	CSC 7	*C. fasciculare*	Bashagard	26º 45 30' N	55º 46' 48' E	-	-	-
8	CSC 8	*C. fasciculare*	Bashagard	26º 45 30' N	55º 46' 48' E	-	-	-
9	CSC 9	*C. fasciculare*	Bashagard	26º 45 30' N	55º 46' 48' E	-	-	-
10	CSC 10	*C. fasciculare*	Bashagard	26º 45 30' N	55º 46' 48' E	-	-	-
11	CSC 11	*C. fasciculare*	Bashagard	26º 45 30' N	55º 46' 48' E	-	-	-
12	CSC 12	C. fasciculare	Hashtbandi	27°8'41"N	57°27'38"E	-	-	-
13	CSC 13	*C. fasciculare*	Hashtbandi	27°8'41"N	57°27'38"E	-	-	-
14	CSC 14	*C. fasciculare*	Hashtbandi	27°8'41"N	57°27'38"E	-	-	-
15	FDSC 15	*F. decemcellulare*	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	KT935560	KT935548	KT935544
16	FDSC 16	*F. decemcellulare*	Bashagard	26º 45 30' N	55º 46' 48' E	KT935561	KT935549	KT935545
17	FDSC 17	*F. decemcellulare*	Hashtbandi	27°8'41"N	57°27'38"E	-	-	-
18	FDSC 18	*F. decemcellulare*	Hashtbandi	27°8'41"N	57°27'38"E	-	-	-
19	SI-FSSC 20A	FSSC 20	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	KT935556	KT935550	-
20	SI-FSSC 20B	FSSC 20	Bashagard	26º 45 30' N	55º 46' 48' E	KT935557	KT935551	-
21	SI-FSSC 20C	FSSC 20	Hashtbandi	27°8'41"N	57°27'38"E	KT935558	KT935552	-
22	SI-FSSC 20D	FSSC 20	Hashtbandi	27°8'41"N	57°27'38"E	KT935559	KT935553	-
23	SI-FSSC 20E	FSSC 20	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	-	-	-
24	SI-FSSC 20F	FSSC 20	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	-	-	-
25	SI-FSSC 20G	FSSC 20	Bandar Abbas	27º 10' 59' N	56º 16' 59' E	-	-	-

In this research, weak grapevines with small and necrotic leaves, shortened internodes and uneven wood maturity were selected. Infected roots, crown and trunks of diseased grapevines were cut into small disks (1.5 cm). Necrotic wood were surface sterilised with 1% sodium hypochlorite solution for 1 min and then washed twice with sterilised distilled water. The tissues were placed on peptone-pentachloronitrobenzene agar (PPA) and potato dextrose agar (PDA) plates. All the plates were incubated under 12 h alternating light (black/white) at 25±2°C for 1 week and the resulting fungal colonies were transferred to fresh PDA plates and then purified using the single spore isolation technique.

The species were identified on the basis of macroscopic characteristics such as pigmentations and growth rate of the colony on PDA plates, as well as their microscopic features including shape and size of macroconidia, presence of microconidia and chlamydospores. For microscopic observations, all isolates were transferred to carnation leaf agar (CLA) (Fisher et al., 1982) medium. Thirty randomly selected macroconidia and microconidia were measured and analysed by 2-Sample T-Test by using MINITAB® 15. Identification to the species level was based on the descriptions of Halleen et al. (2004) for *Campylocarpon* species, and Leslie and Summerell (2006), Nalim et al. (2011) and Short et al. (2013) for *Fusarium* species.

DNA Isolation, Sequencing and Phylogenetic Analyses

Based on morphological identification, fungal mycelium of selected isolates was grown on PDA with sterile dialysis membranes (Lui et al., 2000) for one week and freeze-dried, and then the DNeasy® Plant Mini Kit (Qiagen) was used to extract total genomic DNA. Portions of the translation elongation factor-1**α** [*tef1*], internal transcribed spacers [ITS rDNA] and β-tubulin genes were amplified by PCR as described previously (Chehri, 2015; Chehri et al., 2015). PCR was performed in a Peltier Thermal Cycler, PTC-100® (MJ Research, Inc. USA). All PCR and sequencing primers used in this study are listed in Table 2. DNA amplification of the *tef1* was performed using the following programme: one cycle of 60 s at 94°C followed by 35 cycles of 30 s at 95°C, 55 s at 59°C, 90 s at 72°C, and a final extension of 10 min at 72°C. The PCR for ITS region was performed at 95°C (one cycle of 120 s) for a hot start, followed by 35 cycles of 60 s at 94°C, 30 s at 56°C, 120 s at 72°C, and a final extension of 72 °C (10 min). The PCR for β-tubulin gene was performed at 94°C (1 cycle of 60 s), followed by 39 cycles of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C, and a final extension of 5 min at 72°C. PCR products were purified using Qiagen columns (QIAquick PCR Purification Kit (250)) according to the manufacturer's protocol and sent for sequencing to a service provider. The maximum parsimony (MP) method, using MEGA4.0 program (Molecular Evolutionary Genetic Analysis software, ver. 4.0; http://www.megasoftware.net) (Tamura et al., 2007), was used to assess the phylogenetic diversity of all three-locus sequences (*tef1*, ITS and β-tubulin) of selected isolates included in the present study. Bootstrap values for the maximum parsimony tree were calculated for 1000 replicates. In order to assess the relationships between the major taxa, ambiguous parts of the *tef1*, ITS and β-tubulin were removed from further analysis and more conserved and alignable parts of the region and gene were used to generate phylogenetic trees containing representative taxa from major groups. The edited *tef1* and ITS (CSC 1,

CSC 2, FDSC 15, FDSC 16, SI-FSSC 20A, SI-FSSC 20B, SI-FSSC 20C, SI-FSSC 20D) and β-tubulin (CSC 1, CSC 2, FDSC 15, FDSC 16) sequences were compared with other available fungal species sequences in the GenBank.

Table 2
Primer sequences used for PCR amplification

ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	White et al. (1990)
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White et al. (1990)
EF1	5'-ATGGGTAAGGAGGACAAGAC-3'	O'Donnell et al. (1998)
EF2	5'-GGAAGTACCAGTGATCATGTT-3'	O'Donnell et al. (1998)
T1	5'-AACATGCGTGAGATTGTAAGT-3'	O'Donnell and Cigelnik (1997)
T2	5'-TAGTGACCCTTGGCCCAGTTG-3'	O'Donnell and Cigelnik (1997)

Pathogenicity Tests

All fungal isolates were used for pathogenicity assays on one-year-old rooted grapevine. The healthy grapevine cultivar, Askari, was used in this experiment. The experiments were carried out in greenhouse conditions maintained at 22 to 28°C, 60-70% RH. The experiments were arranged in a completely randomised design with three replications. The plants were inoculated by dipping the roots in a 1×10^6 conidial suspension for 60 min (Cabral et al., 2012). The control plants were dipped in sterile water. Inoculated plants were planted individually in pots containing sterilised soil:peat moss:vermiculite mixture (2:1:1) and placed in a glasshouse at 22 to 28°C for 90 days. Thirty days after the beginning of the pathogenicity test, the plants were re-inoculated using 50 mL conidial suspension including 10^6 conidia per plant (Alaniz et al*.,* 2007). Starting 90 days after inoculation, the plants were immediately transferred to a mycology laboratory. The roots were washed under running tap water to eliminate soil and debris. Root symptoms of plants were evaluated on the following scale: 0 = healthy, with no lesions; and 1 = discolouration, 1 = discolouration with lesions.

RESULTS

Morpho-Cultural Characteristics

A total of 25 fungal isolates were obtained from two- to four-year old grapevine vineyards located in southern Iran that showed severe symptoms of black disease including necrosis, brown and black streaking on the outside of the foot and roots. Based on their morphological characteristics, all the isolates belonged to *Campylocarpon fasciculare* (14), *F. decemcellulare* (4) and *F. solani* (7). Morphological characteristics including means and ranges of spore dimensions of all the fungal species are summarised in Table 3.

Table 3
Morphological characteristics of selected fungal species isolated from black foot disease of grapevine vineyards collected from southern Iran

Culture No.	Species Identified	Chlamydospores	Types of Conidigenious Cells	Sporodochia Colour	Shape of Basal Cell and Apical Cell	Length x Width of Macroconidia (µm)[a]	
						3- and 4-septate	5-septate
CSC 1	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	40.5 ± 1.5 × 5.2 ± 0.2	44.5 ± 2.5 × 5.2 ± 0.2
CSC 2	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	44.5 ± 2.5 × 4.0 ± 0.5	48.5 ± 2.5 × 5.4 ± 0.2
CSC 3	*C. fasciculare*	+	Monophialidic	White	Rounded, tapered and curved	43.5 ± 1.5 × 5.3 ± 0.2	47.5 ± 2.5 × 5.3 ± 0.2
CSC 4	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	42.5 ± 1.5 × 5.3 ± 0.2	45.5 ± 2.5 × 5.3 ± 0.2
CSC 5	*C. fasciculare*	+	Monophialidic	White	Rounded, tapered and curved	40.5 ± 1.5 × 5.3 ± 0.2	44.5 ± 2.5 × 5.1 ± 0.2
CSC 6	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	43.5 ± 1.5 × 5.3 ± 0.2	47.5 ± 2.5 × 5.3 ± 0.2
CSC 7	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	43.5 ± 1.5 × 5.3 ± 0.2	47.5 ± 2.5 × 5.3 ± 0.2
CSC 8	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	42.5 ± 1.5 × 5.3 ± 0.2	46.5 ± 2.5 × 5.3 ± 0.2
CSC 9	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	39.5 ± 1.5 × 5.3 ± 0.2	45.5 ± 2.5 × 5.2 ± 0.2
CSC 10	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	44.5 ± 1.5 × 5.3 ± 0.2	48.5 ± 2.5 × 5.3 ± 0.2
CSC 11	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	43.5 ± 1.5 × 5.3 ± 0.2	47.5 ± 2.5 × 5.3 ± 0.2
CSC 12	*C. fasciculare*	+	Monophialidic	White	Rounded, tapered and curved	41.5 ± 1.5 × 5.3 ± 0.2	46.5 ± 2.5 × 5.3 ± 0.2
CSC 13	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	43.5 ± 1.5 × 5.3 ± 0.2	47.5 ± 2.5 × 5.3 ± 0.2
CSC 14	*C. fasciculare*	+	Monophialidic	Light brown	Rounded, tapered and curved	44.5 ± 1.5 × 5.3 ± 0.2	48.5 ± 2.5 × 5.3 ± 0.2
FDSC 15	*F. decemcellulare*	-	Monophialidic	Yellow	Foot shaped, blunt	-	61.5 ± 2.5 × 5.6 ± 0.2
FDSC 16	*F. decemcellulare*	-	Monophialidic	Yellow	Foot shaped, blunt	-	65.5 ± 2.5 × 5.6 ± 0.2
FDSC 17	*F. decemcellulare*	-	Monophialidic	Yellow	Foot shaped, blunt	-	66.5 ± 2.5 × 5.7 ± 0.2
FDSC 17	*F. decemcellulare*	-	Monophialidic	Yellow	Foot shaped, blunt	-	66.5 ± 2.5 × 5.7 ± 0.2
SI-FSSC 20A	FSSC 20	+	Monophialidic	Cream	Foot cell and tapered, curved	47.5 ± 2.5 × 5.8 ± 0.5	52.5 ± 2.5 × 5.6 ± 0.5
SI-FSSC 20B	FSSC 20	+	Monophialidic	Cream	Foot cell and tapered, curved	49.2 ± 2.5 × 5.8 ± 0.5	54.5 ± 2.5 × 6 ± 0.5
SI-FSSC 20C	FSSC 20	+	Monophialidic	Cream	Foot cell and tapered, curved	51.2 ± 2.5 × 5.8 ± 0.5	52.5 ± 2.5 × 6 ± 0.5
SI-FSSC 20D	FSSC 20	+	Monophialidic	Cream	Foot cell and tapered, curved	50.2 ± 2.5 × 5.8 ± 0.5	51.5 ± 2.5 × 6 ± 0.5
SI-FSSC 20E	FSSC 20	+	Monophialidic	Cream	Foot cell and tapered, curved	49.2 ± 2.5 × 5.8 ± 0.5	51.5 ± 2.5 × 6 ± 0.5
SI-FSSC 20F	FSSC 20	+	Monophialidic	Cream	Foot cell and tapered, curved	48.2 ± 2.5 × 5.8 ± 0.5	50.5 ± 2.5 × 6 ± 0.5
SI-FSSC 20G	FSSC 20	+	Monophialidic	Cream	Foot cell and tapered, curved	51.2 ± 2.5 × 5.8 ± 0.5	52.5 ± 2.5 × 6 ± 0.5

[a]Mean values of 30 random conidia ± standard deviation

All the studied isolates of *C. fasciculare* produced a cottony surface texture of colonies. The cultures grew slowly, and the growth rate on PDA at 25°C in intermittent light ranged from 4.3 to 4.7 mm/day. All the isolates formed white to light brown sporodochia on the surface of the leaves. Macroconidia arising from sporodochia were straight or slightly curved, with 3-5-septate and mostly 3-septate, tapered and curved apical cells and barely notched or rounded basal cells (Figure 1). All the *C. fasciculare* isolates produced branched conidiophores.

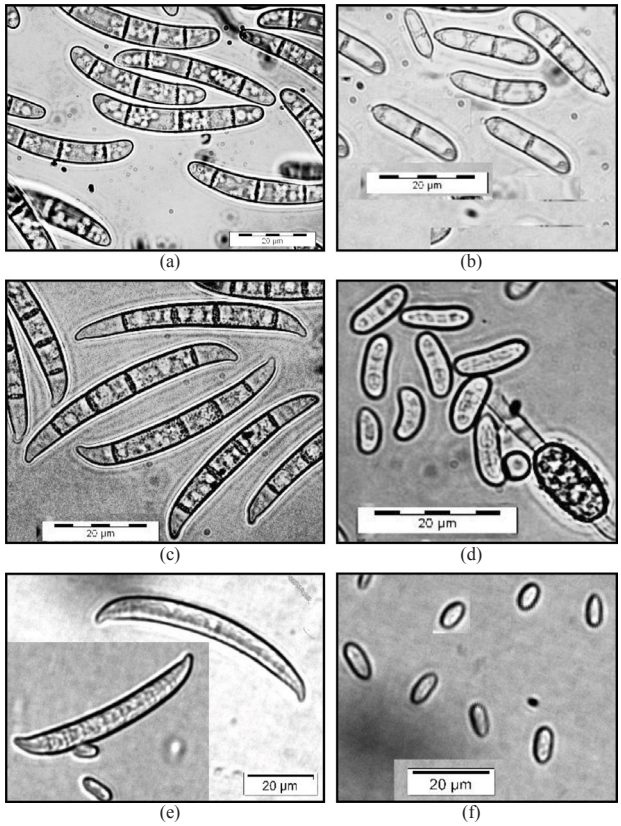

Figure 1. (a) and (b) Campylocarpon fasciculare, (a) macroconidia produced from sporodochia and (b) mesoconidia; (c) and (d) Fusarium solani species complex (FSSC 20), (c) macroconidia produced from sporodochia and (d) microconidia formed on conidiophores in hyphae; and (e) and (f) F. decemcellulare, (e) macroconidia produced from sporodochia and (f) microconidia formed on conidiophores in hyphae. Bar = 20 µm for all pictures

All studied isolates of *F. decemcellulare* produced a cottony surface texture of colonies. The cultures grew slowly, and the growth rate on PDA at 25°C in intermittent light ranged from 3.2 to 3.8 mm/day. All the isolates formed white to yellow sporodochia on the surface of the leaves. Macroconidia arising from sporodochia were very long and thick-walled relative to many other species with even curvature on both sides

of the macroconidia, 5-9-septate with rounded apical cells and foot-shaped basal cells (Figure 1). All the *F. decemcellulare* isolates produced branched monophialidic conidiophores. Oval microconidia were observed in long chains produced from monophialides in branched conidiophores.

All the studied isolates of *F. solani* produced a cottony surface texture of colonies. The cultures grew fast, and the growth rate on PDA at 25°C in intermittent light ranged from 7.8 to 8.6 mm/day. The macroconidia arising from sporodochia were typically falcate and mostly 5-septate with papillate, tapered and curved apical cells and well developed foot cells (Figure 1). Microconidia reniform, elongated oval to sometimes obovoid with a truncate base, were formed in false heads on long monophialides. All the *F. solani* isolates produced chlamydospores.

Molecular Characterisation

Multilocus DNA sequence data were used to assess the phylogenetic relationships and species identification of fungal isolates obtained from diseased grapevines in southern Iran. Based on morphological characteristics, eight isolates were selected for molecular studies (Tables 1 and 3). The aligned partial nuclear ITS regions, *tef1* and β-tubulin gene partitions consisted of 450, 500 and 450 characters, respectively, totalling 1400 bp of aligned DNA sequence per isolates. The edited *tef1*, ITS and β-tubulin sequences were compared with other available fungal species sequences in the GenBank. From similarities searched at the NCBI database, identification of all fungal species was confirmed with statistical significance. Also, this was confirmed by a phylogenetic analysis of the combined dataset (Figure 2). The edited *tef1* and ITS datasets were combined and analysed phylogenetically using MEGA4.0 software for all identified fungal species (Tamura et al., 2007). However, due to highly divergent paralogs, it was thought that the use of β-tubulin gene sequences for phylogeny reconstruction within the members of FSSC could be problematic. So, the individual β-tubulin dataset was used for phylogeny reconstruction for the species of *C. fasciculare* and *F. decemcellulare*.

The phylogenetic trees generated from the combined ITS regions and *tef1* sequences and the individual β-tubulin dataset revealed a monophyly among two isolates (CSC 1 and CSC 2) and *C. fasciculare* (CBS 112613) with strongly supported relationship (Figures 2 and 3). The trees also showed two isolates of FDSC 15 and FDSC 16 with strong bootstrap support placed in distinct lineage of *F. decemcellulare* (Figures 2 and 3). The phylogenetic tree generated from the combined ITS regions and *tef1* dataset showed a monophyly between FSSC 20-c (NRRL 32316) and FSSC 20-a (NRRL 22608) and isolates SI-FSSC 20A, SI-FSSC 20B, SI-FSSC 20C and SI-FSSC 20D (92% MP).

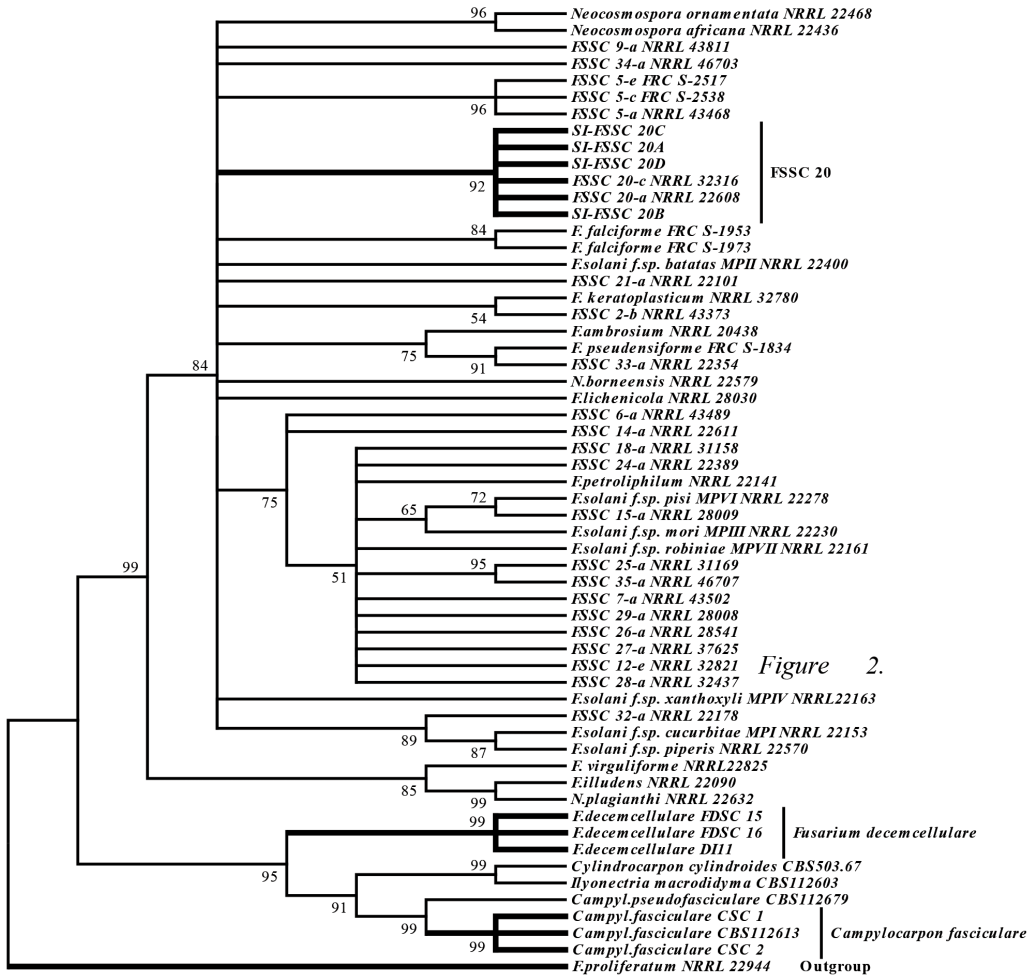

Figure 2. A maximum parsimony phylogeny for 79 taxa of the fungal species inferred from combined ITS and *tef1* sequences. Bootstrap tests were performed with 1000 replications. *Fusarium proliferatum* (NRRL 22944) obtained from GenBank was treated as the outgroup.

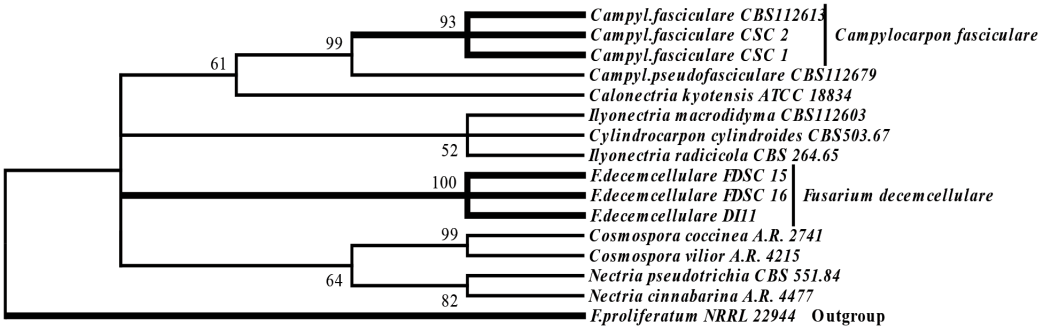

Figure 3. A maximum parsimony phylogeny for eight taxa of the fungal species inferred from partial sequence of β-tubulin gene. Bootstrap tests were performed with 1000 replications. *Fusarium proliferatum* (NRRL 22944) obtained from GenBank was treated as the outgroup.

[a]GenBank numbers for *tef1* and *tub2* genes sequences

Pathogenicity Test

In this experiment, 25 isolates were used for pathogenicity assays on one-year-old rooted grapevine cultivar, Askari. None of the control plants died. Of the 11 isolates belonged to *F. solani* (7) and *F. decemcellulare* (4), isolate SI-FSSC 20D and FDSC 16 showed small lesions on grapevine roots that are considered hypovirulent to grapevine plants. The results of the pathogenicity test also showed that isolates CSC 1, CSC 2 and CSC 13 belong to *C. fasciculare*; the isolates showed external symptoms (long lesions) on the grapevine roots and were considered a virulent group. Re-isolations on PDA medium were attempted for all the isolates.

DISCUSSION

The *Campylocarpon* species are considered the causal agents of black disease of grapevine in different regions of the world (Santos et al., 2014). From the genus *Campylocarpon*, two species were included as the causal agents of black disease in this study i.e. *C. pseudofasciculare*, which has been reported in South Africa (Halleen et al., 2004), Uruguay (Abreo et al., 2010), Brazil (Santos et al., 2014; Correia et al., 2012) and Perú (Álvarez et al., 2012) and *C. fasciculare*, which has been reported in Spain (Alaniz et al., 2011), Turkey (Akgül et al., 2014) and South Africa (Halleen et al., 2004); all the studies were in agreement regarding the result. *Phaeoacremonium (Pm.), Pm. parasiticum*, *Pm. inflatipes*, *Pm. cinereum, Pm. aleophilum, Phaeomoniella chlamydospore, Cylindrocarpon liriodendri*, *Diplodia seriata* and *Neofusicoccum parvum* have been identified to be associated with grapevines, but from other locations in Iran (Mohammadi et al., 2009, 2013). This is the first study reporting on the presence of *C. fasciculare* and *F. decemcellulare* and an unnamed phylogenetic species of FSSC 20 in vineyards in southern Iran.

Phylogenetic studies are very useful in differentiating closely related strains, and they allow clear separation of some morphologically similar species (Leslie & Summerell, 2006; Chaverri et al., 2011; Lombard et al., 2012). Therefore, in this research, in order to define fungal taxa, the combination of different methods namely, morphological and phylogenetic studies based on combined ITS regions and *tef1* datasets, are consistently applied and they support each other. It seems that based on the β-tubulin dataset, highly divergent paralogs were discovered within members of FSSC (O'Donnell, 2000). So, molecular phylogeny based on the β-tubulin dataset was used for phylogeny reconstruction for the species of *C. fasciculare* and *F. decemcellulare*.

Fusarium decemcellulare is usually found in tropical and sub-tropical regions, and has been consistently associated with branch canker and die back of a range of tropical fruit trees (Ploetz et al., 1996). It seems that weather conditions and climate and types of agricultural crop in southern Iran similar to those in subtropical countries

make correct identification of this species very important for management and control of tropical fruit tree diseases in Iran.

Molecular phylogeny demonstrated that *F. solani* isolates in this research formed a monophyletic group with typical strains, FSSC 20-c (NRRL 32316) and FSSC 20-a (NRRL 22608), obtained from GenBank. These strains undoubtedly represent a new species within Clade 3. However, this species should be further studied (in terms of ecological and biological aspects) before its taxonomic status can be drawn.

CONCLUSION

The present study will serve as a basis for future studies of the epidemiology of black disease of grapevine in southern Iran and possibilities for effective management of black foot in Iranian vineyards.

REFERENCES

Abreo, E., Martínez, S., Betucci, L., & Lupo, S. (2010). Morphological and molecular characterization of *Campylocarpon* and *Cylindrocarpon* spp. associated with black foot disease of grapevines in Uruguay. *Australasian Plant Pathology, 39*(5), 446–452.

Agustí-Brisach, C., & Armengol, J. (2013). Black-foot disease of grapevine: An update on taxonomy, epidemiology and management strategies. *Phytopathologia Mediterranea, 52*(2), 245−261.

Akgül, D. S., Savaş, N. G., Önder, S., Özben, S., & Kaymak, S. (2014). First report of *Campylocarpon fasciculare* causing black foot disease of grapevine in Turkey. *Plant Disease, 98*(9), 1277-1277.

Alaniz, S., Agustí-Brisach, C., Gramaje, D., Aguilar, M. I., Pérez-Sierra, A., & Armengol, J. (2011). First report of *Campylocarpon fasciculare* causing black foot disease of grapevine in Spain. *Plant Disease, 95*(8), 1028–1029.

Alaniz, S., Leon, M., García-Jiménez, J., Abad, P., & Armengol, J. (2007). Characterization of *Cylindrocarpon* species associated with black-foot disease of grapevine in Spain. *Plant Disease, 91*(9), 1187–1193.

Álvarez, L. A., Tamayo, D., Castilla, C., Munive, C., Agustí-Brisach, C., Gramaje, D., & Armengol J. (2012). Occurrence of grapevine trunk pathogens in nurseries and vineyards in the northern and southern coast of Peru. *Phytopathologia Mediterranea, 51*, 425.

Auger, J., Esterio, M., & Pérez, I. (2007). First report of black foot disease of grapevine caused by *Cylindrocarpon macrodidymum* in Chile. *Plant Disease, 91*(4), 470-470. doi:10.1094/PDIS-91-4-0470A.

Cabral, A., Rego, C., Crous, P. W., & Oliveira, H. (2012). Virulence and cross-infection potential of *Ilyonectria* spp. to grapevine. *Phytopathologia Mediterranea, 51*(2), 340–354.

Casieri, L., Hofstetter, V., Viret, O., & Gindro, K. (2009). Fungal communities living in the wood of different cultivars of young *Vitis vinifera* plants. *Phytopathologia Mediterranea, 48*(1), 73–83.

Chaverri, P., Salgado, C., Hirooka, Y., Rossman, A. Y., & Samuels, G. J. (2011). Delimitation of *Neonectria* and *Cylindrocarpon* (Nectriaceae, Hypocreales, Ascomycota) and related genera with *Cylindrocarpon*-like anamorphs. *Studies in Mycology, 68*, 57–78.

Chehri, Kh. (2015). First report of postharvest fruit rot of tomato caused by *Fusarium oxysporum* in Iran. *Archives of Phytopathology and Plant Protection, 48*(6), 537–544.

Chehri, K., Salleh, B., & Zakaria, L. (2015). Morphological and phylogenetic analysis of *Fusarium solani* species complex in Malaysia. *Microbial Ecology, 69*(3), 457–471

Correia, K. C., Câmara, M. P. S., Barbosa, M. A. G., Sales, Jr. R., Agustí-Brisach, C., Gramaje, D. … & Michereff, S. J. (2012). Fungal species associated with trunk diseases of table grapes in Northeastern Brazil. *Phytopathologia Mediterranea, 51*, 427.

Fisher, N. L., Burgess, L. W., Toussoun, T. A., & Nelson, P. E. (1982). Carnation leaves as a substrate and for preserving *Fusarium* species. *Phytopathology, 72*(1), 151–153.

Grasso, S., & Magnano, D. S. L. (1975). Infezioni di *Cylindrocarpon obtusisporum* su piante di vite in Sicilia. *Vitis, 14*, 36–39.

Halleen, F., Fourie, P. H., & Crous, P. W. (2006). A review of black foot disease of grapevine. *Phytopathologia Mediterranea, 45*(4), 55–67.

Halleen, F., Schroers, H. J., Groenewald, J. Z., & Crous, P. W. (2004). Novels species of *Cylindrocarpon* (*Neonectria*) and *Campylocarpon* gen. nov. associated with black-foot disease of grapevines (*Vitis* spp). *Studies in Mycology, 50*(2), 431–455.

Leslie, J. F., & Summerell, B. A. (2006). *The Fusarium laboratory manual*. UK: Blackwell Publishing Ltd.

Lombard, L., Shivas, R. G., To-Anun C., & Crous, P. W. (2012). Phylogeny and taxonomy of the genus *Cylindrocladiella*. *Mycological Progress, 11*(4), 835–868.

Lui, D., Coloe, S., Baird, R., & Pedersen, J. (2000). Rapid mini-preparation of fungal DNA for PCR. *Journal of Clinical Microbiology, 38*(1), 471–477.

Menkis, A., & Burokiene, D. (2012). Distribution and genetic diversity of the root-rot pathogen *Neonectria macrodidyma* in a forest nursery. *Forest Pathology, 42*(1), 79–83.

Mohammadi, H., Alaniz, S., Banihashemi, Z., & Armengol, J. (2009). Characterization of *Cylindrocarpon liriodendri* associated with black foot disease of grapevine in Iran. *Journal of Phytopathology, 157*(10), 642–645.

Mohammadi, H., Banihashemi, Z., Gramaje, D., & Armengol, J. (2013). Fungal pathogens associated with grapevine trunk diseases in Iran. *Journal of Agricultural Science and Technology, 15*(1), 137–150.

Nalim, F. A., Samuels, G. J., Wijesundera, R. L., & Geiser, D. M. (2011). New species from the *Fusarium solani* species complex derived from perithecia and soil in the old world tropics. *Mycologia*, *103*(6), 1302–1330.

O'Donnell, K. (2000). Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. *Mycologia, 92*(5), 919–938.

O'Donnell, K., & Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution, 7*(1), 103–116.

O'Donnell, K., Kistler, H. C., Cigelnike, E., & Ploetz, R. C. (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Sciences, 95*(5), 2044-2049.

Ploetz, R., Vazquez, A., & Benscher, D. (1996). First report of *Fusarium decemcellulare* as a pathogen of mango in the United States. *Plant Disease, 80*(10), 1207.

Santos, R. F. D., Blume, E., Muniz, M. F. B., Harakawa, R., Garrido, L. D. R., & Rego, C. (2014). Characterization of *Campylocarpon pseudofasciculare* associated with black foot of grapevine in southern Brazil. *Phytopathologia Mediterranea, 53*(3), 272–281.

Schroers, H. J., Zerjav, M., Munda, A., Halleen, F., & Crous, P. W. (2008). *Cylindrocarpon pauciseptatum* sp. nov., with notes on *Cylindrocarpon* species with wide, predominantly 3-septate macroconidia. *Mycological Research, 112*(1), 82–92.

Short, D. P. G., O'Donnell, K., Thrane. U., Nielsen, K. F., Zhang, N., Juba, J. H., & Geiser, D. M. (2013). Phylogenetic relationships among members of the *Fusarium solani* species complex in human infections and the descriptions of *F. keratoplasticum* sp. nov. and *F. petroliphilum* stat. nov. *Fungal Genetic and Biology, 53*, 59–70.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution, 24*(8), 1596–1599.

Úrbez-Torres, J. R., Peduto, F., & Gubler, W. D. (2012). First report of *Ilyonectria macrodidyma* causing root rot of olive trees (*Olea europaea*) in California. *Plant Disease, 96*(9), 1378.

Vitale, A., Aiello, D., Guarnaccia, V., Perrone, G., Stea, G., & Polizzi, G. (2012). First Report of root rot caused by *Ilyonectria* (=*Neonectria*) *macrodidyma* on avocado (*Persea americana*) in Italy. *Journal of Phytopathology 160*(3), 156–159.

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Food and Feeding Habits and Allometric Relationship of *Synodontis schall* in Lower Ogun River, Akomoje, Ogun State, Nigeria

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ABSTRACT

Overfishing and depletion of wild fish stock are a major problem facing the fisheries sector in Nigeria, hence the need to carry out an extensive research on the biology of wild species. The aim of this study was to provide contributory information on the biology of *Synodontis schall* from Ogun River, Nigeria. Ninety fish samples were obtained from local fishermen bi-monthly between February and August, 2015. The length and weight of the specimen were measured and length-weight relationship was determined using the formula W=aLb. Gut content of the specimens was studied using the frequency of occurrence and numerical method. Index of stomach fullness was also determined monthly. Gut content revealed both plants and animal items consisting mainly of insects, rotifers, crustaceans, fish parts, algae etc. indicating omnivorous feeding habit. Percentage stomach fullness was higher during the peak of the wet season (July and August). Total length, standard length and weight of specimens were ranged between 15.4 and 29.0 cm, 10.1 and 23.5 cm and 50 g and 198 g, respectively. Length-weight analyses gave the values of 'b', 'a', and 'r' as 1.520, 1.115 and 0.8967, respectively, exhibiting a negative allometric growth pattern. The study therefore, shed light on the biology of the species and thus, contributes to documented facts about the species from the Akomoje reservoir, with a view towards developing the aquaculture of the reservoir.

Keywords: Allometric growth pattern, frequency of occurrence, length-weight relationship, negative allometric growth, numerical method, Ogun River, stomach fullness, *Synodontis schall*

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INTRODUCTION

Synodontis schall, commonly referred to as upside-down catfish, is a widely distributed catfish species in African freshwater sources (Paugy & Roberts, 1992). The genus has over 112 species (Daget et al., 1991). Reed et al. (1967) described 20 species found in Northern Nigeria. Studies on food and feeding habits of different fish species have been carried out to determine the dietary requirements of these different species and their place in the food chain for the sole purpose of domestication, culture and for defining the relationships between organisms in their habitat and the environment (predator-prey relationships) (Lopez-Peralta & Arcila, 2002, pp. 23–29). The feeding habits of many fish in different water bodies have been investigated and documented as dietary composition may be due to availability, season, age and size.

Length-Weight Relationship (LWR) has been reported to provide information on fish stock composition, mortality, growth rate, production, life expectancy and relative wellbeing of fish populations as an indicator of habitat conditions according to Foulton's condition factor 'K' (Soyinka & Ebigbo, 2012, pp. 392–401), which is a vital tool in biology, ecology, physiology and fisheries assessment (Oscoz, Campos, & Escala, 2005, pp. 73-74). Thus, studies on food and feeding and length-weight relationship among others cannot be overemphasised in the assessment and management of fish (Atama et al., 2013, pp. 82–86).

Various studies have been conducted on some aspects of the biology of this species. Akombo, Akanga, Adikwu and Araoye (2013, pp. 42–48) studied length-weight relationship, condition factors and feeding habits of *Synodontis schall* in Benue River. Shinkafi, Argungu and Akanbi (2010, pp. 304–307) studied the food and feeding habits of catfish (Synodontis nigrita) in Rima River, while Sokoto, Olojo, Olurin and Osikoya (2003, pp. 21–24) studied the food and feeding habits of catfish (S. nigrita) in the Osun River, Osun State. Although some authors have conducted research into this species, there is still need for research into this species from the Ogun River specifically due to its high economic importance, which has resulted in exploitation. In addition, little has been documented on this species from this particular source. Hence, this study was aimed at contributing to information on some aspects of the biology of this species from the Ogun River specifically.

MATERIALS AND METHOD

Description of Study Area

The Ogun River, with a latitude of 6.63 ($6^0$371 60N) and longitude of 3.45 ($3^0$271 0E), is a forest reserve located in Ogun State, Nigeria, West Africa. The location is situated 6.25 km south west (234^0) of the approximate centre of Nigeria and 526 km south west (239^0) of the capital, Abuja, measured with a compass. A 10-square kilometre area around the Ogun River has an approximate population of 313,439

(0.003134) persons per square metre and an average elevation of 40 m above sea level. It discharges into the Lagos lagoon and rises in Oyo state near Shaki at coordinates 8^{0}4110N, 302810E and flows through Ogun state, precisely at the Abeokuta south west local government area of Ogun State and into Lagos state (Ayoade, Sowunmi, & Nwachukwu, 2004, pp. 171–175).

Collection of Specimen

A total of 90 specimens of the fish were procured from artisanal fishermen every fortnight for six months (March-August, 2015) at the bank of the lower Ogun River, Akomoje. The specimens were serially labelled by number, after which they were transported to the laboratory in ice boxes. The use of ice boxes was in order to reduce posthumous digestion to the barest minimal before subsequent analyses. These collections were done in the morning on each occasion to avoid serious heat effect of the sun that could have caused faster spoilage or shrinkage in spite of the use of ice boxes because of distance. Identification was done using the keys of Reed, Burchard, Hopson and Yaro (1967, p. 226) and Holden and Reed (1972, pp. 46–49).

Length-Weight Measurement

The standard length (SL) of the fish samples were measured using a measuring board. The anterior tip of each fish was placed against a stop at the beginning of the measuring scale with the fish's mouth closed. SL was taken as the length from the tip of the fish′s mouth to the hidden base of the tail fin rays and this was measured to the nearest 0.1 cm. Total body weight (TBW) was measured using a digital electronic weighing balance (Adams AFP 4100L). This was read to the nearest 0.1 g.

Sex Determination

The gender of the Synodontis species can only be identified after dissection. Thus, the fish were dissected and their gonads were inspected using the keys of Nikolsky (1963). In the young males, the testes were thin and thread-like with very small projections, whitish in colour and extended to about one third of the abdominal cavity. In the adult males, the testes were creamy in colour with very conspicuous granules. The young females had thin, pink to white tubular structures occupying about one fifth of the abdominal cavity. In the adult females that were about to spawn eggs, the ovaries were readily discernible as they had increased in size and filled most of the abdominal cavity (Bagenal, 1978; Halim & Guma′a, 1989).

Laboratory Procedure

Individual fish specimens were dissected using a sterile scalpel blade and a pair of scissors from a dissecting kit. The fish were opened up ventrally from the anal opening to the head.

The fish were dissected and their stomachs removed with care so that the content remained intact and its fullness was

individually observed and recorded and preserved immediately in 4% formalin for subsequent examination of the food items. The stomachs were scored 0, ¼, ½, ¾ or full according to their fullness (Table 1). Each stomach sample was then opened and the content emptied in a petri dish. Some food items such as sand grains and insect parts were identified with the naked eye, while others were identified with the aid of a microscope. Slide preparation was made and the samples were examined under a light microscope using objectives X10, X40 and X100.

Table 1
Stomach fullness description

Stomach size	Description
Full stomach (1)	The stomach bulges considerably with food
¾ full stomach	The stomach is almost full but not bulging
½ full stomach	Food occupies about 50% of the stomach volume
¼ full stomach	Stomach wall very flabby, can seem empty at times
Empty stomach (0)	No visible food in the stomach when dissected and examined under the microscope

Source. Olatunde (1978, pp. 197–207)

Numerical Abundance Method

The number of individual food types in each stomach was counted and expressed as a percentage of the total number of food items in the sample studied.

$$\% \text{ Number of a food item} = \frac{\text{Total number of the particular food item}}{\text{Total number of all food items}} \times 100$$

Frequency of Occurrence Method

The number of stomachs in which each item occurred was recorded and expressed as a percentage of the total number of stomachs examined.

$$\% \text{ Occurrence of food item} = \frac{\text{Total no. of stomachs particular food items}}{\text{Total number of stomachs with food}} \times 100$$

Length-Weight Relationship

The LWR of the fish was calculated using the equation

$$W = aL^b \quad \ldots\ldots [1]$$

where W =the observed weight of the fish
L=the observed length
a and *b*=constants

b is the slope usually between 2 and 4 and *a* is the intercept on the length axis (Bagenal, 1978, p.).

The logarithmic transformation of equation (1) gives a straight-line relationship:

$$\text{LogW} = \text{Log}a + b\text{LogL} \quad \ldots\ldots [2]$$

When $Log_{10}W$ is plotted against $Log_{10}L$, the regression coefficient is 'b', and Log a represents the intercept on the y-axis.

Statistical Analysis

The dietary items in the stomach of all the specimens were pooled together and presented based on the frequency of occurrence and numerical abundance or importance. They were further statistically summarised in charts according to the size of the fish and according to the monthly occurrence.

RESULTS

Stomach Fullness

Results of the study showed 20 specimens (22.22%) with empty stomachs that was 0 and ¼ fullness representing an empty stomach, while 57 (63.3.78%) had a full stomach, which included ¾ and 1 stomach fullness (Table 2). Monthly variation in stomach fullness showed the specimen had a full stomach more often in the rainy season (Table 3).

Table 2
Percentage total stomach fullness of Synodontis schall

Stomach fullness	No of samples	Percentage
0	14	15.56
¼	6	6.67
½	13	14.44
¾	23	25.55
1	34	37.78
TOTAL	**90**	**100**

Note. Fullness includes full stomach 1 and ¾ full, half full stomach ½, Emptiness includes ¼ full and 0 stomach

Table 3
Monthly categorisation of percentage fullness

MONTH	Specimen examined	Empty stomach	% empty	Full stomach	% fullness
MARCH	6	3	50	3	50
APRIL	12	7	58.33	5	41.67
MAY	9	5	55.56	4	44.44
JUNE	13	3	23.08	10	76.92
JULY	24	1	4.17	23	95.83
AUGUST	26	1	3.85	25	96.15

Food and Feeding

The results of the stomach content analysis of *S. schall* examined during the study period (March-August, 2015) revealed that the fish was an omnivore that fed on a variety of food items comprising different types of algae, plant materials, insect parts and larvae, fish scales, rotifers, diatoms, crustaceans, detritus and many unidentified food items (Table 4). Algae appear to be the most abundant and important food item both in number and occurrence, representing about nine of the total food items identified in the specimens' stomach, with Polycystis sp. contributing 97.1% and 17.99%, respectively in number and occurrence (Figure 1).

Table 4
Food items in the stomach of S. schall

Food items	Occurrence	% Occurrence	Numerical	% Numerical
ALGAE				
Phormidium sp.	22	31.4	41	0.82
Coelospharium sp.	17	24.2	25	0.50
Polycystis sp.	68	97.1	899	17.99
Aphanocapsa sp.	23	32.8	128	2.56
Oscillatoria sp.	8	11.4	52	1.04
Closterium sp.	51	72.8	83	1.66
Oedogonium sp.	12	17.1	33	0.66
Spirogyra sp.	47	67.1	64	1.28
Unidentified algae	5	7.14	68	1.36
DIATOMS				
Diatoma sp.	63	90	315	6.31
Synedra sp.	52	74.2	184	3.68
INSECTS				
Insect parts	38	54.3	506	10.13
Insect larvae	50	71.4	471	9.44
CRUSTACEAN				
Daphnia sp.	14	20	258	5.13
Ceriodapnia sp.	8	11.4	43	0.86
Copepods sp.	5	7.14	36	0.76
ROTIFERS				
Philodina sp.	29	41.4	48	0.96
Epiphines sp.	25	35.7	62	1.24
Parts of plants	**32**	**45.7**	**511**	**10.23**
Fish scale	**43**	**61.4**	**203**	**4.06**
Detritus	**56**	**80**	**715**	**14.31**
Unidentified items	**41**	**58.5**	**248**	**4.96**

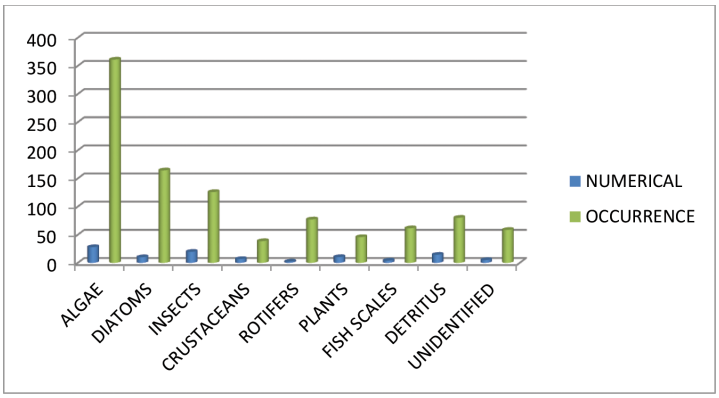

Figure 1. Percentage occurrence and numerical importance of food items in *S. schall*

Length-Weight Relationship of *S. schall*

Figure 2 below shows the log transformed length-weight relationship (LWR) of *S. schall* specimen during the study period. The morphometric features, which include Standard Length (SL), Total Length (TL) and the Total Body Weight (TBW), were calculated in order to determine the LWR. SL ranged from 10.1 to 23.5 cm with mean (16.56±3.52cm); TL ranged from 15.4 to 29.0 with mean (21.8±3.68 cm) and TBW ranged from 50 g to 198 g with mean (117.99±37.9 g).

The results showed *b*, *a*, and *r* values for *S. schall* to be 1.520, 1.115 and 0.8967, respectively.

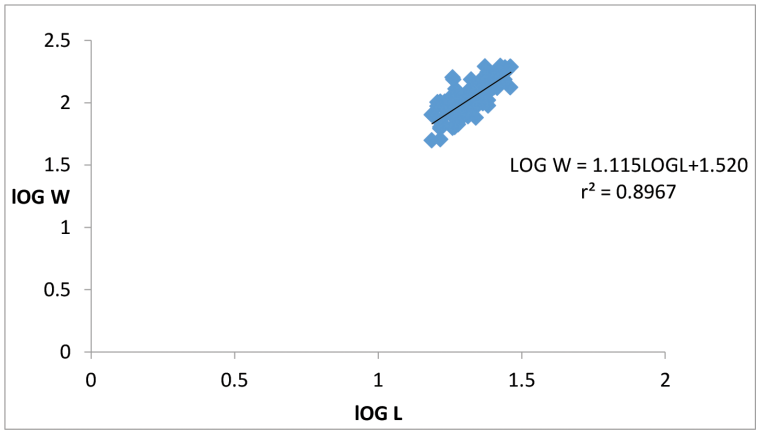

Figure 2. Length-Weight relationship of *S. schall* in Lower Ogun River, Akomoje

DISCUSSION

Monthly variation in stomach content in terms of fullness of this species of fish was as expected. Percentage of empty stomach was observed to be highest during the dry season compared with the rainy months. This could be attributed to the abundance of food items of choice for *S. schall* during the rainy season. This abundance is likely due to the high availability of high organic matter emptied into the water body resulting in high productivity in the waters, which in turn resulted in high phytoplankton production and subsequent abundance of zooplankton and nekton production. This high number of empty stomachs may also have been due to the method employed during harvesting of the specimens. This corroborates the findings of Olojo et al. (2003, pp. 21–24), who reported similar finding in the Osun River for S. nigrita and posited that there was variation in the feeding habits of *S. schall* according to the seasons. Akombo et al. (2014, pp. 42–48) reported similar findings i.e. better condition factors for *S. schall* during the rainy months attributed to availability and sufficiency of food items. Generally, *S. schall* could be said to have a wide choice of food items to consume during the rainy spell when there are more aquatic plants and insects in the water than in the dry months.

S. schall during the study was observed to feed on varieties of food items that included plankton, invertebrates and plant material. This proved the omnivorous feeding nature of the fish as revealed by other researchers (Akombo et al., 2014, pp. 42–48; Olojo et al., 2003, pp. 21–24). The diversity of natural food items found in the stomach of *S. schall* specimens was an indication that the feeding habits of the species was euryphagous, feeding on a broad range and variety of food available in the environment. Natural food items found in the species such as detritus, insect parts, crustaceans and fish scales showed that the species was an omnivorous bottom feeder, which could be due to the position of the mouth in this species. Hassan (2007) in his study posited that *S. schall* in the Nile was an omnivorous bottom-feeding fish species as sand, mud and fish remains were present in its stomach upon dissection. The preference of *S. schall* for phytoplankton, detritus, plant materials, insect parts and larvae in this study could probably have been due to the predominance of these food items in the environment. This finding was corroborated by Olojo et al. (2003, pp. 21–24), who reported high occurrence of similar food items in the stomach of *S. nigrita* in the Osun River.

The LWR of the species during the study period gave a straight linear graph as shown, revealing negative allometry for the species in the water body with a 'b' value less than 3. This result is similar to that of previous research on *Synodontis* sp. from different water bodies. Adeyemi (2010, pp. 69–74) and Akombo et al. (2014, pp. 42–48) also observed negative allometry in the growth pattern exhibited by the different species of this family. The LWR of fish, also known as its growth index, is very

important in fishery management as it is used in the estimation of average weight per length group (Abowei, 2010, pp. 16–21). However, the 'b' values obtained in this study fell outside the 2-4 range reported by Bagenal and Tesch (1978).

CONCLUSION

The findings of this study provided baseline information on some biological aspects of this species in the lower Ogun River. It also confirmed that the fish species was benthic, omnivorous and euryphagous in nature although the species can fit into different trophic levels in the food chain. It also affirmed that the *S. schall* in the Ogun River were positively correlated with the 'b' values, indicating a negative allometric growth pattern.

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REFERENCES

Abowei J. F. N. (2010). Condition factor, length-weight relationship and abundance of Elops senegalensis (Regan, 1909) from Nkoro River, Niger Delta, Nigeria. *Advance Journal of Food Science and Technology*, *2*(1), 16–21. ISSN: 2042-4876

Adebisi A. A. (1981). The physicochemical properties and hydrology of a tropical seasonal river-Upper Ogun River. *Hydrobiologia*, *79*(2), 157–165. http://dx.doi.org/10.1007/BF00006124

Adeyemi S. O. (2010). Food and feeding habits of *Protopterus annectens* (Owen) (Lungfish) at the Idah Area of River Niger, Nigeria. *PAT*, *6*(2), 69–74. ISSN: 0794-5213

Akombo P. M., Akanga E. T., Adikwu I. A., & Araoye P. A. (2014). Length-weight relationship, condition factor and feeding habits of *Synodontis schall* (Bloch & Schneider, 1801) in the river Benue at Makurdi, Nigeria. *International Journal of Fisheries Aquatic Studies*, *1*(3), 42–48.

Atama, C. I., Okeke, O. C., Ekeh, F. N., Ezenwaji, N. E., Onah, I. E., Ivoke, N., ... & Eyo, J. E. (2013). Length-weight relationship and condition factor of six Cichlid (Cichilidae: Perciformis) species of the Anambra River, Nigeria. *Journal of Fisheries and Aquaculture*, *4*(2), 82–86. ISSN: 0976-9927 & E-ISSN: 0976-9935

Ayoade, A. A., Sowunmi, A. A., & Nwachukwu, H. I. (2004). Gill asymmetry in *Labeo ogunensis* from the Ogun River, Southwest Nigeria. *Revista Biological Tropical*, *52*(1), 171–175. doi: http://dx.doi.org/10.15517/rbt.v52i1.14821

Bagenal, T. B. (1978). *Methods of the assessment of fish production in fresh waters*. Oxford: Blackwell Scientific Publications.

Bagenal, T. B., & Tesch, A. T. (1978). *Conditions and growth patterns in fresh water habitats*. Oxford: Blackwell Scientific Publications.

Hassan, A. A. (2007). *Studies on population dynamics of two fresh water fish species: Synodontis schall and Mormyrus kannume from the Nile at Assiut, Egypt*. (M.Sc. Thesis). Assiut University, Assiut, Egypt.

Holden, M., & Reed, W. (1972). *West Africa Freshwater Fish*. London: Longman International Education.

Lopez-Peralta, R. H., & Arcila, C. A. T. (2002). Diet composition of fish species from the Southern Continental shelf of Colombia. *Naga World Fish Cent. Quarterly*, *25*(3-4), 23–29.

Olatunde, A. A. (1978). The food and feeding habits of *Eutropius niloticus* (Ruppell), Family Schilbeidae (Osteichthyes: Siluriformes) in Lake Kianji, Nigeria. *Hydrobiologia*, *57*(3), 197–207.

Olojo, E. A. A., Olurin, K. B., & Osikoya, O. J. (2003). Food and feeding habits of *Synodontis nigrita* from the Osun River, SW Nigeria. *NAGA, WorldFish Center Quarterly*, *26*(4), 21–24.

Oscoz, J., Campos, F., & Escala, M. C. (2005). Weight-length relationships of some fish species of the Iberian Peninsula. *Journal of Applied Ichthyology*, *21*(1), 73–74.

Reed, W. J., Burchard, A. J., Hopson, J., & Yaro, I. (1967). *Fish and fisheries of Northern Nigeria*. Northern Nigeria: Ministry of Agriculture.

Shinkafi, B. A., Argungu, L. A., & Akanbi, H. S. (2010). Food and feeding habits of catfish (*Synodontis nigrita* Cuvier and Valenciennes) in River Rima, Sokoto, Nigeria. *Nigeria Journal of Basic and Applied Sciences, 18*(2), 304–307.

Soyinka, O. O., & Ebigbo C. H. (2012). Species diversity and growth pattern of the fish fauna of Epe Lagoon, Nigeria**.** *Journal of Fisheries and Aquatic Science, 7*(6), 392–401.

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Comparative Evaluation of Growth Functions in Three Broiler Strains of Nigerian Chickens

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ABSTRACT

This study was conducted to compare five growth functions (Brody [B), Logistic [L], Gompertz [G], Von Bertalanffy [VB] and Richards [R]) in describing the body weight changes across age in three broiler ecotypes. Each chick was wing-tagged at day old and weighed on a weekly basis up to 10 weeks of age. Aforementioned non-linear growth models were fitted to appraise age-body weight relationship using procedure NLIN of the S.A.S (Version 9.1). G, L and VB functions converged with a low number of iterations ranging from 8 to 33 in Marshal, 7 to 10 in Naked Neck and 5 to 9 in Normal Feathered chickens. VB had the highest number of iterations (33, 10 and 9) for Marshal, Naked Neck and Normal Feathered chickens, respectively. The G, VB and L models fitted the growth curves of all the chicken ecotypes very well, and the fitting degrees R^2 were all above 99.89. Based on all the criteria used for comparing these models in the three ecotypes, it can be established that the L function gave the best fit for the age-body relationship although G and VB functions were equally good in predicting the growth curves of the chickens. B and R functions were not good in fitting chicken growth data in this study with respect to parameter estimates, convergence criteria and p values.

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INTRODUCTION

Broilers are strains of chickens used for the purpose of producing a huge quantity of chicken meat in a short period. Broiler chickens are raised from six to 10 weeks

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in poultry farms in Nigeria. Capability to raise big broilers in a short period requires only a small amount of money, thereby creating cheap and nutritious meat for the populace. The growth process of these broiler chickens with respect to parameters having a genetic interpretation is explained using growth curves. These parameters allow biological growth processes to be explained. Selection for growth rate can modify these parameters (Blasco et al., 2003). Selection on this growth curve could aid genetic gain on body weight of broiler chickens. Growth curve parameters describe the age-body weight relationship in chickens, and these traits are heritable (Mignon-Grasteau et al., 1999).

The contributions of research to the poultry industry in past years have led to the production of bigger chickens today. Gueye (1998) reported that traditional chickens had contributed a substantial amount to meat production (25-70%) and eggs (12-36%), and accounted for approximately 80% of the total chicken population in sub-Saharan Africa. Some Nigerian indigenous chickens have been genetically selected over time and they are about double the size of the strains (strains not subjected to genetic selection) that are scavengers in rural areas. Many reports are available on growth curves in livestock such as cattle (Brown et al., 1976; Kratochvilova et al., 2002), rabbit (Blasco et al., 2003), turkey (Seng & Küraz, 2005), chicken (Mignon-Grasteau et al., 2000; Norris et al., 2007). However, there is little information available on the evaluation and analysis of growth curves of Nigerian locally adapted broiler chickens. Growth curves, like other traits that are necessary for development, are important for the understanding and design of breeding plans because they change in response to selection (Gwaze et al., 2002).

Non-linear models have been used broadly to describe variations in body weight with age, so the genetic potential of chickens for growth can be assessed. A number of non-linear models have been compared and used to evaluate the growth curves of different chicken breeds (Stephan et al., 1987; Knizetova et al., 1991, 1995; Roush & Branton, 2005). These models include Brody (Brody, 1945), Gompertz (Winsor, 1932), Von Bertalanffy (Von Bertalanffy, 1957), Logistic (Nelder, 1961) and Richards (Richards, 1959). An appropriate growth function, therefore, summarises this information provided by observation on chickens and mathematically expresses its life time growth course (Kratochvilova et al., 2002). The aim of this study was to estimate growth curve parameters using different growth models to determine the age-body weight relationships of three chicken ecotypes.

MATERIALS AND METHOD

The research was carried out at the Poultry Breeding Unit of the Federal University of Agriculture, Abeokuta, (FUNAAB) located in latitude 7˚10 N in Odeda Local Government Area, Ogun State, South-Western Nigeria. The ambient temperature during the period ranged from 26.9˚C in June to 27.1˚C in December with an average relative humidity of 80%, while the

vegetative site was an inter-phase between the tropical rainforest and the derived savannah (AGROMET, FUNAAB, 2015).

Experimental Animals and Their Management

One hundred and twenty broilers (40 per ecotype) from a commercial strain (Marshal) and two Nigerian indigenous chicken ecotypes (Normal-Feathered and Naked Neck) generated from a hatchery in Abeokuta were used for the study. The chicks were raised for 10 weeks and fed *ad libitum* on a broiler starter diet from day-old to 4 weeks old and a broiler finisher diet for the remaining weeks. All the necessary vaccines for broiler chicks were administered at the appropriate ages. The chickens were wing-banded at 1 day of age and the body weight of the birds was recorded on a weekly basis up to 10 weeks of age. Widely used non-linear growth models, Brody, Gompertz, Logistic, Von Bertalanffy and Richards, were fit to estimate the mean age-body weight relationship using procedures (NLIN) of the S.A.S (Version 9.1). To examine the accuracy of the model used, the fitting criteria were coefficient of determination (R^2) and standard error of prediction. Coefficient of determination (R^2) accounts for amount of the total variation due to the explanatory variable. The models were given as follows:

Gompertz: $y_t = Ae^{-b\exp(-kt)} + \varepsilon_t$

Logistic: $y_t = A/(1 + e^{-kt}) + \varepsilon_t$

Brody: $y_t = A(1 - be^{-kt}) + \varepsilon_t$

Richards: $y_t = A(1 - be^{-kt})^m + \varepsilon_t$

Von Bertalanffy: $y_t = A(1 - be^{-kt})^3 + \varepsilon_t$

where y_t represented the weight of the animal at a given age (t); parameter A was the asymptotic weight, if $t \rightarrow \infty$; when the adult weight of the animal was not reached, this reflected in an estimate of the weight of the last weighing; b was a constant without biological interpretation, but it was important to model the sigmoidal format of the growth curve from birth (t=0) up to the adult age of the animal ($t \rightarrow \infty$); K was the maturity index, which expressed the ratio of the maximum growth rate in relation to the adult size, where lower k values indicated delayed maturity and higher k values indicated accelerated maturity; M was the parameter that shaped the curve; e was the natural base logarithm; the L parameter had no biological meaning, but together with K constituted b, which had the function of modelling the sigmoidal curve; and ε represented the residue error associated with each weighing.

RESULTS AND DISCUSSION

The fitting of the Brody, Gompertz, Logistic, Von Bertalanffy and Richards functions offered no computational difficulty for any of the three chicken ecotypes considered in terms of computational time and convergence as these three curves converged to solutions at a short time interval for the three chicken ecotypes. In the Brody and Richards curves, convergence solutions were not attained for all the chicken ecotypes. The Gompertz, Logistic and Von Bertalanffy functions achieved convergence with a low number of iterations ranging from 8

to 33 in Marshal, 7 to 10 in Naked Neck and 5 to 9 in Normal Feathered chickens. The Von Bertalanffy function had the highest number of iterations (33, 10 and 9) for Marshal, Naked Neck and Normal Feathered chickens, respectively. This lack of convergence in all ecotypes for the Brody and Richards functions indicated lack of usefulness of the functions (Lopez de Torre et al., 1992) in this study because the models showed inadequacy in fitting all the growth data reasonably.

The means and their standard errors for the parameters estimated were used as a basis for the comparison of the models in all the chicken ecotypes. The means and their standard errors for the parameters estimated for the growth constant of each function in Marshal chickens are shown in Table 1. Average mature weight (A) values from the Logistic function was the closest to the observed values, followed by the Von Bertalanffy then the Gompertz functions. The Brody function overestimated A, while the Richards function underestimated A. The value of *b* for the Marshal ecotype ranged from -1.0377 to 29.7607. The highest value of *k* was observed in the Gompertz function (0.3930). The larger estimates of A were generally more associated with smaller estimates of *k* in the Brody function than were found in other models. The results of this study tend to corroborate the views and observations of Brown et al. (1976) that a non-positive correlation between *k* and A implies that early maturing animals tend to grow to a lower mature weight. The Gompertz, Logistic and Von Bertalanffy functions were superior to the Richards and Brody functions in terms of the values of the coefficient of determination (R^2), standard errors of the estimated parameters, convergence criteria and *p* values.

Table 1
Means and standard errors of the parameter estimates and coefficient of determination of five growth models fit for Marshall chickens

Model	Parameters	Mean	SE	R^2 (%)	Convergence	*p* value
Brody	A	10927.3	710274.0	66.91	Not converged	Not significant (0.05)
	b	0.997	0.148			
	k	0.006	0.407			
Gompertz	A	4656.4	546.2	99.90	Converged	Significant (0.0001)
	b	4.657	0.0673			
	k	0.121	0.0007			
Logistic	A	1823.4	173.4	99.96	Converged	Significant (0.0001)
	b	29.7607	2.3952			
	k	0.3930	0.026			
Von Bertalanffy	A	3566.53	1966.78	99.99	converged	Significant (0.0001)
	b	1.8984	0.0157			
	k	0.277	0.0068			
Richards	A	1.2681	30434.5	52.65	Not converged	Significant (0.05)
	b	-1.0377	188552			
	k	-2.5822	341868			
	m	0.1283	16987.9			

SE=standard error, R^2= coefficient of determination, A=Asymptotic weight, *b*=integration constant, *k*=maturity rate, *m*=point of inflection

Table 2 shows the growth curve parameters and their R^2 values for Naked Neck chickens. All the models had considerably high values of R^2 while the Von Bertalanffy function had the highest values for R^2 (99.98), followed by the Gompertz and Logistic functions that shared the same value of R^2 (99.97). The high R^2 for the models were close to unity and indicated a good overall measure of fitness (Lopez et al., 2000). Also, the Gompertz, Logistic and Von Bertalanffy functions were superior to the Richards and Brody functions in terms of the values of coefficient of determination, standard errors of the estimated parameters, convergence criteria and *p* values. When comparing the Gompertz, Von Bertalanffy and Logistic models whose *p* values were highly significant and converged within a short time, the Logistic function was preferable in terms of closeness of average mature weight and its standard error to the observed values.

Table 2
Means and standard errors of the parameter estimates and coefficient of determination of five growth models fit for Naked Neck chickens

Model	Parameters	Mean	SE	R^2 (%)	Convergence	*p* value
Brody	A	8848.7	338863	85.15	Not converged	Not significant (0.05)
	b	0.9975	0.0909			
	k	0.0053	0.2097			
Gompertz	A	1206.00	128.6	99.97	Converged	Significant (0.0001)
	b	3.4796	0.0894			
	k	0.1712	0.0162			
Logistic	A	810.6	67.2784	99.97	Converged	Significant (0.0001)
	b	15.4213	1.9833			
	k	0.4056	0.0396			
Von Bertalanffy	A	1885.9	363.9	99.98	Converged	Significant (0.0001)
	b	0.7428	0.0106			
	k	0.0905	0.0139			
Richards	A	361.6	119261	70.36	Not converged	Significant (0.05)
	b	0.0379	3669.5			
	k	0.055	162.6			
	m	7.6693	749304			

SE=standard error, R^2=coefficient of determination, A=Asymptotic weight, b=integration constant, k=maturity rate, m=point of inflection

Table 3 shows the growth function parameters and their R^2 values for Normal Feathered chickens. All the models had considerably high values of R^2 while the Gompertz model had the highest values for R^2 (99.99), followed by the Von Bertalanffy model (99.97). The Gompertz, Von Bertalanffy and Logistic models fit the growth curves of the three chicken ecotypes very well, and the fitting degree values, R^2, were all above 99.89. This is in consonance with reports of Eleroglu et al. (2014) and Zhenhua et al. (2015), who observed similar reports with the Gompertz, Von Bertalanffy and Logistic models in slow-growing chicken genotypes and indigenous chicken breeds in China, respectively.

Table 3
Means and standard errors of the parameter estimates and coefficient of determination of five growth models fit for Normal Feathered chickens

Model	Parameters	Means	SE	R^2 (%)	Convergence	*p* value
Brody	A	7791.6	159320	92.38	Not converged	Not significant (0.05)
	b	0.9986	0.0241			
	k	0.0064	0.1361			
Gompertz	A	1232.8	95.4757	99.99	Converged	Significant (0.0001)
	b	3.7746	0.064			
	k	0.1685	0.0108			
Logistic	A	785.2	51.7345	99.95	Converged	Significant (0.0001)
	b	18.8210	1.9498			
	k	0.4172	0.0307			
Von Bertalanffy	A	2159.1	500.5	99.97	Converged	Significant (0.0001)
	b	0.7781	0.0103			
	k	0.0821	0.0139			
Richards	A	841.0	1722.7	81.65	Not converged	Significant (0.05)
	b	-0.0194	21.514			
	k	0.2627	1.8705			
	m	-121.7	133491			

SE=standard error, R^2=coefficient of determination, A=Asymptotic weight, b=integration constant, k=maturity rate, m=point of inflection

General differences in fit of the five models in all the ecotypes are illustrated in Figures 1, 2 and 3. Straight lines observed in Figures 1 and 2 indicated that the Richards model projected the growth data of Naked Neck and Marshall chickens poorly but did better in projecting the growth data of Normal Feathered chickens. The fit of curves obtained from using the Richards and Brody models varied from those of other models over the different time periods. This is an important consideration from the stand point of choosing an appropriate model. Models that yield differences between predicted and actual weight at short intervals are preferred over models that yield deviations at longer intervals.

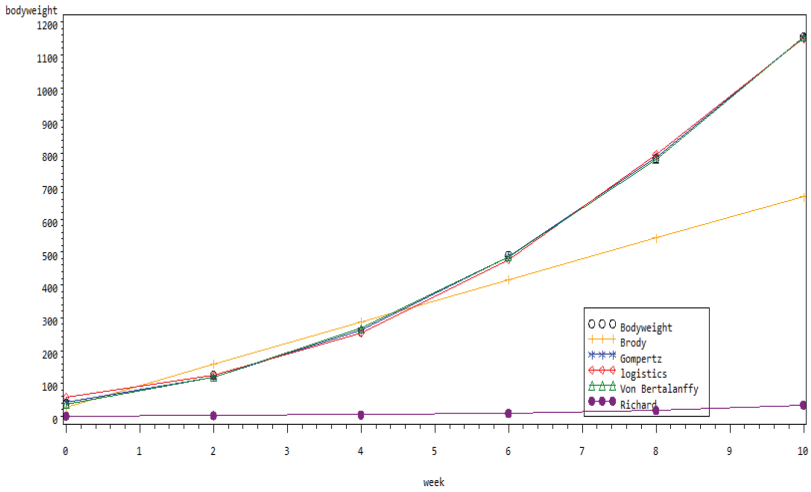

Figure 1. Estimates of growth, in g, of Marshall chicken ecotype according to age, in weeks, obtained by the models Brody, Gompertz, Logistic, Von Bertalanffy and Richards and observed mean weight

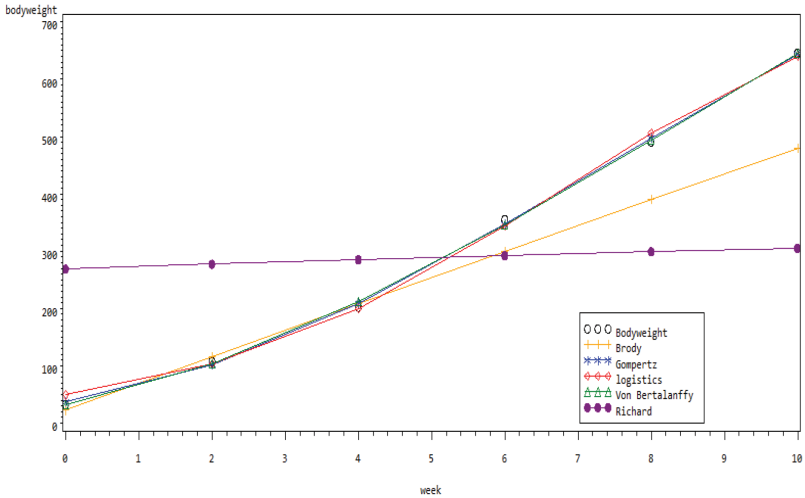

Figure 2. Estimates of growth, in g, of Naked Neck chicken ecotype according to age, in weeks, obtained by the models Brody, Gompertz, Logistic, Von Bertalanffy and Richards and observed mean weight

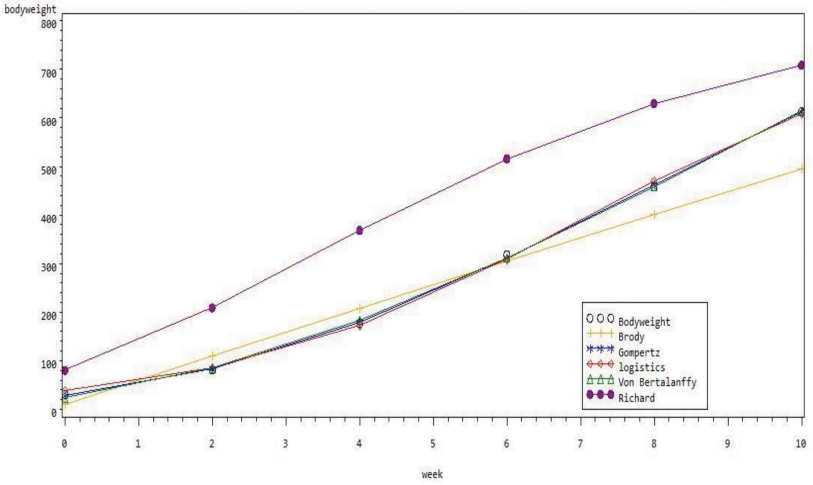

Figure 3. Estimates of growth, in g, of Normal Feathered chicken ecotype according to age, in weeks, obtained by the models Brody, Gompertz, Logistic, Von Bertalanffy and Richards and observed mean weight

CONCLUSION

Based on all the criteria used for comparing these models in the three genetic groups (Marshal, Naked Neck and Normal Feathered chickens), it could be established that the Logistic function used in this study gave the best fit for the data analysed although the Gompertz and Von Bertalanffy functions were equally good in predicting the growth curves of the chickens. The Brody and Richards functions were not suitable for fitting chicken growth data in this study with respect to parameter estimates, convergence criteria and *p* values. Also, based on growth curve parameters, the Marshal genetic group had a better rate of maturing and greater mature weight, followed by Naked Neck and then Normal Feathered chickens. Hence, Naked Neck chickens are recommended over Normal Feathered ecotypes for broiler production in breeding programmes based on the growth curve parameters for the five models considered in this study.

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REFERENCES

AGROMET, FUNAAB, (2015). Department of Water Resource Management and Agricultural Meteorology Federal University of Agriculture, Abeokuta, Nigeria.

Blasco, A., Piles, M., & Varona, L. (2003). A Bayesian analysis of the effect of selection for growth rate on growth curves in rabbits. *Genetics Selection and Evolution, 35*(1), 21–41.

Brody, S. (1945). *Bioenergetics and growth.* New York: Rheinhold Publishing.

Brown, J. E., Fitzhugh, H. A. Jr., & Cartwright, T. C. (1976). A comparison of nonlinear models for describing weight–age relationships in cattle. *Journal of Animal Science, 42*(4), 810–818.

Eleroglu, H., Yildirim, A., Sekeroglu, A., Çoksöyler, F. N., & Duman, M. (2014). Comparison of growth curves by growth models in slow–growing chicken genotypes raised the organic system. *International Journal of Agriculture and Biology, 16*(3), 529–535.

Guèye, E. F. (1998). Village egg and fowl meat production in Africa. *World's Poultry Science Journal, 54,* 73–86.

Gwaze, D. P., Bridgwater, F. E., & Williams, C. G. (2002). Genetic analysis of growth curves for a woody perennial species, Pinus taeda 1. *Theoretical and Applied Genetics, 105*(4), 526–531.

Knizetova, H., Hyanek, J., Hyankova, L., & Belicek, P. (1995). Comparative study of growth curves in poultry. *Genetic Selection and Evolution, 27*(4), 365–375.

Knizetova, H., Hyanek, J., Knize, B., & Roubicek, J. (1991). Analysis of growth curves of fowl I. Chickens. *British Poultry Science, 32*(5), 1027–1038.

Kratochvilova, M., Hyankova, L., Knizetova, H., Fiedler, J., & Urban, F. (2002). Growth curves analysis in cattle from early maturity and mature body size viewpoints. *Czech. Journal Animal Science, 47*(4), 125–132.

Lopez, S., France, J., Gerrits, W. J. J., Dhanoa, M. S., Humphries, D. J. & Dijkstra, J. (2000). A generalized michaelis-menten equation for the analysis of growth. *Journal of Animal Science, 78*(7), 1816–1828.

Lopez De Torre, G., Candotti, J. J., Reverter, A., Bellido, M. M., Vasco, P., Garcia, L. J., & Brinks, J. S. (1992). Effects of growth functions. *Archives Tierzucht, 39,* 121–127.

Mignon-Grasteau, S., Beaumont, C. E., Poivey, J. P., De Rochambeau, H., & Ricard, F. H. (1999). Genetic parameters of growth curve parameters in male and female chickens. *British Poultry Science, 40*(1), 44–51.

Mignon-Grasteau, S., Piles, M., Varona, L., De Rochambeau, H., Poivey, J. P., Blasco, A., & Beaumont, C. (2000). Genetic analysis of growth curve parameters for male and female chickens resulting from selection on shape of growth curve. *Animal Science Journal, 78*(10), 2515–2524.

Nelder, J. A. (1961). The fitting of a generalization of the logistic curve. *Biometrics, 17*(1), 89–110.

Norris, D., Ngambi, J. W., Benyi, K., Makgahlela, M. L., Shimelis, H. A., & Nesamvuni, E. A. (2007). Analysis of growth curves of indigenous male Venda and Naked Neck chickens. *South African Journal of Animal Science, 37*(1), 21–26.

Richards, J. F. (1959). A flexible growth functions for empirical use. *Journal of Experimental Botany, 10*(2), 290–300.

Roush, W. B., & Branton, S. L. (2005). A comparison of fitting growth models with a genetic algorithm and nonlinear regression. *Poultry Science, 84*(3), 494–502.

Seng, T. L., & Küraz, S. (2005). Non-Linear models for growth curves in large white turkeys. *Turkey Journal of Veterinary and Animal Science, 29*(2), 331–337.

Stephan, R. R., Pesti, G. M., & Marks, H. L. (1987). Comparison of three nonlinear regression models for describing broiler growth curves. *Growth, 51*(2), 229–239.

Von Bertalanffy, L. (1957). Quantitative laws in metabolism and growth. *Quarterly Reviews of Biology, 32*(3), 217–231.

Winsor, C. P. (1932). The Gompertz curve as a growth curve. *Proceedings of the national academy of sciences, 18*(1), 1–8.

Zhenhua, Z., Shoufeng, L., Huayun, H., Chunmiao, L., Qianbao, W., & Longgang, X. (2015). Comparative study on growth and developmental model of indigenous chicken breeds in China. *Open Journal of Animal Sciences, 5*(02), 219–223.

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Short-term Heat Exposure Effect on *PSII* Efficiency and Growth of Rice (*Oryza sativa* L.)

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ABSTRACT

An increase in temperature is predicted to have a negative effect on plant growth and development, thereby leading to its loss of yield. Higher air temperature has damaging effects on plant physiology, including its photosynthetic mechanism. Therefore, this study aims to investigate the effects of short-term high temperatures on photosynthesis at PSII, and the growth of rice cultivars at the heading stage. The experiment was carried out with two rice cultivars, namely, PT60 and Dular under 45ºC for 30 minutes. The results showed that panicles and leaf tips of rice cv. PT60 had chlorophyll bleaching, and at day 5 after heat treatment (DAH), the symptoms were very severe. On the other hand, all plant parts of cv. Dular remained green at 5th DAH. Fluorescence parameters in cv. Dular remained unchanged after heat treatment. In contrast, cv. PT60 exhibited a significant decrease in all fluorescence parameters. Also, the electron transport rate in cv. PT60 drastically declined after exposure to heat. The growth of cv. PT60 was inhibited by heat stress as indicated by a slight reduction in plant height, whereas cv. Dular continued growing after heat exposure. Therefore, the cultivar PT60 was susceptible to heat, and the cultivar Dular seemed to be tolerant to heat during the heading stage.

Keywords: High temperature stress, fluorescence parameter, height, rice

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INTRODUCTION

Rice (*Oryza sativa* L.) is one of the main food staple for people worldwide. However, climate change has led to an increase in global temperatures causing a reduction in yields (Baker et al., 1992). An increase in air temperature of 1ºC causes

a 10% reduction in rice yield (Peng et al., 2004). A study showed high temperatures have an effect on phenology, physiology, cellular and molecular response of rice plants (Wahid et al., 2007). Prasad et al. (2011) suggested that high temperatures at night induced the reduction of chlorophyll content to 8% and the photosynthetic rate to 22%. When the temperature is increased by approximately 10–15^{o}C above the normal growth temperature, photosynthetic pigments change, reducing the rate of photosynthesis . Additionally, the reduction in photosynthesis as a result of exposure to high temperatures leads to photoinhibition of *PSII* and thylakoid disorganisation. Photosystem II is very sensitive to high temperatures compared with other chloroplast functions (Martinazzo et al., 2012; Ambavaram et al., 2014). Thus, high temperatures damage the organelle membranes, leading to the loss of their functions. As a result, it affects cellular permeability and the photosystem embedded in the thylakoid membrane (Martinazzo et al., 2012). High temperatures cause Mn-stabilising proteins in the reaction centre of *PSII* to break down, which damage its D1 and D2 proteins (Wahid et al., 2007). Chlorophyll a fluorescence changing and significantly decreased *Fv/Fm* is an indicator of *PSII* efficiency (Brestic & Zivcak, 2013). Stress from high temperatures affects plant growth and development. Additionally, high temperatures can have a greater effect on the reproductive stage in rice plants by decreasing pollination, increasing sterility and decreasing the pollen viability percentage, thereby decreasing yield (Poli et al., 2013). In this study, the effects of short-term heat exposure on growth and photochemical efficiency of *PSII* were investigated in two rice cultivars differing in responses to heat stress to understand heat tolerance mechanisms of rice.

MATERIALS AND METHODS

Plant materials and stress conditions

Seeds of two rice cvs. Dular and PT60 were surface sterilised with 70% C_2H_5OH (ethanol) for 1 minute, followed by 5% NaOCl (sodium hypochlorite) for 5 minutes to protect against fungal invasiveness, and then rinsed with distilled water three times. Sterilised seeds were soaked with distilled water for 24 hours and placed on wet paper until the emergence of the shoot and radical. Seedlings were transferred to pots which contained plain soil and grown in a greenhouse at the Faculty of Agriculture, Khon Kaen University, Thailand. At the heading stage, rice plants were exposed to temperatures between 25^{o}C and 45^{o}C in a temperature-controlled chamber. After 30-minute exposure at 45^{o}C, the temperature was cooled down to 25^{o}C and then the rice plants were returned to the greenhouse. The *PSII* efficiency and growth measurements were performed at 0 (before heat exposure), 1, 2, 3, 4 and 5 days after heat exposure (DAH). Flag leaf of the same plant was used to measure *PSII* efficiency.

Determination of *PSII* efficiency

Chlorophyll fluorescence parameters were investigated before and after the plants were treated with high temperatures until the rice plants showed severe symptoms. A PAM fluorometer (Mini-PAM, Walz, Effeltrich, Germany) was used for chlorophyll fluorescence measurements. Fluorescence parameters were investigated from 8.00 to 11.00 am: basic fluorescence in the dark-adapted state (*Fo*), steady-state fluorescence under natural irradiation (*Fs*), the maximal fluorescence in the dark-adapted state (*Fm*), and the maximal fluorescence under natural irradiation (*Fm*). The maximal quantum yield of *PSII* photochemistry (*Fv/Fm*) and effective quantum yield of *PSII* photochemistry (*ΔF/Fm*) were calculated based on Schreiber (2004).

Determination of plant growth

The height of rice cvs. Dular and PT60 were measured before and after treatment with high temperatures until the rice plants showed severe symptoms. The height of rice plants was measured from the above ground surface to the tip of the flag leaf.

Statistical analysis

The experiment was a completely randomised design (CRD) with four replications. Difference of photosynthetic and growth data between the two rice cultivars were statistically analysed by using t-test method with SPSS window version 11.0.

RESULTS AND DISCUSSIONS

Plant characteristics exposed to heat were examined after a 30-min heat treatment (45°C). Two previous studies have reported that rice cv. Dular showed heat tolerance during the reproductive stage (Tenorio et al., 2013; Manigbas et al., 2014). Figure 1 shows heat tolerance in all plant parts, and rice cv. Dular remained green after short-term heat treatment (45°C, 30 min). In contrast, heat stress symptoms were exhibited in rice cv. PT60. Chlorophyll bleaching was found in some parts of the panicles and some leaf tips of rice cv. PT60 after a 30-min heat treatment. Additionally, rice cv. PT60 showed leaf wilting and drying symptoms (Figure 1). Moreover, severe symptoms developed after 5 DAH in cv. PT60. In contrast to cv. PT60, cv. Dular did not show any symptoms.

Figure 1. The photographic view of rice cvs. Dular (A) and PT60 (B) exposed to high temperature at 45°C for 30 min

In general, the reproductive stage of rice was affected by elevated temperatures. Booting and flowering are the most heat-sensitive stages of rice growth (Tenerio et al., 2013). Plant physiological processes such as photosynthesis are the most sensitive to high temperature stress (Yin et al., 2010). An air temperature higher than 35°C reduced rice photosynthesis by 50% (Restrepo-Diaz & Garces-Vanron, 2013). High temperature stress influences rubisco activity, leaf chlorophyll content, maximum quantum yield of *PSII* efficiency (*Fv/Fm*), effective quantum yield of *PSII* efficiency (*ΔF/Fm'*) and non-photochemical quenching in rice plants (Cao et al., 2009; Yin et al., 2010). Chlorophyll fluorescence is light re-emitted by chlorophyll molecules during the return from an excited state to a ground state. This is useful for determining photosynthetic performance and as a non-invasive tool for studying plant responses to environmental stress (Sayed, 2003; Baker, 2008; Murchie and Lawson, 2013), including low and high temperature stress.

In the present study, chlorophyll fluorescence parameters including *Fv/Fm, ΔF/Fm', Fo, Fm, Fs and Fm',* were measured before and after exposure to heat treatment in rice cvs. Dular and PT60. Figures 2 and 3 show that all chlorophyll fluorescence parameters in rice cv. Dular remained unchanged after treatment with high temperature stress. Nevertheless, rice cv. PT60 showed a significant decline in all fluorescence parameters. The value of *Fv/Fm* in cv. Dular was in the range of 0.73–0.81. However, rice cv. PT60 exhibited *Fv/Fm* of 0.8 before heat treatment, but after heat treatment, the *Fv/Fm* values were drastically decreased within the range of 0.00–0.15. *Fv/Fm* is useful for estimating the maximum quantum yield of *PSII* photochemistry. *Fv/Fm* value of ~0.83 is markedly considered for non-stressed leaves (Baker, 2008; Murchie and Lawson, 2013). Thus, we suggested that the leaves of rice cv. PT60 were severely affected by heat exposure at 45°C for 30 min.

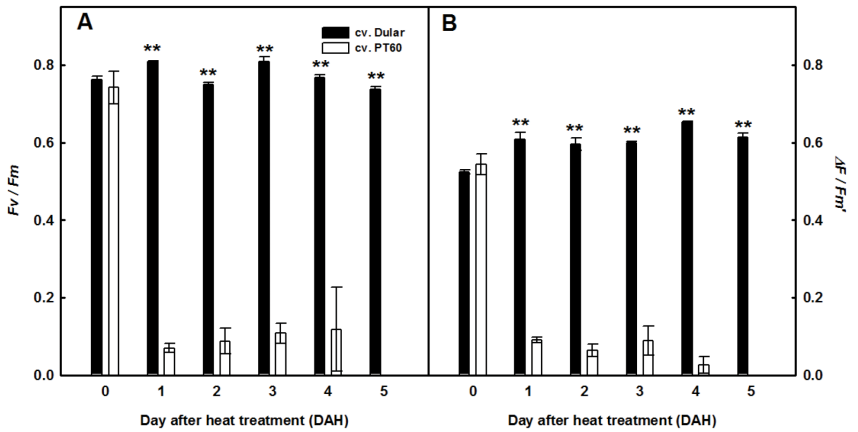

Figure 2. Fv/Fm (A) and *ΔF/Fm* (B) in rice cvs. Dular and PT60 at 0, 1, 2, 3, 4 and 5 DAH. The values are means ± SE (n=4). ** represents the means significantly different at p≤0.05

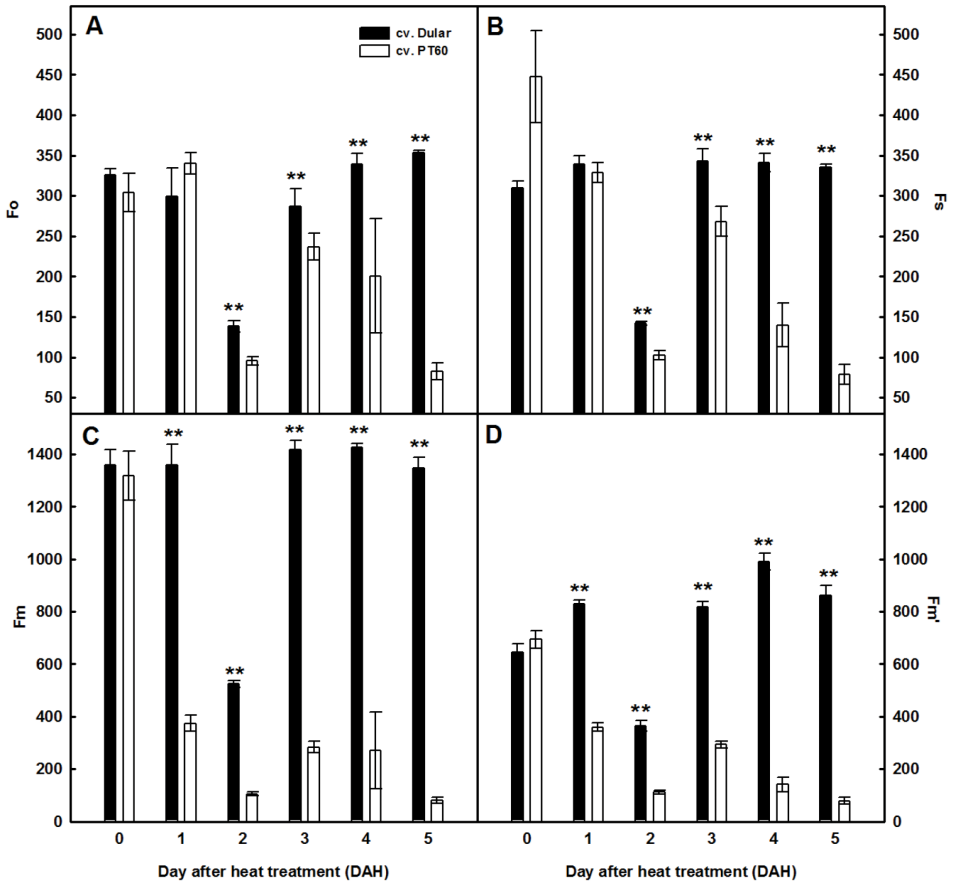

Figure 3. *Fo* (A), *Fs* (B), *Fm* (C) and *Fm* (D) in rice cvs. Dular and PT60 at 0, 1, 2, 3, 4 and 5 DAH. The values are means ± SE (n=4). ** represented the means significantly different at $p \leq 0.05$

Electron transport rate values of rice cv. PT60 markedly declined after heat treatment (Figure 4). On the other hand, cv. Dular exhibited an increasing trend in ETR values. At fifth day after heat treatment, the ETR value of cv. Dular was approximately 414.44 ± 6.33 µmol $m^{-2}s^{-1}$, in contrast to cv. PT60 in which ETR values fell to zero. The ETR can be used as an estimator of the relative photosynthetic electron transport rate (Schreiber et al., 2011) and is calculated using the *ΔF/Fm'* values, which reflect the noncyclic electron transport rate through *PSII* (Baker, 2008). This therefore suggests that the photosynthetic apparatus of cv. Dular is highly resistant to heat stress, and electron transport activities are able to completely recover 5 days after heat treatment. In contrast, rice cv. PT60 is highly sensitive to heat stress that the photosynthetic components were severely

damaged, leading to a drastic reduction in *PSII* efficiency and electron transport activity. Additionally, the plant height of cv. PT60 was affected by heat stress, but cv. Dular still had an increase in height after heat treatment.

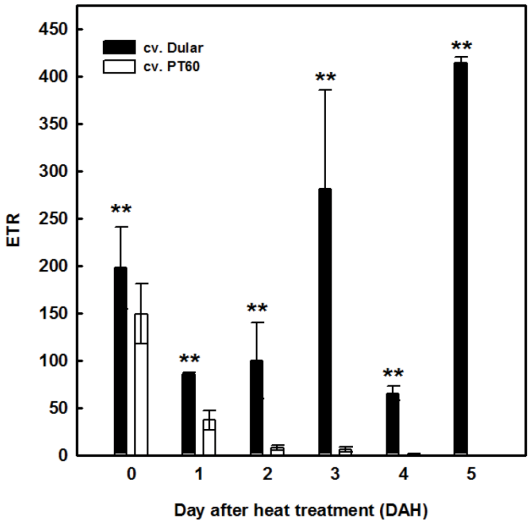

Figure 4. ETR in rice cvs. Dular and PT60 at 0, 1, 2, 3, 4 and 5 DAH. The values are means ± SE (n=4). ** represented the means significantly different at p≤0.05

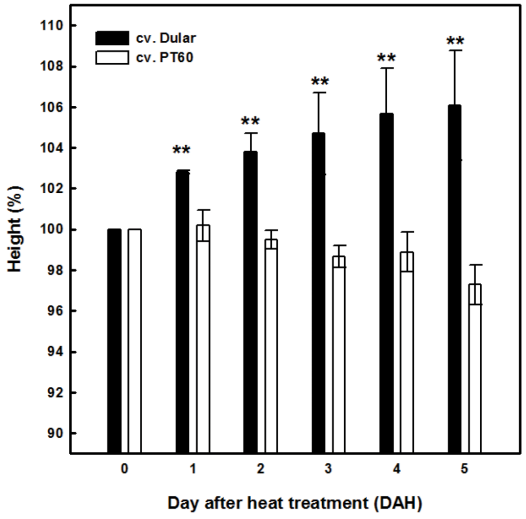

Figure 5. Height in rice cvs. Dular and PT60 before and after heat exposure. The values are means ± SE (n=4). ** represented the means significantly different at p≤0.05

CONCLUSIONS

This experiment showed that short-term high temperature stress during the heading stage affected leaf photosynthesis of *PSII* efficiency. PT60 was susceptible to high temperatures as indicated by the bleaching of chlorophyll, decrease in quantum yield of *PSII* efficiency and lack of growth, whereas the cultivar Dular was high temperature tolerant, as indicated by its green colour and high photosynthetic efficiency of *PSII*.

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REFERENCES

Ambavaram, M. M. R., Basu, S., Krishnan, A., Ramegowda, V., Batlang, Rahman, L., Baisakh, N., & Pereira, A. (2014). Coordinated regulation of photosynthesis in rice increases yield and tolerance to environmental stress. *Nature communication, 5*, 1-14. doi: 10.1038/ncomms6302.

Baker, J. T., Allen Jr, L. H., & Boote, K. T. (1992). Response of rice to Carbon dioxide and temperature. *Agricultural and forest meteorology*, *60*(3-4), 153-166.

Baker, N. R. (2008). Chlorophyll Fluorescence: A Probe of Photosynthesis *In Vivo*. *Annual Review of Plant Biology*, *59*, 89-113.

Berry, J. A., & Raison, J. K. (1981). Responses of macrophytes to temperature. In O. L. Lange, P. S. Nobel, C. B. Osmond & H. Ziegler (Eds.), *Encyclopedia of Plant Physiology*. Berline, Heidelberg, New York: Springer.

Brestic, M., & Zivcak, M. (2013). PSII fluorescence techniques for measurement of drought and high temperature stress signal in crop plants: protocols and applications. In *Molecular stress physiology of plants* (pp. 87-131). India: Springer.

Cao, Y. Y., Duan, H., Yang, L. N., Wang, Z. Q., Liu, L. J., & Yang, J. C. (2009). Effect of high temperature during heading and early filling on grain yield and physiological characteristics in Indica Rice. *Acta Agronomica Sinica, 35*(3), 512–521.

Manigbas, N. L., Lambio, L. A. F., Madrid, L. B., & Cardenas, C. C. (2014). Germplasm Innovation of heat tolerance in rice irrigated lowland conditions in Philippines. *Rice science*, *21*(3), 162-169.

Martinazzo, E. G., Ramm, A., & Bacarin, M. A. (2012). The chlorophyll fluorescence as an indicator of the temperature stress in the leaves of *Prunus persica*. *Brazilian society of plant physiology, 24*(4), 237-246.

Murchie, E. H., & Lawson, T. (2013). Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *Journal of Experimental Botany*, *64*(13), 3983-3998.

Prasad, P. V. V., Pisipati, S. R., Momčilović, I., & Ristic, Z. (2011). Independent and combined effects of high temperature and drought stress during grain filling on plant yield and chloroplast EF-Tu Expression in spring wheat. *Journal of Agronomy Crop Science*. *197*(6), 430-441.

Peng, S., Huang, J., Sheehy, J. E., Laza, R. C., Visperas, R. M., Zhong, X., Centeno, G. S., Khush, G. S., & Cassman, K. G. (2004). Rice yields decline with higher night temperature from global warming. *Proceeding of the international academy of sciences of the United State of America*, *101*(27), 9971-9975.

Poli, Y., Basava, R. K., Panigrahy, M., Vinukonda, V. P., Dokula, N. R., Voleti, S. R., Desiraju, S., & Neelamraju, S. (2013). Characterization of a Nagina22 rice mutant for heat tolerance and mapping of yield traits. *Rice*, *6*(36), 1-9.

Restrepo-Diaz, H., & Garces-Varon, G. (2013). Response of rice plants to heat stress during initiation of panicle primordia or grain-filling phases. *Journal of Stress Physiology & Biochemistry*, *9*(3), 318-325.

Sayed, O. H. (2003). Chlorophyll fluorescence as a tool in cereal crop research. *Photosynthetica*, *41*(3), 321-330.

Schreiber, U. (2004). Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method: An overview. In G. Papageorgiou & C. Govindjee (Eds.), *Chlorophyll a Fluorescence. A Signature of Photosynthesis: Advances in Photosynthesis and Respiration* (Vols 19). Dordrecht, Netherlands: Springer.

Schreiber, U., Klughammer, C., & JörgKolbowski, J. (2011). High-end chlorophyll fluorescence analysis with the MULTI-COLOR-PAM. I. Various light qualities and their applications. *PAM Application Notes*, *1*, 1-12.

Singh, A. K., & Singhal, G. S. (1999). Specific degradation of D1 protein during exposure of thylakoid membranes to high temperature in the dark. *Photosynthetica*, *36*(3), 433-440.

Taniyama, T., Subbaiah, S. V., Rao, M. L. N., & Ikeda, K. (1988). Cultivation and Ecophysiology of Rice Plants in the Tropics, 3: Photosynthesis of Rice Cultivars of India, Measured by the Tsuno Simple Method. *Japanese Journal of Crop Science*, *57*(1), 184-190.

Tornolio, F. R., Ye, C., Redonas, E., Sierra, S., Laza, M., & Argayoso, M. A. (2013). Screening rice genetic resources for heat tolerance. *SABROU journal of breeding and genetics*, *45*(3), 371-381.

Wahid, A., Gelani, S., Ashraf, M., & Foolad, M. R. (2007). Heat tolerance in plants, An Overview. *Environmental and experimental botany*, *61*(3), 193-223.

Yin, Y., Li, S., Liao, W., Lu, Q., Wen, X., & Lu, C. (2010). Photosystem II photochemistry, photoinhibition, and the xanthophyll cycle in heat-stressed rice leaves. *Journal of Plant Physiology*, *167*(12), 959-966.

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Pleurotus Ostreatus: Its Effect on Carcass, Serum Metabolites and Meat Lipoprotein Content of Broiler Chickens

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ABSTRACT

High demand by consumers for poultry meat and products devoid of residue of antibiotics and micro-organisms has been the drive for recent research into the Nigerian poultry industry. This study was carried out to investigate the potential effect of *Pleurotus ostreatus* (oyster mushroom) on carcass, blood and meat cholesterol of broiler chickens. A total of 90 two-week old broiler chickens were randomly allotted to three levels of administration of ethanolic extract of *Pleurotus ostreatus*: control (0 ppm), 7.5 ppm and 15 ppm. Data obtained on carcass characteristics, serum metabolites and meat lipid profiles were subjected to a completely randomised design. Most carcass indices considered were not influenced ($p>0.05$) except those for the legs and breast. Breast meat (%) was highest in 15 ppm group. Meat lipoprotein content was significantly ($p<0.05$) affected by oyster mushroom administration except for triglycerides and very low density lipoprotein (LDL). High density lipoprotein (HDL) was highest in the 15-ppm dosed group, while LDL was lowest in meat obtained from the 15-ppm dosed group. In conclusion, the use of oral administration of *Pleurotus ostreatus* at 15 ppm in water can increase HDL and decrease the concentrations of LDL, VLDL and triglycerides in thigh meat.

Keywords: Carcass characteristics, oyster mushroom, antibiotics, health indices, lipoproteins, growth promoters, blood serum, meat quality

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INTRODUCTION

Antibiotics are growth promoters commonly used in the poultry industry. At present the convention of using antibiotics as growth promoters in the diets of birds

has been proscribed due to fears about their possible residue/filtrate in animal tissue (Dipeolu, 2004; Simon & Baxter, 2006). The prohibition on the use of most synthetic antimicrobial growth promoters (AGP) and their relatively negative residual effects has led to preference for natural ingredients such as oyster mushroom extracts (*Pleurotus ostreatus*) over synthetic antimicrobial growth promoters (SAGP).

Mushrooms have long been cherished as a vital source of bioactive compounds of medicinal value (Breene, 1990). Fungi have a wide range of activities that have found their use in combatting outbreaks of disease (Chang & Buswell, 1996). Mushrooms and their different derivatives contain a variety of active substances like ergothioneine (Dubost et al., 2007) phenolic antioxidants, variegatic acid and dibiviquinone (Kasuga et al., 1995). These biologically active components found in mushroom possess antifungal, antibacterial, antioxidant and antiviral properties (Barros et al., 2009) and have been found admissible as insecticides and nematicides.

The quality of carcass (meat) produced by the poultry industry has a direct consequence on consumers' health. Many consumers, therefore, endeavour to eat more healthy products (Bickel & Wetscherek, 2005) with the supposition that chicken meat has low fat content compared to beef or pork. Numerous attempts have been made to produce meat, especially broilers, free of synthetic drugs, lower in cholesterol and fat content and without antibiotics. This was to prevent drug residue in meat samples and subsequent introduction of emerging antibiotic resistant strains (Saleha et al., 2009). These attempts were made with no priority given to attendant consequences on the performance of the animals.

The poultry industry in Nigeria at present has set no ban on antibiotic usage; however, researchers have taken on the challenge of seeking substitutes that can replace synthetic drugs and also satisfy consumer demand for residue-free products. Currently, plant-based products have been identified as replacements for synthetic drugs because they possess phyto-nutrients with excellent antioxidant and anti-cholesterolemic properties. Of particular interest is mushroom (*Pleurotus ostreatus*). The chemical evaluation of *Pleurotus ostreatus* revealed it to be a biological plant capable of inducing several bio-medicinal functions and activities in animals e.g. immunomodulation, anti-tumour causing, antidiabetic, antibiotic or antiparasitic, hypercholesterolemic, hepatoprotective, antipathogenic, detoxicant and antioxidant (Guo et al., 2004; Wasser, 2010). The present study was, therefore, conducted to determine the possible effect of *Pleurotus ostreatus* extract on carcass yield, serum biochemical and the carcass lipid profile of broiler chickens.

MATERIALS AND METHOD

Area Depiction

The experiment was carried out at the Poultry Unit of the Directorate of University Farms (DUFARMS), Federal University of Agriculture, Abeokuta, Ogun

State, Nigeria located on latitude 7° 15' N, longitude 3° 26' E and 76 m above sea level (Google Earth, 2014).

Experimental Birds and Management

A total of 90 two-week old Arbor Acre broiler chicks were randomly divided into three treatment groups i.e. T1, T2, T3 containing 30 birds per treatment. T1 served as the control group, where the birds were given medication (coccidiostat and antibiotics), while T2 and T3 were administered different levels of oyster mushroom extract in water. Each group was further subdivided into 3 replicates of 10 birds.

Experimental Period

The duration of the experiment was six weeks. The study was carried out between July and September 2015.

Processing of Experimental Materials

Fresh oyster mushroom (*Pleurotus ostreatus*) was purchased and the extraction process was carried out at the Processing Laboratory, Department of Animal Production and Health, Federal University of Agriculture Abeokuta, Ogun State, Nigeria.

Procedure

A fresh sample of oyster mushroom was properly rid of dirt and was subsequently weighed using a digital scale. Both stalks and pliae were rendered into bits and soaked in ethanol to the ratio of 1:2 (w/v). The mushroom was soaked in a determined volume of ethanol for 24 h after which the extract was poured out. Subsequent soaking was done until a clear solution was obtained. The extract was then concentrated using a water bath at 50^{o}C until a semi-liquid solution was obtained.

Details. Antibiotics with scheduled medication and vaccination was given to the birds in the control experimental group (T1) while mushroom (*Pleurotus ostreatus*) extract was given in water i.e. orally to birds in the other two groups (T2, T3) at 7.5 ppm and 15 ppm twice a week throughout the duration of the study.

Diet. The birds were fed *ad lib* from the starting phase i.e. 14-28 days on a commercial starter and a finisher ration from Days 29-56.

Haematological analysis. On the 28th and 56th day of the experiment, blood samples (2 ml each) were collected from three birds per replicate into Ethylene Diamine Tetra-Acetate (EDTA) bottles for haematological analysis. A heparine tube was used for the serum constituents. The blood samples were analysed for Packed Cell Volume (PCV), Red Blood Cells (RBC), White Blood Cells (WBC) and Haemoglobin (Hb). Standard methods were used for haematological parameters (Schalm, 1986), while serum total protein, albumin and globulin were analysed colourimetrically using a diagnostic reagent kit.

Determination of carcass yield. At the end of the experiment, four birds per replicate were randomly selected, weighed,

slaughtered and eviscerated. The dressed weight was determined while cut-up parts such as the head, neck, thigh, legs, drumstick, back, wings and breast were weighed using a digital scale. The organs such as liver, gizzard and heart were also weighed. The averages of weight were calculated and the parts mentioned were expressed as percentages of the live weight (Sogunle et. al., 2009).

Muscle pH. At 20 min post-mortem, the breast muscle pH was determined at a depth of 2.5 cm below the surface using a Model PH-211 meter equipped with a spear electrode.

Meat Cholesterol Profile

A known quantity (50 g) of meat was sampled from the thigh region of dressed carcasses and analysed to determine the amount of total cholesterol, triglyceride, High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL) and Very Low Density Lipoprotein (VLDL).

Determination of Total Cholesterol

Serum cholesterol was determined spectrophotometrically according to the methods of Allain et al. (1974). The reagent was made up of three enzymes, cholesterol esterase (C, E), cholesterol oxidase (CO) and peroxidase (POD) and two substrates, 4-aminoantipyrine (4-AA) and phenol.

Procedure. Three clean test tubes labelled blank (B), standard (S) and test (T) were arranged in a test tube rack. A volume of 10ml of distilled water, standard cholesterol and serum was added to each test tube, respectively. A volume of 1ml of reagent was added to all the test tubes. The reaction mixtures were mixed and incubated for 10 min at room temperature and the absorbance of the sample was read at 550 mm wavelength against the blank.

$$\text{Cholesterol Concentration in Serum } \left(\frac{\text{mg}}{\text{dl}}\right) = \frac{(\text{Absorbance of test} \times \text{Concentration Of STD})}{\text{Absorbance of STD}}$$

Determination of HDL

Three clean test tubes marked and labelled blank (B), standard (S) and test (T) were arranged in a test tube rack. To each of these tubes were added 1.0 ml of working reagent while 0.05 ml distilled water, HDL standard and super nutrient were added to each test tube. The reaction mixtures were incubated at 37^{o}C for 5 min. The absorbance of the standard (Abs. S) and test samples (Abs. T) against the blank were measured within 60 min using a spectrophotometer. Below is the calculation for measuring HDL cholesterol in the samples.

$$\text{HDL cholesterol (mgldl)} = \frac{\text{Abs. T x con. of STD}}{\text{Abs. S}}$$

Determination of LDL-C

Three clean test tubes marked or labelled blank (B), standard (S) and test (T) were arranged in a test tube rack. A volume of 1000 ml of cholesterol reagent was added to each test tube, 50 ml of water, cholesterol stand and super nutrient were added to each test tube. The reaction mixture were incubated for 10 min at 25^{o}C, and the

absorbance of the test (Abs. T) and standard (Abs. S) samples were measured against the blank (B). Below is the calculation for measuring the concentration of cholesterol in the samples.

$$\text{Conc. of cholesterol} = \frac{\text{Abs. T x conc. of STD}}{\text{Abs. S}}$$

Determination of VLDL-Cholesterol

The concentration of very low density lipoprotein (VLDL) cholesterol was calculated by modification of Freidwald's formulae, as shown below.

$$\text{VLDL} - \text{Cholesterol} = \text{triglyceride value divide by 5}$$

Data Analysis

Data obtained were subjected to a completely randomised design and the significant differences among the treatment means were separated using the Dunnett Multiple Comparisons Test contained in the Statistical Analysis Software (SAS) (2009) package.

RESULTS AND DISCUSSION

Effects of *Pleurotus ostreatus* (Oyster Mushroom) Extract at Different Levels on Carcass Yield of Broiler Chicken

The effect of the ethanolic *Pleurotus ostreatus* (oyster mushroom) extract on carcass yield of broiler chickens is presented in Table 1. All parameters considered were not significantly ($p>0.05$) affected. However, the wings, back, thigh and drumstick were numerically higher in the 15-ppm dosed group. The drumstick, a prime cut of carcass that provides the greatest portion of edible meat in broilers (Sogunle et al., 2009), was highest in the 15-ppm group compared to the other treatment groups. This confirmed the report of Guo et al (2003) and Giannenas et al. (2010) that mushroom was capable of enhancing the growth performance of birds, a phenomenon that was reflected in the carcass composition, especially in the meaty cut-parts.

Table 1
Effects of Pleurotus ostreatus *(oyster mushroom) extract on carcass yield of broiler chickens*

Parameter	0 ppm	7.5 ppm	15 ppm	SEM
Live weight (g)	1566.70	1433.30	1466.70	44.44
Carcass (g)	1360.00	1233.30	1363.30	50.16
[1]Cut-up parts (%)				
Head	2.81	2.94	2.92	2.26
Neck	5.02	4.33	4.91	0.77
Wings	8.02	8.26	8.14	8.29
Back	14.36	13.58	14.98	13.65
Legs	5.11[a]	4.40[b]	4.43[b]	0.17
Thigh	9.28	9.21	9.61	0.81
Drumstick	10.64	10.18	10.73	0.28
Breast	17.43[ab]	15.90[b]	18.69[a]	0.58
PH	6.58[b]	6.73[a]	6.60[ab]	0.15
[2]Organ (%)				
Gizzard	2.18	2.57	2.03	0.07
Liver	1.62	1.66	1.78	1.51
Abdominal Fat	0.00	0.00	0.00	0.00
Heart	0.41	0.44	0.45	0.33

[a,b]: means in the same row with different superscript differ significantly ($p<0.05$)
[1,2]: values are expressed as percentage of live weight.

The non-significant effect of *Pleurotus ostreatus* (oyster mushroom) extract on organs of birds in the present study follows asimilar trend of occurrence as found by Guo et al. (2004), who used fungus (Mushroom: *Lentinus edodes* and *Tremella fuciformis*) as alternatives for antibiotics in broilers. The extract exhibited growth promoting capacity as did the antimicrobial growth promoters used in the control group. Alternative growth promoting options of plant origin like turmeric rhizome (Emadi & Kermanshahi, 2006) have been documented to improve carcass quality and cut prime, decrease the abdominal fat pad and increase liver weight and whole goblet weight (Durrani et al., 2006; Dhama et al., 2015). The insignificant effect of oyster mushroom extract on relative weight of organs also aligns with the reports of Denli et al. (2003) and Daneshmad et al. (2012), who used combinations of oyster mushroom and other phytobiotic options.

Effects of *Pleurotus ostreatus* (Oyster Mushroom) Extract at Different Levels on Meat Cholesterol of Broiler Chicken

The effect of *Pleurotus ostreatus* (Oyster mushroom) extracts at different levels of inclusion on the cholesterol of broiler meat is shown in Table 2. All parameters considered were influenced ($p<0.05$) by oyster mushroom administration except triglyceride and VLDL-cholesterol. Total cholesterol was highest ($p<0.05$) in the 15-ppm group, while statistically lower and similar values were obtained in the control and the 7.5-ppm group.

Table 2
Effects of Pleurotus ostreatus *(oyster mushroom) extract at different levels on meat cholesterol of broiler chickens*

Parameter	Control	7.5 ppm	15 ppm	SEM
Total cholesterol (mg/dl)	64.50^{b}	61.00^{b}	74.50^{a}	2.33
Triglyceride (mg/dl)	101.00	112.50	92.50	13.67
High density lipoprotein (mg/dl)	30.30^{b}	27.50^{b}	46.50^{a}	3.01
Low density lipoprotein (mg/dl)	14.25^{a}	11.00^{b}	9.50^{c}	1.40
Very low density lipoprotein (mg/dl)	20.20	22.50	18.50	2.73

^{abc}: means in the same row with different superscript differ significantly ($p<0.05$)

The HDL cholesterol concentration of meat increased as the level of administration of oyster mushroom increased, with the highest value obtained in the 15-ppm group. This increase indicated the ability of oyster mushroom extract in aggregation HDL-c components in meat, favouring its consumption by people with no predisposition to adverse disease conditions, thereby meeting the increasing demand for organically produced food.

The LDL-cholesterol content of meat sampled was lowest in the 15-ppm dosed group, with the highest value obtained in

the control group. The VLDL-cholesterol was numerically lowest in the 15-ppm group although statistically similar to the other treatment groups. This confirms the ability of plant extracts to influence the post-mortem quality of meat, particularly its cholesterol concentration (Wallace et al., 2010).

Effects of *Pleurotus ostreatus* (Oyster Mushroom) Extract on Some Serum Metabolites of Broiler Chickens

All serum metabolites determined presented in Table 3 were not significantly ($p>0.05$) affected except for triglyceride at 28 days of age. There were no marked changes in the concentration of serum for total protein, albumin and globulin for all the groups; however, the percentage increase in albumin was greatest in the control group. Mushroom contains basic antioxidant compounds, namely ascorbic acid, Vitamin C, Vitamin E, β carotene and phenolic compounds (Yang et al., 2002), therefore its usage will enhance the immunity of the birds.

Table 3
Effects of Pleurotus ostreatus *(oyster mushroom) extract on some serum metabolites of broiler chickens*

Parameter		Control	7.5 ppm	15 ppm	SEM
Total Protein (g/dl)	**56 days**	4.95	5.20	5.40	0.13
	28 days	4.60	4.90	5.10	0.21
Albumin (g/dl)	**56 days**	2.95	2.85	2.95	0.12
	28 days	2.30	2.80	2.85	0.22
Globulin (g/dl)	**56 days**	2.00	2.35	2.45	0.11
	28 days	2.30	2.10	2.25	0.07
Cholesterol (mg/dl)	**56 days**	92.00	85.50	82.00	4.10
	28 days	83.50	80.00	85.50	2.88
Triglyceride (mg/dl)	**56 days**	94.00	84.00	87.50	2.57
	28 days	96.00[a]	87.50[c]	107.50[a]	4.03

[abc]: means in the same row with different superscript differ significantly ($p<0.05$)

There was an increase in the serum cholesterol of the control and the 7.5-ppm dosed group (10.18 and 6.43 %, respectively), while a 4.01% reduction was observed in birds administered the highest dosage of 15 ppm in water. Plants and phytochemicals alter cholesterol metabolism in animals (Wallace et al., 2010). *Pleurotus ostreatus* (oyster mushroom) contains phytogenic

substances, the most prominent being phenolic compounds which have hypo-cholesterolemic effect (Ikeda et al., 1992; Daneshyar et al., 2011) and are positively responsible for the reduction observed in serum total cholesterol. The possible pathway to this reduction may be the ability of the extract to inhibit the activity of HMG-CoA reductase in the liver (enzyme responsible for cholesterol synthesis) at the highest level of administration due to lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme, a reductase (HMG-CoA reductase) that interferes with mevalonate production. The concentration of lovastatin was high enough to bind with HMG-KoA reductase; this prevents mevalonat acid, a compound of cholesterol biosynthesis, from forming, an action that inhibits the formation of cholesterol (Cheung et al., 1993).

Triglyceride value (on Day 28) was statistically highest at 15 ppm, which was the least value obtained in the 7.5-ppm group. In addition, the percentage reduction in triglyceride value from Day 28 to 56 was greatest in the 15-ppm group compared to the control and the 7.5-ppm treatment. The depression in serum triglyceride may be due to altering and lowering of the hepatic lipogenesis effect of oyster mushroom extract since it is produced in the liver. It affected the triglyceride aggregation (Daneshyar et al., 2011) in meat sampled from the three treatment groups with least effect (numerically) in the 15-ppm dosed group. Since lipid content and composition of meat can greatly influence its dietetic, sensory and storage attributes (Chartrin et al., 2005), meat samples higher in HDL and lower in both LDL and VLDL are suitable for consumption with no possible predisposition to disease conditions.

CONCLUSION

The results of the study showed that application of *Pleurotus ostreatus* (oyster mushroom) (applied via drinking water at 15 ppm) could be considered a potential natural growth promoter and a cholesterol-lowering agent in producing organically acceptable meat to the populace without adverse effects on the health of people and the health indices of birds.

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REFERENCES

Allain, C. C., Poon, L. S., Chan, C. S., Richmond, W., & Fu, P. C. (1974). Enzymatic determination of total serum cholesterol. *Clinical Chemistry, 20*(4), 470-475.

Barros, L., Venturini, B. A., Baptista, P., Estevinho, L. M., & Ferreira, C. F. R. (2008). Chemical composition and biological properties of Portuguese wild mushrooms: A comprehensive study. *Journal of Agriculture and Food Chemistry, 56*(10), 3856–3862.

Bickel, S., & Wetscherek, W. (2005). Influence of fat source on the performance of broilers and on relevant carcass characteristics for consumers (3rd report): Effects of the use of rape seed oil ad animal fat in poultry fattening on fatty acid quality of grilled broiler meat. *Bodenkultur, 56*(1/4), 39–45.

Breene, W. M. (1990). Nutritional and medicinal value of specialty mushrooms. *Journal of Food Protection, 53*(10), 883–894.

Chang, S. T., & Buswell, J. A. (1996). Mushroom nutriceuticals. *World Journal of Microbiology Biotechnology, 12*(5), 473–476.

Chartrin, P., Schiavone, A., Bernadet, M. D., Guy, G., Mourot, J., Duclos, M. J., & Baeza, E. (2005). Effect of genotype and overfeeding on lipid deposition in myofibres and intramuscular adipocytes of breast and thigh muscles of ducks. *Reproduction Nutrition Development, 45*(1), 87–99.

Daneshmand, A., Sadeghi, G. H., & Karimi, A. (2012). The effects of a combination of garlic, oyster mushroom and propolis extract in comparison to antibiotic on growth performance, some blood parameters and nutrient digestibility of male broilers. *Brazilian Journal of Poultry Science, 14*(2), 141–147.

Denli, M., Okan, F., & Celik, K. (2003). Effect of dietary probiotic, organic acid and antibiotic supplementation. *Pakistan Journal Nutrition, 2*(2), 89–91.

Dharma, K., Latheef, S. K., Mani, S., Samad, H. A., Karthik, K., Tiwari, R., … & Tufarelli, V. (2015). Multiple beneficial applications and modes of action of herbs in poultry health and production. *International Journal of Pharmacology, 11*(3), 152–176.

Dipeolu, M. A. (2004). Problems and prospects of antibiotics residues in meat products in Nigeria. *Vom Journal Veterinary Science, 1*(1), 63–67.

Dubost, N. J., Ou, B., & Beelman, R. B. (2007). Quantification of polyphenols and ergothioneine in cultivated mushrooms and correlation to total antioxidant capacity. *Food Chemistry, 105*(2), 727–735.

Durrani, F. R., Ismail, M., Sultan, A., Suhail, S. M., Chand, N., & Durrani, Z. (2006). Effect of different levels of feed added turmeric (Curcuma longa) on the performance of broiler. *American Journal of Agriculture and Biological Sciences, 1*(2), 9–11.

Emadi, M., & Kermanshahi, H. (2006). Effect of turmeric rhizome powder on performance and carcass characteristics of broiler chickens. *International Journal of Poultry Science, 5*(11), 1069–1072.

Giannenas, I., Pappas, I. S., Mavridis, S., Kontopidis, G., Skoufos, J., & Kyriazakis, I. (2010). Performance and antioxidant status of broiler chickens supplemented with dried mushrooms (*Agaricus bisporus*) in their diet. *Poultry Science, 89*(2), 303–311.

Google Maps. (2014). *Federal University of Agriculture*.Retrieved from https://earth.google.com/web/@7.22330744,3.44033719,137.84884575a,1046.69760578d,35y,100.57030218h,44.99999706t,-0r/data=Cm4abBJmCiUweDEwM2EzN2MwMDA4ZDY4MDk6MHg2Mjc3MWVhYzUyMmQyNzM0GWy0vTOq5BxAIalRGZXRhQtAKitGZWRlcmFsIFVuaXZlcnNpdHkgb2YKQWdyaWN1bHR1cmUsIEFiZW9rdXRhGAIgAQ.

Guo, F. C., Savelkoul, H. F. J., Kwakkel, R. P., Williams, B. A., & Verstegen, M. W. A. (2003). Immunoactive, medicinal properties of mushroom and herb polysaccharides and their potential use in chicken diets. *World's Poultry Science Journal, 59*(4), 427–440.

Guo, F. C., Williams, B. A., Kwakkel, R. P., Li, H. S., Li, X. P., Luo, J. Y., … & Verstegen, M. W. A. (2004). Effects of mushroom and herb polysaccharides, as alternatives for an antibiotic, on the cecal microbial ecosystem in broiler chickens. *Poultry Science, 83*(2), 175–182.

Ikeda, I., Imasato, Y., Sasaki, E., Nakayama, M., Nagao, H., Takeo, T., … & Sugano, M. (1992). Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. *Biochimica et Biophysica Acta, 1127*(2), 141–146.

Kasuga, A., Aoyagi, Y., & Sugahara, T. (1995). Antioxidant activity of fungus Suihs bovinus (L: Fr.) O. Kuntze. *Journal of Food Science, 60*(5), 1113–1115.

Saleha, A. A., Myaing, T. T., Ganapathy, K. K., Zulkifli, I., Raha, R., & Arifah K. (2009). Possible effect of antibiotic-supplemented feed and environment on the occurrence of multiple antibiotic resistant Escherichia coli in chickens. *International Journal Poultry Science, 8*(1), 28–31.

SAS Institute Inc. (2009). *SAS/STAT® 9.2. User's Guide* (2nd Ed.). Cary, North Carolina: SAS Institute Inc.

Simon, A. H., & Baxter, G. A. (2006). Biosensor screening for veterinary drug residues in food stuffs. *Journal of AOAC International, 89*(3), 862–867.

Sogunle, O. M., Ekunseitan, D. A., Adeoti, M. T., Iyanda, A. I., & Fanimo, A. O. (2009). Performance and carcass characteristics of two strains of broiler chickens fed three different commercial feeds. *Journal of Applied Agricultural Research, 1*, 53–59.

Wallace, R. J., Oleszek, W., Franz, C., Hahn, I., Baser, K. H. C., Mathe, A., & Teichmann, K. (2010). Dietary plant bioactives for poultry health and productivity. *British Poultry Science 51*(4), 461–487.

Wasser, S. P. (2010). Current findings, future trends and unsolved problems in studies of medical mushrooms. *Applied Microbiology and Biotechnology, 89*(5), 451–58.

Weiss, D. J., & Wardrop, K. J. (Eds.). (1986). *Schalm's Veterinary Hematology* (6th Ed.). United States of America, USA: John Wiley & Sons.

Yang, J. H., Lin, H. C., & Mau, J. L. (2002). Antioxidant properties of several commercial mushrooms. *Food Chemistry, 77*(2), 229–235.

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Nutritional Values and Amino Acid Profiles of *Clinacanthus nutans* (Belalai Gajah/ Sabah Snake Grass) from Two Farms in Negeri Sembilan, Malaysia.

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ABSTRACT

The objective of the study was to determine the content of moisture, ash, protein, fat, carbohydrate, crude fibre, total sugar and amino acid profile for the medicinal herb *Clinacanthus nutans*. Three-month-old Sabah Snake Grass/Belalai Gajah (*C. nutans*) was collected from You Dun Chao Herb Farm (YDC) and Yik Poh Ling Herb Farm (YPL) in Negeri Sembilan, Malaysia. All the experiments were conducted in triplicate. Total crude fibre was found significantly higher in the stem samples. A comparison of non-shaded and shaded samples from YDC revealed higher ($p<0.05$) moisture, protein, ash, total crude fibre and total sugar content in the shaded samples for both leaves and the stem. Total fat was higher ($p<0.05$) in the shaded leaves than in the non-shaded leaves but it was the opposite for the stem. In comparing non-shaded samples from the two different farms for moisture, protein, ash, fat and total sugar content, the YPL leaves and stems showed significantly higher amounts than the YDC samples. The leaves of *C. nutans* contained more amounts of all essential and non-essential amino acids than the stem. Aspartic acid exhibited significantly higher amounts in both leaves (3.48, 1.08 and 2.13% of dry weight sample for YPL, YDC non-shaded and shaded samples, respectively) and stem (2.17, 0.95 and 1.96% of dry weight sample for YPL, YDC non-shaded and shaded samples, respectively) than the other amino acids. Geographical factors and planting conditions revealed different nutritive composition.

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INTRODUCTION

Clinacanthus nutans (Burm.f.) Lindau is a native medicinal herb that grows in tropical

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climate, and is mainly found in Malaysia. *C. nutans* is commonly called Sabah Snake Grass or 'Belalai Gajah'. However, there is limited information on this plant and its benefits have been under-cultivated. *C. nutans* has been listed as a research target in NKEA Malaysian Herbal Monograph 2012 and EPP#1 Research Grant Scheme. *C. nutans* belongs to the domain *Eukaryote*, kingdom *Plantae*, sub-kingdom *Viridaeplantae*, phylum *Tracheophyta*, subphylum *Euphyllophytin*a, infraphyllum *Radiatopses*, class *Magnoliopsida*, subclass *Asteridae*, superorder *Lamianae* and order *Lamiales*, family *Acanthaceae* and genus *Clinacanthus* with the specific epithet *nutans*. It is a shrub plant, which can be grown by stem propagation method. *C. nutans* is used in home decoration, teas and the bath (Siew et al., 2014).

The washed leaves of *C. nutans* can be freshly eaten or blended with apple and drunk as fruit juice. *C. nutans* is also used to treat skin affections, insect and snakebites and swellings due to a fall or boils (Chiwapreecha, Janprasert, & Kongpakdee, 2014). The phytochemical compounds that can be found in *C. nutans* (Burm.f.) Lindau plants are flavonoids, betulin, phytosterols such as stigmasterol, lupeol and β-sitosterol, saponin and diterpenes, which contribute to antimicrobial and anti-inflammatory properties (Sakdarat, Shuyprom, Pientong, Ekalaksananan, & Thongchai, 2009; Yang, Peng, Madhavan, Shukkoor, & Akowuah, 2013). The phenolic acids and flavonoids that are found in the *C. nutans* contribute to antioxidant activities. Petroleum ether extract of *C. nutans* has the radical scavenging activity of 82.0±0.02%, compared with ascorbic acid and α-tocopherol corresponding values of 88.7±0.0 and 86.6±0.0%, respectively (Arullappan, Rajamanickam, Thevar, & Kodimani, 2014). Chloroform leaf extract of *C. nutans* shows good antioxidant activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and galvinoxyl radicals, but is less effective in negating nitric oxide and hydrogen peroxide radicals (Yang et al., 2013, pp. 349–355). No study was found covering the nutritive aspects of *C. nutans* such as moisture, ash, protein, fat, carbohydrate, crude fibre, total sugar and amino acid profile. The objective of this study was to identify the quantity of nutrients in *C. nutans* in two farms in Malaysia, You Dun Chao Herb Farm (YDC; shaded and non-shaded samples) and Yik Poh Ling Herb Farm (YPL; non-shaded samples) in Negeri Sembilan.

MATERIALS AND METHOD

Three-month-old Sabah Snake Grass/ Belalai Gajah (*C. nutans*) was collected from You Dun Chao Herb Farm (YDC) and Yik Poh Ling Herb Farm (YPL) located in Negeri Sembilan, Malaysia. YDC farm had planted *C. nutans* in two conditions, shaded and non-shaded, while YPL farm had planted *C. nutans* only under the non-shaded condition. The YPL farm was located in a hilly area, while the YDC farm was situated on flat land. The samples were collected in triplicate. The leaves and stem were washed with water to remove sand and

dust particles. The leaves and stem were then freeze-dried using the ALPHA freeze dryer (Hampshire UK) and homogenised to 0.5 mm using the Universal cutting mill (FRITSCH, Germany) before analysis. All the chemicals used were from Sigma-Aldrich, USA. All the experiments were conducted in triplicate.

Determination of Moisture Content

About 5 g of dried leaves and stem of *C. nutans* samples were weighed using an aluminium dish and placed overnight in an oven at 105°C. Then, the samples were cooled down in a dessicator. The samples were weighed after they had attained room temperature and then re-weighed. The process was repeated until the difference in two successive weighings was less than 1 mg (Nielsen, 1994). The percentage of moisture was calculated using the formula below:

$$\text{Percentage of moisture (\%)} = \frac{(W1 - W2) \times 100}{S}$$

where
W1 = sample weigh (g) before drying
W2 = sample weigh (g) after drying
S = sample weigh (g)

Determination of Ash Content

About 5 g of dried leaves and stem of *C. nutans* samples was weighed using tared silica. The samples were heated on an electric heating mantle (Favorit®, Malaysia) in the fume hood till fumes were no longer produced. The sample was transferred onto a muffle furnace (Nabertherm, Germany) and the temperature was set at 550°C until the ash was free of carbon (overnight). The sample was cooled down in a dessicator and weighed. The process was repeated until the difference between two successive weighings was less than 1 mg (Nielsen, 1994). The formula below was used to determine the ash content of the sample:

$$\text{Total ash (g)} = \text{Weight of tared silica with ash} - \text{Weight of tared silica}$$

$$\text{Percentage of total ash (\%)} = \frac{\text{Weight of ash} \times 100}{\text{Weight of sample}}$$

Determination of Protein Content

About 0.5 g of dried leaves and stem of *C. nutans* samples was placed in a digestion tube (Favorit®, Malaysia). About 3 to 5 g of catalyst (mixture of potassium sulphate and copper sulphate in 10:1 ratio) and about 12 to 13 ml of concentrated sulphuric acid was added into the digestion tube. Then, the digestion tube was shaken gently and placed in a Tecator™ Digestor (FOSS, Denmark). The sample was digested until the colour green was observed. The homogenised sample was distilled using the Kjeltec™ 8200 Auto Distillation Unit (FOSS, Denmark). The green coloured receiver solution was titrated with 0.096N of hydrochloric acid, HCl, until the colour changed to red. The volume of used hydrochloric acid was noted (AOAC, 1990). The percentage of nitrogen present in the sample was calculated using the formula below:

$$\text{Percentage of nitrogen (\%)} = \frac{0.1 \times (S - B) \times 14 \times 100}{W \times 1000}$$

where

S = Volume of used hydrochloric acid for sample

B = Volume of used hydrochloric acid for blank

W = Weight of sample

Percentage of crude protein (%) = percentage of nitrogen X 6.25

Determination of Fat Content

About 1 to 2 g of the dried leaves and stem of *C. nutans* samples (W1) was weighed using Whatman filter paper wrapped neatly and placed in a thimble. The thimble was placed on a thimble holder. A thin layer of cotton was placed on the top of the sample. The thimble was moved to a thimble support. The pre-dried extraction cup was weighed (W2). About 50 ml of hexane was poured into an extraction cup using a measuring cylinder, after which the extraction cup was placed on a cup holder that was attached to the Soxtec™ 2043 Extraction Unit (FOSS, Denmark). After that, the extraction cup was transferred to an oven and dried at 105°C for 1 hr. The extraction cup was cooled down in a dessicator until it reached room temperature. The extraction cup with fat was weighed (W3) (AOAC, 1997). The percentage of crude fat in the sample was calculated using the formula below:

$$\text{Percentage of crude fat (\%)} = \frac{W3 - W2 \times 100}{W1}$$

Determination of Carbohydrates

The percentage of carbohydrates in the sample was calculated using the formula below:

Total carbohydrates = 100 − (moisture + proteins + fat + ash)

where moisture, proteins, fat and ash stand for their masses, respectively, expressed in units of 1 g.

Determination of Total Crude Fibre

The capsule cap and lid were weighed (*W1*). Next, 1 g of the sample was weighed (W2) into the capsule and sealed. One empty capsule was sealed with no sample due to correction (W4). The sealed capsule was placed on a hot plate FiberCap 2022 (FOSS, Tecator™ Technology, Denmark) and 350 mL of 1.25% sulfuric acid (H_2SO_4) was added. The heating temperature was set to 100°C. After 30 min, the capsule was re-washed in hot distilled water several times and 350 mL of 1.25% sodium hydroxide (NaOH) was added. The temperature was set to 100°C and the reaction time was set to 30 min. Afterwards, the samples were re-washed using hot distilled water and 350 mL of 1% hydrochloride acid (HCl). The heating temperature was set to 100°C. After 30 min, the capsule was re-washed in hot distilled water several times. Subsequently, the samples were kept in an oven at 105°C for 5 h and after cooling in the desiccator, weighed (W3). Finally, all the capsules were burnt in a muffle furnace at 550°C for 5.5 h and after cooling in the desiccator,

they were weighed (*W5*). The crude fibre content was calculated as follows (AOAC, 1997):

$$\% \text{ Crude fibre} = \frac{W3 - (W1 \times C) - (W5 - W4 - D) \times 100}{W2}$$

where
W1 = Initial capsule weight (g)
W2 = Sample weight (g)
W3 = Capsule + residue weight (g)
W4 = Crucible weight (g)
W5 = Total ash + crucible (g)
C = Blank correction for capsule solubility
D = Blank capsule ash (g)

Determination of Total Sugar

A total of 5 g of dried leaves and stem of *C. nutans* samples was refluxed with 20 ml of 80% ethanol for 1-2 h. Then, the ethanol was removed by evaporation using a rotary evaporator (Heidoplph, America) and the aqueous portion was transferred to a 100-ml volumetric flask and diluted to volume with distilled water and filtered with filter paper. The standard glucose stock solution prepared was of 1 mg/ml concentration (100 mg of glucose with 100 ml of distilled water). The volumes of standard glucose solution (0, 200, 400, 600, 800 and 1000 µl) were pipetted into test tubes and distilled water was added to make up a volume of 1000µl each. A 1000-µl of the sample was added into the test tubes. Each test tube had 1000 µl phenol and 5 ml concentrated sulphuric acid added to it. The test tubes were left for 15-30 min and then the optical density was measured spectrophotometrically (SpectrOstar^{Nano}, Germany) at 490 nm (AOAC, 1997).

$$\text{Total sugar (µg/ g)} = R \times (TV/SV) \times (DF/Wt)$$

where
R = Sugar content from standard curve (µg)
TV = Sample volume from extraction step
SV = Volume of sample used for spectrophotometric measurement
Wt = Sample weight
DF = Dilution factor

Determination of Amino Acid Profile

Acid hydrolysis (6 N HCl) of the freeze-dried sample was performed according to the AOAC (1995) method. Approximately 0.3 g of sample was weighed onto a glass-toppered test tube and hydrolysed with 5 mL of 6 N HCl at 110°C for 24 h. Samples were cooled to room temperature before they were filtered using filter paper (Sartorius grade 292) into a 100-mL volumetric flask. Mobile phase A (AccQ Taq Eluent A) was prepared by diluting 100 mL of AccQ Taq Eluent A with 1000 mL deionised water and filtered via a nylon cellulose membrane (size 0.45 µm). The diluted mobile phase A was later digested in an ultrasonic bath for 15 min. Mobile phase B was prepared by diluting 600 mL HPLC grade acetonitrile with 400 mL of deionised water and then filtered via a nylon cellulose membrane (size 0.45 µm). The internal standard (400 µL) (50 µmoL mL-1 α-Aminobutyric Acid (AABA) in 0.1 M HCl) was added and

made up to 100 mL with distilled water. The aliquot was filtered through 0.20 mm polytetrafluoroethylene microfilter (Merck, Germany). As for derivatisation, 10 µL of filtered hydrolysated samples or standard was transferred to a 1.5 mL glass vial and 70 µL of borate buffer solution was added to it and mixed well. Then, 20 µL of AccQ Flour Reagent (3 mg/mL in acetonitrile) was added to the mixture and thoroughly mixed through vortex for several seconds. After that, amino acid analysis was performed on high performance liquid chromatography (Shimadzu LC-10 AD, Shimadzu Corporation, Japan) and the samples were analysed on AccQ Tag type column (3.9 x 150 mm) at a constant flow rate of 1 ml/min. The amino acids from the samples were derivatised with *AccQ Flour Reagents* detected using a fluorescent detector and the data were integrated using an integrator model C-R7A (Shimadzu chromatopac data processor), where two channels were used simultaneously at a wavelength of 250 nm, a bandwidth of 5 nm and another wavelength of 395 nm.

Statistical Analysis

The data obtained were statistically analysed using SPSS Version 22 (Chicago, Inc.) and one-way ANOVA followed by Duncan's Multiple Range test. A p value of <0.05 was considered statistically significant for all the statistical tests conducted.

RESULTS AND DISCUSSION

For YPL samples and YDC samples, the leaves of *C. nutans* exhibited higher moisture, ash, protein, fat content and total sugar than the stem (Table 1 & 2). The total protein of *C. nutans* leaves for YPL and YDC non-shaded and shaded samples was 16.14%, 8.79% and 15.76% respectively. The value of protein content in all the samples was lower than the protein content found in the leaves of *Jatropha curcas* (26.00±0.47%) (Asuk, Agiang, Dasofunjo, & Willie, 2015). Comparing *C. nutans* with date fruit, *Phoenix dactylifera* (dried/*tamar* stage), date fruit revealed significantly higher moisture (15-21%) than *C. nutans*. However, *C. nutans* contained significantly higher ash content and protein than date fruit (ash content: 1.0-2.0%; protein: 1.8-8.0%) (Al-Harrasi et al., 2014).

In YPL samples, the content of ash in the leaves and stem were 12.71% and 5.39%, respectively. For YDC samples, the content of ash in non-shaded leaves, non-shaded stem, shaded leaves and shaded stem were 6.33%, 4.57%, 10.25% and 5.82%, respectively. All the tested samples exhibited higher amounts of the substance than cooked whole eggs (3.7%), okra leaf (2.44%), young fruit (1.38%) and mature fruit (1.17%) (Kassis, Beamer, Matak, Tou, & Jaczynski et al., 2010; Nwachukwu, Nulit, & Go, 2014). Compared with a previous study on saffron, *Crocus sativus*, some of the samples contained lower ash content than the saffron whole flower (7.39±0.12%) (Serrano-Díaz, Sánchez, Martínez-Tomé, Winterhalter, & Alonso, 2013).

The total fat in YPL leaves and stem were 4.82 and 0.85%, respectively. For YDC samples, the content of fat in non-shaded leaves, non-shaded stem, shaded

leaves and shaded stem were 2.08%, 0.84%, 3.53% and 0.52%, respectively. All the tested samples were more than 10 times lower in fat than cooked whole eggs (42.3%) and lower than saffron stigma (8.76±0.16%) and stamen (10.73±0.38%) (Serrano-Díaz et al., 2013, pp. 101–108).

The total crude fibre analysis comprised acid and alkaline hydrolysis. Acid hydrolysis was performed using sulfuric acid for the extract of sugars and starch from the samples, while alkaline hydrolysis was done using sodium hydroxide to remove the proteins, hemi-cellulose and lignin. Total crude fibre was found significantly higher in both leaves and stem of YDC shaded samples, with a value of 13.65% and 43.35%, respectively compared with the other treatments. Stem samples comprised more total crude fibre than the leaf samples in all areas. Total crude fibre was ranged between 10.72-13.65% in the leaf samples and between 32.80% and 43.35% for stem samples. The current results showed much higher fibre than recorded in previous studies done on flakes (made up of commercial white wheat: 1.68%; Dickopf wheat: 2.09%; red wheat: 1.87%; Kamut: 1.77%; Spelta: 1.24%) and muesli (made up of commercial whiter wheat: 4.76%; Dickopf wheat: 5.72%; red wheat: 5.40%; Kamut: 5.35%; Spelta: 3.62%) (Sumczynski, Bubelova, Sneyd, Erb-Weber, & Micek, 2015). Moreover, the *C.nutans* leaves and stem contained more total crude fibre compared to what was discovered in previous studies done on Sri Lankan rice varieties (Pokkali: 0.9%; Kalu heenati: 0.9%; Kahawanu: 1.0%; Sudu murunga: 1.1%; Unakola Samba: 0.9%; Gurusinghe wee: 1.0%) (Kariyawasam, Godakumbura, Prashantha, & Premakumara, 2016).

In comparing non-shaded and shaded samples of YDC, higher ($p<0.05$) moisture, protein, ash, total crude fibre and total sugar content was observed in the shaded samples for both leaves and stem. Total fat was higher ($p<0.05$) in the shaded leaves than in the non-shaded leaves but not so for the stem. Comparing the content of the leaves and stem from the two different farms for the non-shaded samples, it was found that the moisture, protein, ash, fat and total sugar content of YPL leaves and stem samples was significantly higher than that of the YDC samples.

Table 1
Percentage of moisture, ash, protein, fat, carbohydrate, crude fibre content and total sugar in leaves of Clinacanthus nutans

Nutritional parameter	**YPL Non-Shaded** Mean ± SD	**YDC Non-Shaded** Mean ± SD	**YDC Shaded** Mean ± SD
Moisture (%)	7.91 ± 0.01^{a}	6.72 ± 0.19^{c}	7.64 ± 0.38^{b}
Ash (%)	12.71 ± 0.00^{a}	6.33 ± 0.25^{c}	10.25 ± 0.14^{b}
Protein (%)	16.14 ± 0.04^{a}	8.79 ± 0.02^{c}	15.76 ± 0.02^{b}
Fat (%)	4.82 ± 0.10^{a}	2.08 ± 0.09^{c}	3.53 ± 0.49^{b}
Carbohydrate (%)	58.42 ± 0.12^{c}	76.08 ± 0.02^{a}	62.82 ± 0.04^{b}
Crude fibre (%)	11.35 ± 0.26^{b}	10.72 ± 0.02^{c}	13.65 ± 0.81^{a}
Total sugar (mg/g)	34.23 ± 0.17^{c}	52.31 ± 0.13^{a}	42.31 ± 0.02^{b}

± = Standard deviation of triplicate samples (n=3)

$^{a-c}$: Values with different superscripts in the same row are significantly different at $p<0.05$.

Table 2
Percentage of moisture, ash, protein, fat, carbohydrate, crude fibre content and total sugar in stems of Clinacanthus nutans

Nutritional parameter	**YPL Non-Shaded** Mean ± SD	**YDC Non-Shaded** Mean ± SD	**YDC Shaded** Mean ± SD
Moisture (%)	6.28 ± 0.33^{a}	5.06 ± 0.09^{c}	5.72 ± 0.74^{b}
Ash (%)	5.39 ± 0.17^{b}	4.57 ± 0.15^{c}	5.82 ± 0.29^{a}
Protein (%)	7.58 ± 0.12^{b}	5.36 ± 0.02^{c}	10.40 ± 0.02^{a}
Fat (%)	0.85 ± 0.02^{a}	0.84 ± 0.12^{b}	0.52 ± 0.08^{c}
Carbohydrate (%)	79.90 ± 0.23^{b}	84.17 ± 0.15^{a}	77.54 ± 0.13^{c}
Crude fibre (%)	32.80 ± 0.13^{c}	36.23 ± 1.23^{b}	43.35 ± 0.31^{a}
Total sugar (mg/g)	24.12 ± 0.67^{b}	33.43 ± 0.15^{a}	23.69 ± 0.14^{c}

± = Standard deviation of triplicate samples (n=3)
$^{a-c}$: Values with different superscripts in the same row are significantly different at $p<0.05$.

The leaves of *C. nutans* contained higher amounts of all essential and non-essential amino acids than its stem. Aspartic acid exhibited a significantly higher amount in both leaves (3.48%, 1.08% and 2.13% of dry weight sample for YPL, YDC non-shaded shaded samples, respectively) and stem (2.17%, 0.95% and 1.96% of dry weight sample for YPL, YDC non-shaded and shaded samples, respectively) than other amino acids (Table 3 & 4). However, the value was about three times less than the content of aspartic acid in cooked whole eggs (9.04%) (Kassis et al., 2010). The content of aspartic acid in all samples in the current study was higher compared to that in wild and cultivated *Panax* ginseng, with a value of 6.40 mg/g or 0.64% and 5.24mg/g or 0.52% (Sun et al., 2016). Furthermore, the current result revealed significantly higher amounts than in genuine Dong Chong Xia Cao (corpus: 1.70%; fruiting part: 1.84%), fermented preparations of *Cordyceps sinensis* (mycelium: 1.05%; supernatant of broth: 0.31%) and counterfeit Dong Chong Xia Cao (corpus: 0.62%; fruiting body: 0.82%) (Hsu,Shiao, Hsieh, & Chang, 2002), while methionine exhibited the lowest amount in both leaves (0.17%, 0.13% and 0.19% of dry weight sample for YPL, YDC non-shaded and shaded samples, respectively) and stem (0.08%, 0.08% and 0.04% of dry weight sample for YPL, YDC non-shaded and shaded samples, respectively). The content of methionine in all the samples except for YDC shaded stem samples exhibited a higher amount than in wild and cultivated *Panax* ginseng with a value of 0.71 mg/g or 0.07% and 0.55 mg/g or 0.05% (Sun et al., 2016). Glutamine and aspartate are major metabolic fuels for the small intestine. They regulate neurological functions along with glycine. Leucine activates mammalian targets of rapamycin to stimulate protein synthesis and inhibits proteolysis, while tryptophan

modulates neurological and immunological functions through multiple metabolites, including serotonin and melatonin (Wu, 2010). Dietary supplementation with one or a mixture of amino acids may be beneficial for ameliorating health problems at various stages of the life cycle such as fetal growth restriction, neonatal morbidity and mortality, weaning-associated intestinal dysfunction and wasting syndrome, obesity, diabetes, cardiovascular disease, metabolic syndrome and infertility. In addition, dietary supplementation optimises efficiency of metabolic transformations to enhance muscle growth, milk production, egg and meat quality and athletic performance, while preventing excess fat deposition and reducing adiposity (Wu, 2009). Glycine, histidine, isoleucine, leusine, lysine phenylalanine, threonine, valine, serine, glutamic acid, arginine, alanine and proline exhibited higher concentrations in all the samples compared to cultivated *Panax* ginseng (3.29 mg/g or 0.33% for glycine; 0.94 mg/g or 0.09% for histidine; 1.41 mg/g or 0.14% for isoleucine; 2.68 mg/g or 0.27% for leucine; 2.13 mg/g or 0.21% for lysine; 1.48 mg/g or 0.15% for phenylalanine; 1.88 mg/g or 0.19% for threonine; 1.92 mg/g or 0.19% for valine; 2.13 mg/g or 0.21% for serine; 8.09 mg/g or 0.81% for glutamic acid; 10.87 mg/g or 1.09% for arginine; 2.66 mg/g or 0.27% for alanine; 2.15 mg/g or 0.22% for proline) (Sun et al., 2016).

Table 3
Amino acid percentage in dry leaves of Clinacanthus nutans

Essential Amino Acid (% of dry weight sample)	**YPL Non-Shaded**	**YDC Non-Shaded**	**YDC Shaded**
	Mean ± SD	Mean ± SD	Mean ± SD
His	0.36 ± 0.01^{o}	0.32 ± 0.01^{l}	0.35 ± 0.01^{m}
Ile	0.74 ± 0.01^{l}	0.61 ± 0.01^{f}	0.73 ± 0.03^{k}
Leu	1.45 ± 0.01^{c}	1.05 ± 0.02^{b}	1.32 ± 0.06^{c}
Lys	1.15 ± 0.10^{e}	0.95 ± 0.01^{c}	1.03 ± 0.02^{f}
Met	0.17 ± 0.01^{p}	0.13 ± 0.01^{n}	0.19 ± 0.01^{n}
Phe	0.93 ± 0.01^{i}	0.72 ± 0.01^{e}	1.10 ± 0.24^{d}
Thr	0.82 ± 0.01^{j}	0.61 ± 0.01^{f}	0.73 ± 0.03^{k}
Val	0.98 ± 0.01^{g}	0.82 ± 0.01^{d}	0.94 ± 0.04^{h}
Non-Essential Amino Acid (% of dry weight)			
Ala	1.03 ± 0.01^{f}	0.34 ± 0.03^{j}	1.00 ± 0.08^{g}
Arg	0.47 ± 0.72^{n}	0.23 ± 0.03^{m}	0.85 ± 0.01^{i}
Asp	3.48 ± 0.08^{a}	1.08 ± 0.08^{a}	2.13 ± 0.08^{a}
Glu	2.29 ± 0.03^{b}	0.56 ± 0.07^{g}	1.84 ± 0.07^{b}
Gly	0.95 ± 0.01^{h}	0.35 ± 0.04^{i}	1.00 ± 0.07^{g}
Pro	1.18 ± 0.01^{d}	0.51 ± 0.02^{h}	1.06 ± 0.07^{e}
Ser	0.81 ± 0.02^{k}	0.33 ± 0.04^{k}	0.78 ± 0.05^{j}
Tyr	0.65 ± 0.01^{m}	0.13 ± 0.02^{n}	0.66 ± 0.01^{l}

± = Standard deviation of triplicate samples (n=3)
$^{a-p}$: Values with different superscripts in the same column are significantly different at $p<0.05$.

Table 4
Amino acid percentage in dry stem of Clinacanthus nutans

Essential Amino Acid (% of dry weight)	**YPL Non-Shaded**	**YDC Non-Shaded**	**YDC Shaded**
	Mean ± SD	Mean ± SD	Mean ± SD
His	0.18 ± 0.01^{n}	0.11 ± 0.01^{n}	0.14 ± 0.02^{m}
Ile	0.34 ± 0.04^{l}	0.25 ± 0.01^{l}	0.22 ± 0.03^{l}
Leu	0.56 ± 0.06^{f}	0.44 ± 0.01^{d}	0.39 ± 0.05^{i}
Lys	0.58 ± 0.02^{e}	0.34 ± 0.02^{g}	0.39 ± 0.04^{i}
Met	0.08 ± 0.01^{p}	0.08 ± 0.01^{o}	0.04 ± 0.01^{n}
Phe	0.35 ± 0.04^{k}	0.29 ± 0.01^{i}	0.25 ± 0.03^{k}
Thr	0.40 ± 0.07^{j}	0.26 ± 0.01^{k}	0.25 ± 0.03^{k}
Val	0.31 ± 0.16^{m}	0.35 ± 0.01^{f}	0.31 ± 0.04^{j}
Non-Essential Amino Acid (% of dry weight)			
Ala	0.60 ± 0.06^{c}	0.34 ± 0.01^{g}	0.90 ± 0.02^{d}
Arg	0.42 ± 0.19^{i}	0.27 ± 0.02^{j}	0.71 ± 0.02^{f}
Asp	2.17 ± 0.36^{a}	0.95 ± 0.02^{a}	1.96 ± 0.16^{a}
Glu	1.12 ± 0.15^{b}	0.67 ± 0.01^{b}	1.58 ± 0.01^{b}
Gly	0.46 ± 0.08^{g}	0.36 ± 0.01^{e}	0.82 ± 0.01^{e}
Pro	0.59 ± 0.12^{d}	0.48 ± 0.04^{c}	1.46 ± 0.44^{c}
Ser	0.45 ± 0.07^{h}	0.32 ± 0.01^{h}	0.67 ± 0.01^{g}
Tyr	0.15 ± 0.01^{o}	0.17 ± 0.01^{m}	0.55 ± 0.03^{h}

± = Standard deviation of triplicate samples (n=3)
$^{a-p}$: Values with different superscripts in the same column are significantly different at $p<0.05$.

CONCLUSION

The leaves of *C. nutans* exhibited more moisture, ash, protein, fat content and total sugar than its stem in all the tested samples. However, total crude fibre was found significantly higher in the stem samples. In comparing the non-shaded and shaded samples of YDC, higher ($p<0.05$) moisture, protein, ash, total crude fibre and total sugar content was observed in the shaded samples for both leaves and stem. Total fat was higher ($p<0.05$) in the shaded leaves than in the non-shaded leaves but this was not so for the stem. Comparing the non-shaded samples of the two different farms for moisture, protein, ash, fat and total sugar content showed that YPL leaves and stem had significantly ($p<0.05$) higher amounts of these substances than the YDC samples. The leaves of *C. nutans* contained more amounts of all essential and non-essential amino acids than the stem. Aspartic acid exhibited a significantly ($p<0.05$) higher amount in both leaves (3.48%, 1.08% and 2.13% of dry weight sample for YPL, YDC non-shaded and shaded samples, respectively) and stem (2.17%, 0.95% and 1.96% of dry

weight sample for YPL, YDC non-shaded and shaded samples, respectively) than other amino acids. Geographical factors and planting conditions revealed different nutritive composition.

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REFERENCES

Al-Harrasi, A., Rehman, N. U., Hussain, J., Khan, A. L., Al-Rawahi, A. A., Gilani, S. S., … & Ali, L. (2014). Nutritional assessment and antioxidant analysis of 22 date palm (*Phoenix dactylifera*) varieties growing in Sultanate of Oman. *Asian Pacific Journal of Tropical Medicine*, *7*(1), 591–598.

AOAC. (1990). *Official methods of analysis* (15th Ed.). Washington D.C.: AOAC International.

AOAC. (1995). *Official methods of analysis.* Washington D. C.: AOAC International.

AOAC. (1997). *Official methods of analysis* (16th Ed.). Washington D.C.: Association of Official Analaytical Chemist.

Arullappan, S., Rajamanickam, P., Thevar, N., & Kodimani, C. C. (2014). In vitro screening of cytotoxic, antimicrobial and antioxidant activities of *Clinacanthus nutan*s (Acanthaceae) leaf extracts. *Tropical Journal of Pharmaceutical Research*, *13*(9), 1455–1461.

Asuk, A. A., Agiang, M. A., Dasofunjo, K., &Willie, A. J. (2015). The biomedical significance of the phytochemical, proximate and mineral compositions of the leaf, stem bark and root of *Jatropha curcas*. *AsianPacific Journal of Tropical Biomedicine, 5*(8), 650–657.

Chiwapreecha, B., Janprasert, K., & Kongpakdee, C. (2014). Comparative anatomy of three medicinal plants in Acanthaceae. *Acta Horticulturae, 1023,* 229–232.

Fennema, O. R. (1996). *Food chemistry.* New York, NY: Marcel Dekker.

Hsu, T. H., Shiao, L. H., Hsieh, C., & Chang, D. M. (2002). A comparison of the chemical composition and bioactive ingredients of the Chinese medicinal mushroom Dong Chong Xia Cao, its counterfeit and mimic, and fermented mycelium of *Cordyceps sinensis*. *Food Chemistry, 78*(4), 463–469.

Kariyawasam, T. I., Godakumbura, P. I., Prashantha, M. A. B., & Premakumara, G. A. S. (2016). Proximate composition, calorie content and heavy metals (As, Cd, Pb) of selected Sri Lankan traditional rice (*Oryza Sativa* L.) Varieties. *Procedia Food Science, 6,* 253–256.

Kassis, N. M., Beamer, S. K., Matak, K. E., Tou, J. C., & Jaczynski, J. (2010). Nutritional composition of novel nutraceutical egg products developed with omega-3-rich oils. *LWT – Food Science and Technology, 43*(8), 1204–1212.

Nielsen, S. S. (1994). *Introduction to the chemical analysis of foods.* Boston: Jones and Bartlett Publishers, Inc.

Nwachukwu, E. C., Nulit, R., & Go, R. (2014). Nutritional and biochemical properties of Malaysian okra variety. *Advancement in Medicinal Plant Research, 2*(1), 16–19.

Sakdarat, S., Shuyprom, A., Pientong, C., Ekalaksananan, T., & Thongchai, S. (2009). Bioactive constituents from the leaves of *Clinacanthus nutans Lindau. Bioorganic and Medicinal Chemistry*, *17*(5), 1857–1860.

Serrano-Díaz, J., Sánchez, A. M., Martínez-Tomé, M., Winterhalter, P., & Alonso, G. L. (2013). Original contribution to nutritional studies on *Crocus sativus* flowers and their value as food. *Journal of Food Composition and Analysis, 31*(1), 101–108.

Siew, Y. Y., Zareisedehizadeh, S., Seetoh, W. G., Neo, S. Y., Tan, C. H., & Koh, H. L. (2014). Ethnobotanical survey of usage of fresh medicinal plants in Singapore. *Journal of Ethnopharmacology, 155*(3), 1450–1466.

Sumczynski, D., Bubelova, Z., Sneyd, J., Erb-Weber, S., & Micek, J. (2015). Total phenolics, flavonoids, antioxidant activity, crude fibre and digestibility in non-traditional wheat flakes and muesli. *Food Chemistry, 174,* 319–325.

Sun, H., Liu, F. B., Sun, L. W., Liu, J. Z., Wang, M. Y., Chen, X. N., … & Jiang, R. (2016). Proteomic analysis of amino acid metabolism differences between wild and cultivated *Panax ginseng. Journal of Ginseng Research, 40*(2), 113–120.

Wu, G. Y. (2009). Amino acids: Metabolism, Functions, and nutrition. *Amino Acids, 37*(1),1–17.

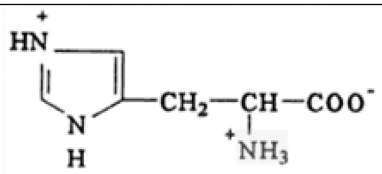
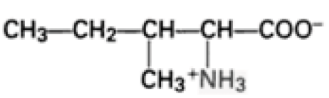
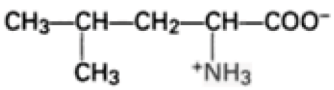
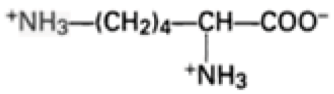
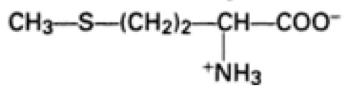
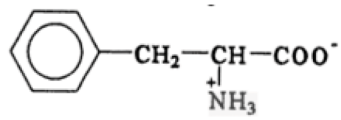
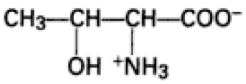
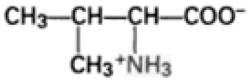
Wu, G. Y. (2010). Functional amino acids in growth, reproduction, and health. *Advances in Nutrition, 1*(1), 31–37.

Yang, H. S., Peng, T. W., Madhavan, P., Shukkoor, M. S. A., & Akowuah, G. A. (2013). Phytochemical analysis and antibacterial activity of methanolic extract of *Clinacanthus nutans* leaf. *International Journal of Drug Development and Research*, *5*(3), 349–355.

ABBREVIATIONS

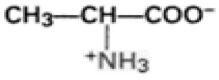
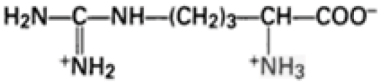
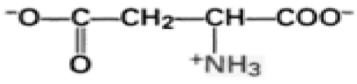
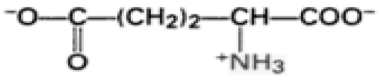
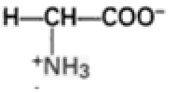
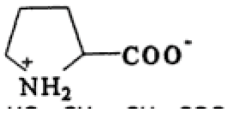
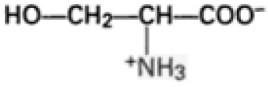
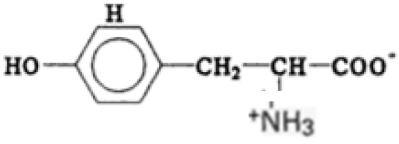
Supplementary 1

Essential amino acids (Fennema, 1996)

Name	Symbol	Structure at neutral pH
Histidine	His	
Isoleucine	Ile	
Leucine	Leu	
Lysine	Lys	
Methionine	Met	
Phenylalanine	Phe	
Threonine	Thr	
Valine	Val	

Supplementary 2

Non-essential amino acids (Fennema, 1996)

Name	Symbol	Structure at neutral Ph
Alanine	Ala	
Arginine	Arg	
Aspartic acid	Asp	
Glutamic acid	Glu	
Glycine	Gly	
Proline	Pro	
Serine	Ser	
Tyrosine	Tyr	

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Integrated Management of Stem Canker and Black Scurf of Potato

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ABSTRACT

The study was conducted to evaluate the efficacy of integrated management of stem canker and black scurf disease affecting the potato plant. The integrated management options were: T_1 = Poultry manure (PM) + seed trématent (ST) with Azoxystrobin (0.05%), T_2 = PM + ST-Azoxystrobin (0.10%), T_3= PM+ST-Boric acid (3.0%), T_4 = PM + ST-Carboxin (0.20%), T_5 = PM + ST- Carbendazim (0.10%), T_6 = PM + soil drenching (SD) - Azoxystrobin (0.05%), T_7 = PM + SD-Azoxystrobin (0.10%), T_8 = PM + SD-Carboxin (0.20%), T_9 = PM + SD-Carbendazim (0.10%) and T_{10} = Untreated control. The integrated management significantly influenced the disease incidence, yield attributes and yield of potato. The lowest disease incidence (11.2%) and percentage of disease index (4.58) were found in T_7 (poultry manure at 5 t ha^{-1} before 25 days of planting, DAP + soil drenching with Azoxystrobin at 0.10% during sowing and 45 DAP) followed by T_6 (PM 5 t ha^{-1} + soil drenching with Azoxystrobin at 0.05%). The minimum weight of russet (480 g plot^{-1}), deformed (450 g plot^{-1}) and Sclerotia infected (150 g plot^{-1}) tubers were also recorded in T_7. The highest healthy tuber (1900.05 g plot^{-1}) and tuber yield (22.4 t ha^{-1}) were found in the same treatment. Therefore, poultry manure 5 t ha^{-1} before 25 DAP + soils drenching with Azoxystrobin at 0.10% during sowing and 45 DAP can be recommended to produce healthy tubers and maximum tuber yield of potato.

Keywords: Potato, stem canker, black scurf, fungicide, poultry manure

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INTRODUCTION

Potato (*Solanum tuberosum*) is an important food crop in Bangladesh and has contributed to the nutrition of the poor in Bangladesh, who suffer from malnutrition. Potato has also contributed to the recent development of the Agro and Food industries in Bangladesh (Islam et al., 2013). The area under this crop is increasing gradually and farmers are adapting it as a cash crop. During 2014-2015, about 9.25 million tons of potato were produced from 0.47 million hectares of land and the national yield was 19.65 metric tons per hectare (BBS, 2015), which is lower compared to that of other potato-growing countries like North America and the Netherlands (Swaminathan, 2000). The main limiting factors for production of potato in Bangladesh are poor quality seeds, management factors, insect-pests and diseases. BARI Alu-7 (cv *Diamant*), the most popular variety of potato in Bangladesh, was used as a test variety; however, its disease resistance capacity is similar to that of other varieties. This variety is also susceptible to late blight disease.

In Bangladesh, a total of 39 diseases (both biotic and abiotic) of potato have been recorded (Ali & Khan, 1990). The major soil and tuber-borne diseases are black scurf, stem canker, bacterial wilt and common scab. Among them, stem canker and black scurf caused by *Rhizoctonia solani* (Kuhn) is the most common and destructive disease (Bains et al., 2002) and the pathogen is widespread in all potato-growing countries (Ali & Dey, 1994; Jager et al., 1996; Bains et al., 2002; El-Bakali & Martin, 2006). *Rhizoctonia solani* infects the underground stem and produces necrosis called stem canker, while tuber infection produces symptoms on the skin in the form of black sclerotia, called black scurf. Canker is commonly known as black scurf. In Bangladesh, fungicides on potato crops generally show little effect on *R. solani* attack under field conditions. Therefore, Azoxystrobin (trade name Amistar), a broad-spectrum fungicide from the strobilurin group, was tested along with poultry manure. Djelbali and Belhassen (2010) reported that the applications of Pencycuron and Azoxystrobin on seed potato and/or in-furrow have reduced the percentage of infection of Nicola progeny tubers by *R. solani* black scurf. The application of Azoxystrobin on seed potato and in-furrow proved to be of superior efficacy in reducing the percentage and the level of infection of Nicola progeny tubers by *R. solani* black scurf.

The common scab of potato (*Streptomyces scabies*) was effectively managed by an organic amendment through poultry manure (@ 2 t ha^{-1}) with bio-agent *Pseudomonas fluorescens* (Chaudari et al., 2003). The weight of black scurf tubers was minimum when the integrated approaches of poultry manure (PM) + seed Carbendazim (0.1%) and drenching was applied. Integration of PM + Carboxin (0.2%) soil drenching was found to be effective (Hossain et al., 2009). Hossain et al. (2007) reported that use of poultry

manure and seed treatment with Carboxin and poultry manure and soil drenching with Carbendazim showed better performance in reducing stem canker and black scurf disease of potato. *Rhizoctonia* does not contest very well with other microbes in the soil. Increasing the rate of poultry manure decomposition decreases the growth rate of *Rhizoctonia*. Poultry manure decomposition also releases carbon dioxide, which reduces the competitive ability of the pathogen. The fungus is not an efficient cellulose decomposer, so soil populations are greatly reduced by competing microflora and less disease is observed (Phillip & Elisabeth, 2017).

Many researchers have attempted to control black scurf and stem canker of potato. The single approach of a control measure in many cases was not adequate for controlling the disease. In Bangladesh, there is no report on the research of any aspect of integrated management of the disease. Under the circumstances, developing a package of integrated management of the diseases is of prime need. Economic and eco-friendly methods of controlling black scurf and stem canker of potato are urgently needed. Considering the above facts, the present study was initiated to formulate integrated management of stem canker and black scurf of potato.

MATERIALS AND METHOD

Experimental Location and Crop Characteristics

An experiment was conducted at Tuber Crops Research Centre, Bangladesh Agricultural Research Institute (BARI), Bogra during the 2009-2010 cropping season. The experimental site was located in Tista Meander Floodplain Soil (AEZ-3) at about N-24^0 78′ and E-89^0 35′; it has a mean elevation of 22 m above sea level. The experiment location was on high land and had sandy loam soil. The soil was acidic (pH 5.6) in nature. Potato is grown here in the Rabi season (November to March). The test crop was potato (*Solanum tuberosum* L.) cv. BARI Alu-7 (Diamant), collected from the Breeder Seed Production Centre, Debigonj. BARI Alu-7 tubers are white, oval, medium-to-large, smooth skinned, light yellow in flesh, shallow eyed. Its yield is 25-35 t ha^{-1} and it is the most popular variety of potato in Bangladesh**.** It is susceptible to late blight disease.

Experimental Design, Fertilizer Application, Treatment and Intercultural Operation

The experiment was laid out in a randomised complete block design (RCBD) with four replications. Urea, triple super phosphate (TSP), muriate of potash (MoP), gypsum, zinc sulphate and boric acid were applied at the rate of 360, 220, 250, 120, 14 and 6 kg per hectare, respectively. The entire amount of TSP, MoP, gypsum, zinc sulphate, boron and half of urea were applied at the time of final land preparation. The remaining amount of urea was applied at 30 DAP (days after planting). The integrated treatments were: T_1 = Poultry manure (PM) + seed trématent (ST) with Azoxystrobin (trade name Amistar) (0.05%), T_2 = PM +

ST-Azoxystrobin (0.10%), T_3 = PM + ST-Boric acid (3.0%), T_4 = PM + ST-Carboxin (trade name Provax) (0.20%), T_5 = PM + ST-Carbendazim (trade name Bavistin) (0.10%), T_6 = PM + SD-Azoxystrobin (0.05%), T_7 = PM + SD-Azoxystrobin (0.10%), T_8 = PM + SD-Carboxin (0.20%), T_9 = PM + SD-Carbendazim (0.10%) and T_{10} = Untreated control. The chemicals were used both as seed treatment (ST) and soil drenching (SD). After treatment, the tubers were kept in the shade for 24 hours. The chemicals were drenched just after sowing in furrows at 45 DAP (days after planting). The poultry manure (5 tha^{-1}) was incorporated into the soil and mixed properly before 25 DAP. Intercultural operations such as weeding and mulching were done as and when required. Irrigation was carried out four times during the whole growing season. The first irrigation was light, applied at 7 DAP to ensure proper germination. The second irrigation was carried out at 30 DAP followed by earthing and side-dressing (urea fertilisers). The third and fourth irrigation was done at 48 and 63 DAP, respectively. Dithen M45 2 g L^{-1} was sprayed at 50 DAP to prevent late blight disease.

Potato Tuber Planting

The study was conducted in previously *Rhizoctonia solani* infested soil. Potato seed tubers were planted with a spacing of 60 cm × 25 cm on 20 November, 2009 and a crop was was harvested on 25 February, 2010.

Data Collection

The germination percentage, number of stems per hill and plant height were recorded at 30, 50 and 60 DAP, respectively. The yield data were noted at harvest. The disease incidence (%) and percent disease index (PDI) were assessed at 70 DAP. Twenty plants were randomly selected from each unit plot at 70 DAP, uprooted carefully, washed under running tap water and checked for infection to record the disease incidence. The numbers of infected and healthy plants were counted and the percent disease incidence was calculated based on the total number of plants checked according to the formulae:

$$Disease\ incidence\ (\%) = \frac{\text{Number of infected plants}}{\text{Total number of plants checked}} \times 100$$

At 70 DAP, the severity of stolon infection was indexed on a 0-6 indexing scale (Bakr et al., 2010), where 0 = No symptom on stolon, 1 = Minute brown lesion on stolon or root, 2 = Moderately brown lesion on stolon and curling tendency on central leaf, 3 = Stolon symptom discoloured accompanied by brown discolouration on roots, 4 = Brown to black discolouration on underground parts, tissue discolouration and curling of growing leaves, 5 = Profuse emerging of auxiliary leaves, leaf size reduced markedly and pale green on leaf margin, and 6 = Production of aerial tuber with the colour green. Finally it was converted into percent disease index (PDI) following the formulae outline by Dey et al. (2010).

Statistical Analysis

The analysis of variance (ANOVA) for various crop characteristics and disease incidence was performed following the F test. When F was significant at the $p<0.05$ level, treatment means were separated using the DMRT (Steel & Torii, 1960) test. Data were analysed following standard procedure using SAS software (version 9.3). Computation and preparation of graphs were done using the Microsoft Excel 2003 Programme.

RESULTS

Integrated disease management significantly influenced plant growth, disease incidence, tuber quality and tuber yield of potatoes.

Effect of Integrated Disease Management on Plant Growth

Tuber germination (%) and plant growth were significantly influenced by the integrated management of black scurf and stem canker.

Table 1
Effect of integrated management of black scurf and stem canker on germination and growth parameters of potato

Treatment Combination	Germination (%)	No. of Stem Hill^{-1}	Plant Height (cm)
T_1 = PM + ST-Azoxystrobin (0.05%)	90.41 ^{abc} (71.96)	4.75 ^{abc}	55.17
T_2 = PM + ST-Azoxystrobin (0.10%)	91.25 ^{abc} (72.82)	4.60 ^{abc}	56.40
T_3 = PM + ST-Boric acid (3.0%)	89.16 ^{bc} (70.78)	4.38 ^{abc}	55.70
T_4 = PM + ST-Carboxin (0.20%)	92.08 ^{ab} (73.80)	4.95 ^{abc}	55.80
T_5 = PM + ST-Carbendazim (0.10%)	91.66 ^{abc} (72.42)	4.28 ^{cd}	51.63
T_6 = PM + SD-Azoxystrobin (0.05%)	92.49 ^{abc} (74.38)	4.80 ^{abc}	57.95
T_7 = PM + SD-Azoxystrobin (0.10%)	94.17^{a} (76.27)	5.25 ^{a}	58.65
T_8 = PM + SD-Carboxin (0.20%)	91.66 ^{abc} (72.42)	4.95 ^{abc}	56.95
T_9 = PM + SD-Carbendazim (0.10%)	89.58 ^{abc} (71.17)	4.98 ^{ab}	53.38
T_{10} = Untreated control	87.50 ^{c} (69.39)	3.88 ^{d}	57.50
CV%	1.78	10.59	9.56

Means followed by the same letter within a column did not differ significantly at the 5% level of DMRT.
Note: PM = Poultry manure, ST = Seed tuber treatment, SD = Soil drenching

Maximum germination (94.2%) was found in T_7 (PR + SD-Azoxystrobin, 0.10%), followed by T_6 (PR + SD-Azoxystrobin, 0.05%) and T_4 (PR + SD-Carboxin, 0.20%). Minimum germination (87.5%) was recorded in the untreated control. Similarly, T_7 showed the highest number of stem hill^{-1} (5.25) and plant height (58.65 cm) followed by T_6. The lowest stem hill^{-1} (3.88) and plant height (51.6 cm) were noted in the control (Table 1).

Incidence of Stem Canker and Percent Disease Index (PDI)

The incidence of stem canker and percent disease index (PDI) were significantly varied among the treatments. The highest disease incidence (%) of stem canker (28.75%) was found in the control, which was significantly higher than that of the other treatments (Table 2 and Figure 1).

T_7: Integrated management of poultry manure + T_{10}: Untreated Control soil drenching (Azoxystrobin 0.10%)

Figure 1. Effect of integrated management on the incidence of stem canker

T_3 (PR + ST-Boric acid, 3.0%) showed the second highest incidence of stem canker (22.50%), followed by T_9 (PR + SD Carbendazim, 0.10%). The control showed the highest percent disease index (17.7%), which was significantly higher than that of the other treatment combinations. The second highest percent disease index (11.46%) was also recorded in T_3, which was statistically similar to T_5 (PR + ST-Carbendazim, 0.10%) and T_9. The lowest incidence (%) of stem canker (11.25%) and percent disease index (4.58%) were found in T_7 (Table 2 and Figure 1).

Table 2
Effect of integrated management options on the incidence of stem canker of potato

Treatment Combination	Incidence (%) of Stem Canker	Percent Disease Index (PDI)
T_1 = PM + ST-Azoxystrobin (0.05%)	16.25 ^{cde} (4.02)	7.49 ^{ef}
T_2 = PM + ST-Azoxystrobin (0.10%)	15.00 ^{def} (3.87)	6.24 ^{fg}
T_3 = PM + ST-Boric acid (3.0%)	22.5 ^{b} (4.74)	11.46 ^{b}
T_4 = PM + ST-Carboxin (0.20%)	17.50 ^{cd} (4.18)	8.54 ^{cde}
T_5 = PM + ST-Carbendazim (0.10%)	20.00 ^{bc} (4.46)	10.41 ^{bc}
T_6 = PM + SD-Azoxystrobin (0.05%)	13.33 ^{efg} (3.65)	5.61 ^{g}
T_7 = PM + SD- Azoxystrobin (0.10%)	11.25 ^{g} (3.33)	4.58 ^{g}
T_8 = PM + SD-Carboxin (0.20%)	16.66 ^{cde} (4.08)	7.91 ^{def}
T_9 = PM + SD-Carbendazim (0.10%)	21.66 ^{b} (4.64)	10.21 ^{bcd}
T_{10} = Untreated control	28.75 ^{a} (5.35)	17.71 ^{a}
CV %	7.39	8.75

Means followed by the same letter within a column did not differ significantly at the 5% level of DMRT.

Effect of Integrated Management on Tuber Quality

The data on the number of inflected tubers have different types of symptom. Russet, deformed and sclerotia-bearing tubers were varied significantly due to integrated management option (Table 3). The number of russet-bearing tubers ranged from 7.00 to 16.25, the highest number seen in the control and the lowest (7.00) in T_7. The maximum number of deformed tubers (15.00) was found in the control, followed by T_9 and T_1 (PR + ST with Azoxystrobin, 0.05%). The minimum number of deformed tubers was also noted in T_7. The sclerotia-bearing tubers were significantly varied among the treatments. It ranged from 2.75 to 52.00. The control showed the highest number of sclerotia-bearing tubers (52.00), which was significantly higher than in the other management options. T_3 showed the second highest number of sclerotia-bearing tubers (15.5), which was identical to T_5. The lowest number of sclerotia-bearing tubers (2.75) was found in T_7 (Table 3). Healthy tubers were significantly influenced by the integrated management option. The highest number of healthy tubers (297.1 tubers plot^{-1})

was found in T_7, which was statistically identical to T_6 but different from the rest of the treatments (Table 3 and Figure 2). The lowest number of healthy tubers (159.0 tubers plot^{-1}) was recorded in the control.

T_7: Integrated management with poultry T_{10}: Untreated control manure + soil drenching with Azoxystrobin

Figure 2. Effect of integrated management on the potato tubers

Table 3
Effect of integrated management options on the number of black scurf and healthy tubers

Treatment Combination	Number of Infected Tuber Plot^{-1}			
	Russet Tuber	Deformed Tuber	Sclerotia Bearing Tuber	Total Healthy Tuber
T_1 = PM + ST-Azoxystrobin (0.05%)	12.25 ^{b}	11.25 ^{ab}	5.25 ^{efg}	260.05 c
T_2 = PM + ST-Azoxystrobin (0.10%)	10.75 ^{bc}	9.05 ^{bc}	4.75 ^{efg}	275.05 b
T_3 = PM + ST-Boric acid (3.0%)	10.00 ^{bc}	9.25 ^{bc}	15.50 ^{b}	221.05 ef
T_4 = PM + ST-Carboxin (0.20%)	11.00 ^{bc}	11.75 ^{ab}	7.00 ^{cde}	241.00 d
T_5 = PM + ST-Carbendazim (0.10%)	10.25 ^{bc}	8.75 ^{bc}	13.25 ^{b}	217.03 f
T_6 = PM + SD- Azoxystrobin (0.05%)	8.75 ^{bc}	8.00 ^{bc}	4.00 ^{fg}	291.00 a
T_7 = PM + SD-Azoxystrobin (0.10%)	7.00 ^{c}	6.50 ^{c}	2.75 ^{g}	297.08 a
T_8 = PM + SD-Carboxin (0.20%)	11.50 ^{b}	11.00 ^{b}	6.50 ^{def}	272.10 b
T_9 = PM + SD-Carbendazim (0.10%)	10.25 ^{bc}	12.00 ^{ab}	7.75 ^{cd}	238.00 d
T_{10} = Untreated control	16.25 ^{a}	15.00 ^{a}	52.00 ^{a}	159.00 g
CV %	13.03	13.19	16.16	13.37

Means followed by the same letter within a column did not differ significantly at the 5% level of DMRT.

Effect of Integrated Management on the Weight of Infected and Healthy Tuber

The weight of russet, deformed and sclerotia-bearing tubers was significantly varied due to application of different integrated management options against canker disease of potato (Table 4). The maximum weight of russet tubers (1100 g plot^{-1}), deformed tubers (1330 g plot^{-1}) and sclerotia-bearing tubers (3530 g plot^{-1}) was found in the control (Table 4); the weight was significantly higher than that of the tubers in the other treatments. The minimum weight of russet tubers (480 g plot^{-1}), deformed tubers (450 g plot^{-1}) and selerotia-bearing tubers (150 g plot^{-1}) was observed in T_7. The weight of healthy tubers was also significantly influenced by the different treatments. T_7 showed the highest weight of healthy tubers (1900 g plot^{-1}); this was statistically similar to T_6 and T_8 (PR + SD-Carboxin, 0.20%). The lowest weight of healthy tubers (883 g plot^{-1}) was noted in the control (Table 4).

Table 4
Effect of integrated management options on the weight of black scurf infected tubers

Treatment Combination	Weight of Infected and Healthy Tubers Plot^{-1} (g)			
	Russet Tubers	Deformed Tubers	Sclerotia-Bearing Tubers	Weight of Healthy Tubers
T_1 = PM + ST-Azoxystrobin (0.05%)	800 ^{b}	780 ^{bc}	350 ^{def}	1500.68^{cd}
T_2 = PM + ST-Azoxystrobin (0.10%)	680 ^{bc}	650 ^{bcd}	300 ^{def}	1600.9^{bc}
T_3 = PM + ST-Boric acid (3.0%)	730 ^{bc}	630 ^{bcd}	1080 ^{b}	1400.35^{d}
T_4 = PM + ST-Carboxin (0.20%)	600 ^{bc}	800 ^{b}	500 ^{cd}	1500.27^{cd}
T_5 = PM + ST-Carbendazim (0.10%)	700 ^{bc}	600 ^{bcd}	950 ^{b}	1300.57^{d}
T_6 = PM + SD-Azoxystrobin (0.05%)	600 ^{b}	550 ^{cd}	280 ^{ef}	1800.0^{ab}
T_7 = PM + SD-Azoxystrobin (0.10%)	480 ^{c}	450 ^{d}	150 ^{f}	1900.05^{a}
T_8 = PM + SD-Carboxin (0.20%)	780 ^{b}	750 ^{bc}	450 ^{cde}	1800.8^{ab}
T_9 = PM + SD-Carbendazim (0.10%)	730 ^{bc}	800 ^{b}	600 ^{c}	1600.05^{bc}
T_{10} = Untreated control	1100 ^{a}	1330 ^{a}	3530 ^{a}	883^{e}
CV %	12.16	18.77	17.21	8.78

Means followed by the same letter in the same column did not differ significantly at the 5% level of DMRT.

Effect of Integrated Management on the Tuber Yield of Potato

The potato tuber yield was significantly influenced by the integrated management option of stem canker and black scurf disease. The potato yield ranged from 16.24 to 22.36 t ha^{-1}. The highest tuber yield (22.36 t ha^{-1}) was found in T_7, where soil was drenched with Azoxystrobin (0.10%) and poultry manure was used. T_6 showed the second highest yield (20.60 t ha^{-1}), followed by T2 (20.55 t ha^{-1}). The lowest tuber yield (16.24 t ha^{-1}) was recorded in the untreated control (Table 5).

Table 5
Effect of integrated management options of black scurf and stem canker on the tuber yield of potato

Treatment combination	Tuber yield (t ha^{-1})
T_1 = PM + ST-Azoxystrobin (0.05%)	19.55 ^{abc}
T_2 = PM + ST-Azoxystrobin (0.10%)	20.60 ^{ab}
T_3 = PM + ST-Boric acid (3.0%)	18.58 ^{bcd}
T_4 = PM + ST-Carboxin (0.20%)	19.02 ^{bcd}
T_5 = PM + ST-Carbendazim (0.10%)	17.52 ^{cd}
T_6 = PM + SD-Azoxystrobin (0.05%)	20.55 ^{ab}
T_7 = PM + SD-Azoxystrobin (0.10%)	22.36 ^{a}
T_8 = PM + SD-Carboxin (0.20%)	18.99 ^{bcd}
T_9 = PM + SD-Carbendazim (0.10%)	18.33 ^{bcd}
T_{10} = Untreated control	16.24 ^{d}
CV %	9.28

Means followed by the same letter within a column did not differ significantly at the 5% level of DMRT

DISCUSSION

Stem canker and black scurf caused by *Rhizoctonia solani* is a very common and widespread disease of potato throughout Bangladesh (Ali & Dey, 1994). The management of this disease is not possible by a single control measure approach because of the nature of the soil and the very high degree of survivability of pathogens (Frank & Leach, 1980; Hide et al., 1973; Kumar, 1976). Therefore, the integration of chemical and organic substances is the best approach to control stem canker and black scurf disease of potato. It appeared from the results that incorporation of poultry manure (5 t ha^{-1}) at 25 days before planting, application of Azoxystrobin (0.1%) in furrows during sowing and soil drenching with Azoxystrobin (0.1%) at 45 days after sowing showed the better performance in controlling stem canker and black scurf disease of potato. Djelbali and Belhassen (2010) reported that the application of Pencycuron and Azoxystrobin on seed potato and/or in-furrow reduced the percentage of infection of Nicola progeny tubers by *R. solani* black scurf. The application of Azoxystrobin on seed potato and in-furrow proved to be of superior efficacy in reducing the percentage and level of infection of Nicola progeny tubers by *R. solani* black scurf in two years of experimentation. Integration of soil amendment by organic products and soil application of fungicide was reported by Sharma et al. (1995) as an effective management option against the disease. *Rhizoctonia* is not able to compete remarkably with other microbes in the soil. The growth rate of *Rhizoctonia* decreases with an increase in the decomposition rate of poultry manure. Poultry manure decomposition also releases carbon dioxide, which reduces the competitive

ability of pathogens. *Rhizoctonia* is not an efficient cellulose decomposer, so soil populations are greatly reduced by competing microflora and less disease is observed (Phillip & Elisabeth, 2017). The possible mechanisms for pathogen suppression by poultry manure include inhibition of pathogen growth, pathogen survival and reduction of infection of the host (Hoitink & Grebus, 1994). First, beneficial microbial populations including poultry-manure-derived microorganisms compete for nutrients with plant pathogens in the rhizosphere zone (De Brito et al., 1995; Hoitink & Boehm, 1999). The second mechanism includes production of antibiotic compounds by beneficial microorganisms that are effective in controlling various plant pathogens (Hoitink et al., 1996). The third is parasitism and predation of soil-inhabiting pathogens by poultry-manure-inhabiting beneficial microorganisms (Hoitink & Boehm, 1999). Poultry manure caused a temporary initial increase in soil pH. This increase in pH was accompanied by an increase in ammonia levels and the release of this volatile toxic gas may have been involved in reducing the population levels of *S. scabies* (Conn & Lazarovits, 1999). Conn and Lazarovits also mentioned that application of fresh poultry manure was highly effective in reducing the incidence of Verticillium wilt, potato scab and the population of plant parasitic nematodes. The application of poultry manure to the soil not only reduced disease severity; it also increased the tuber yield of potato in this study. Organic manure may release some hormones or organic compounds that suppress stem canker disease. Organic amendments may exert stimulatory or inhibitory effects on microbial plant pathogen populations and disease development (Rahman et al., 2016). They may either prevent infection by activating soil microflora potentially competitive with or antagonistic to plant pathogens present in the soil or control plant pathogens by producing toxic compounds in the soil when they decompose in the soil (Narayanasamy, 2013; Swain et al., 2006).

Similar findings were also reported by many other researchers (Shaikh & Ghaffar, 2004; Hossain et al., 2007; Banyal et al., 2008) regarding the effects of soil amendment with poultry manure followed by soil drenching with a fungicide. This provides strong support for the results obtained from the integrated management options used in this study. These results were also supported by Naz (2006) and Mian (2007), who paved the way for black scurf disease management through integrated options. Similar observations for soil amendments with indifferent fungicides on other crops have been advocated by Banyal et al. (2008), Hossain et al. (2007), Hossain et al. (2009), Mian (2007), Naz (2006) and Sharma et al. (1975).

CONCLUSION

Integrated management significantly influenced disease incidence, yield attributes and yield of potato in this study. The lowest disease incidence (11.2%) and percent disease index (4.58) were found

in T_7 (poultry manure at 5 t ha^{-1} before 25 days of planting, DAP + soil drenching with Azoxystrobin at 0.10% during sowing and 45 DAP) followed by T_6 (PM 5 t ha^{-1} + soil drenching with Azoxystrobin at 0.05%). The minimum weight of russet, deformed and sclerotia-infected tubers was also recorded in T_7. The highest number of healthy tubers and the highest tuber yield were found in the same treatment. Therefore, poultry manure 5 t ha^{-1} before 25 DAP + soil drenching with Azoxystrobin at 0.10% during sowing and 45 DAP can be recommended for producing healthy tubers and the maximum tuber yield of potato.

REFERENCES

Ali, M. S., & Dey, T. K. (1994). Pathological research on tuber crops in Bangladesh. In *Proceedings of Workshop on Tuber Crops on Transfer of Technology of CDP Crops under Research Extension Linkage Programme* (pp. 159–165). Gazipur, Bangladesh: BARI (Bangladesh Agricultural Research Institute) Training and Communication Section.

Ali, M. S., & Khan, A. L. (1990). Pathological constraints of seed potato production in Bangladesh. In M. M. Rashid, M. A. Siddique, & M. M. Hussain (Eds.), *Proceedings of the International Seminar, Seed Production in Bangladesh* (pp. 187–199). Dhaka, Bangladesh: BADC.

Bains, P. S., Bennypaul, H. S., Lynch, D. R., Kawchuk, L. M., & Schaupmeyer, C. A. (2002). Rhizoctonia disease of potatoes (*Rhizoctonia solani*): Fungicidal efficacy and cultivar susceptibility. *American Journal of Potato Research, 79*(2), 99–106.

Bakr, M. A., Hossain, S., & Ahmed, H. U. (2010). *A guide to disease identification, data recording, scale and grading system of major diseases of important crops*. Gazipur, Bangladesh: Oilseed Research Centre, Bangladesh Agricultural Research Institute, Training and Communication Section.

Banyal, D. K., Mankotia, V., & Sugha, S. K. (2008). Integrated management of tomato collar rot caused by *Sclerotium rolfsii*. *Journal of Mycology and Plant Pathology, 38*(2), 164–168.

BBS (Bangladesh Bureau of Statistics). (2015). *The year book of agricultural statistics of Bangladesh*. Dhaka, Bangladesh: Ministry of Planning.

Chaudhari, S. M., Patel, R. N., Khurana, S. M. P., Patel, K. R., & Ratel, N. H. (2003). Management of common scab of potato. *Journal of the Indian Potato Association, 30*(1/2), 135–136.

Conn, K. L., & Lazarovits, G. (1999). Impact of animal manures on Verticillium wilt, potato scab and soil microbial populations. *Canadian Journal of Plant Pathology, 21*(1), 81–92.

De Brito, A., Gagne, S., & Antoun, H. (1995). Effect of compost on rhizosphere microflora of the tomato and on the incidence of plant growth-promoting rhizobacteria. *Applied Environmental Microbiology*, *61*(1), 194–99.

Dey, T. K. (2010). A guide to disease identification, data recording, scale and grading system of major diseases of important crops. In M. A. Bakr, S. Hossain, & H. U. Ahmed (Eds.), *Tuber crops diseases – Their identification, method of recording, rating scale and grading system* (pp. 28–31). Gazipur, Bangladesh: Oilseed Research Centre, BARI.

Djebali, N., & Bethassen, T. (2010). Field study of the relative susceptibility of eleven potato *(Solanum tuberosum L.)* varieties and the efficacy of two fungicides against *Rhizoctonia solani* attack. *Crop Protection*, *29*(9), 998–1002.

El-Bakali, A. M., & Martin, M. P. (2006). Black scurf of potato. *Mycologist, 20*(4), 130–132.

Frank, J. A., & Leach, S. S. (1980). Comparison of tuber-borne and soil-borne inoculum in the *Rhizoctonia* disease of potato. *Phytopathology*, *70*(5), 51–53.

Hide, G. A., Hirst, J. M., & Stedman, O. J. (1973). Effects of black scurf (*Rhizoctonia solani)* on potatoes. *Annals of Applied Biology*, *74*(2), 139–148.

Hoitink, H. A. J., & Boehm, M. J. (1999). Biocontrol within the context of soil microbial communities: A substrate-dependent phenomenon. *Annual Review of Phytopathology, 37*(1), 427–46.

Hoitink, H. A. J., & Grebus, M. E. (1994). Status of biological control of plant diseases with composts. *Compost Science and Utilization, 2*(2), 6–12.

Hoitink, H. A. J., Stone, A. G., & Grebus, M. E. (1996). Suppression of plant diseases by composts. In M. De Bertoldi, P. Sequi, B. Lemmes, & T. Papi (Eds.), *The science of composting* (Vol. 1, pp. 373–381). Glasgow: Blackie Academic & Professional.

Hossain, M., Dey, T. K., Hossain, M. M., & Rahman, M. M. (2007). Tuber crops disease management of Bangladesh. In M. A. Bakar, H. U. Ahmed, & M. A. Wadud Main (Eds.), *Proceedings of the Workshop on Invention of Plant Pathological Research in Bangladesh* (pp. 296–306). Gazipur, Bangladesh: BARI.

Hossain, M. I., Hossain, M., & Dey, T. K. (2009). Integrated management of stem canker/ black scurf disease of potato. In B. C. Kundu, T. K. Dey, M. Hossain, & H. C. Mohanta (Eds.), *Annual report for 2008-2009* (p. 138). Gazipur, Bangladesh: Tuber Crops Research Centre (TCRC).

Islam, M. R., Mondal, C., Hossain, I., & Meah, M. B. (2013). Compost tea and poultry litter extract: Alternative organic management approaches for stem canker of potato caused by *Rhizoctonia solani. Journal of Agricultural Science, 5*(10), 261–272.

Jager, M. J., Hide, G. A., Van Den Boogert, P. H. J. F., Termorshuizen, A. J., & Van Baarlen, P. (1996). Pathology and control of soil borne fungul pathogens of potato. *Potato Research, 39*(3), 437–69.

Kumar, B. P. (1976). *Studies on root rot and seedling blight of wheat* (*Triticum aestivum L.)*. (PhD Thesis). Kanpur University, Kanpur, India.

Mian, I. H. (2007). *Advances in plant pathological research in Bangladesh*. Gazipur, Bangladesh: Bangladesh Agricultural Research Institute.

Naz, F. (2006). *Integrated management of black scurf disease of potato.* (Ph.D. Dissertation). University of Arid Agriculture Rawalpindi, Pakistan.

Narayanasamy, P. (2013). Cultural practices influencing biological management of crop diseases. In *Biological Management of Diseases of Crops* (vol. 2, pp. 9–56). New York: Springer Publishing.

Rahman, M. M., Ali, M. A., Dey, T. K., Islam, M. M., & Khalequzzaman, K. M. (2016). Effect of organic amendment on stem canker and black scurf disease of potato (*Solanum tuberosum)*. *Bioscience Journal*, *32*(2), 361–370.

Shaikh, A. H., & Ghaffar, A. (2004). Effect of poultry manure and sawdust on survival of sclerotia of *Macrophomina phaseolina* in soil. *Pakistan Journal of Botany, 36*(2), 425–428.

Sharma, V. C., Vashisth, K. S., & Sahai, D. (1975). Black scurf disease of potato. In *25th scientific report for the year 1974-1975.* Simla, India: Central Potato Research Institute (CPRI) Press.

Steel, R. C. B., & Torii, J. H. (1960). *Principles and procedures of statistics*. New York, NY: McGraw Hall.

Swain, S., Harnik, T., Mejia-Chang, M., Hayden, K., Bakx, W., Creque, J., & Garbelotto, M. (2006). Composting is an effective treatment option for sanitization of Phytophthora ramorum - Infected plant material. *Journal of Applied Microbiology*, *101*(4), 815–827.

Swaminathan, M. S. (2000). Potato for global security. In S. M. P. Khurana, G. S. Shekhawat, & S. K. Pandey (Eds.), *Potato global research and development* (p. 302). Shimla, India: Indian Potato Association.

Wharton, P., & Wood, E. (2017). *Rhizoctonia stem canker and black scurf of potato*. Retrieved April 27, 2017, from https://www.cals.uidaho.edu/edcomm/pdf/CIS/CIS1198.pdf

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Characterization of Phytochemical Compounds and Antimicrobial Activity of Crude Alkaloid from Papaya (*Carica papaya* L. var. Eksotika) Leaf Extract

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ABSTRACT

The papaya plant yields a lot of by-products after its fruit is harvested. Papaya leaves contain a bio-active phytochemical that can serve as a potential source of natural antibacterial agents. Phytochemical screening has discovered that papaya leaf extract contains compounds such as alkaloids, tannins and saponins. Crude alkaloid detection by Dragendorff's test was positive. Chemical structure characterisation of extracted crude alkaloids revealed by ^{1}H-NMR spectrum testing was partial, as the spectrum identified only the chemical structure corresponding to aliphatic carbon compounds. Crude alkaloid dissolved in organic solvent dichloromethane showed no antifungal properties towards *Candida albicans*. Antibacterial properties of crude alkaloid dissolved in dichloromethane were selective on both Gram positive and negative bacteria. Kirby Bauer test results showed negative inhibition for both *Staphylococcus aureus* (Gram positive) and *Salmonella typhi* (Gram negative) but inhibition was positive for antibiotic resistant bacteria, MRSA (Gram positive) and *Escherichia coli* (Gram negative) bacteria. Inhibition efficacy increased with the amount of crude alkaloids used. The present study supports the use of papaya by-products as an alternative natural antibiotic for both the community and healthcare personnel.

Keywords: Alkaloid, *Carica papaya* leaves, dichloromethane, ^{1}H-NMR, antibacterial and antifungal properties

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INTRODUCTION

The leaves of the *Carica papaya* plant, locally known as 'betik' in Malaysia have been widely utilised as traditional medicine for centuries in this country. The leaf extract is used traditionally to treat intestinal worm infection, gastric pain, fever and

amoebic dysentery as genito urinary ailment, to relieve symptoms of asthma, as a vermifuge (Teixeira et al., 2007). The leaves of *C. papaya* contain many bio-active phytochemical compounds in different concentrations such as steroidal saponins, flavonols, tannins, phenols and alkaloids as well as organic acids and unsaturated sterols (Head & Lauter, 1956). Phytochemicals are categorised as secondary metabolites, which are organic compounds that are not involved directly in normal growth, development or reproduction of an organism. These phytochemical compounds might independently or synergistically interact to react against pathogens active in the plant.

Alkaloids are naturally occurring chemical compounds and can be found in most plant leaves. Alkaloids in the form of carpaine and carpasemine are reported to be found abundantly in *C. papaya* leaves (Burdick, 1971; Chávez-Quintal, González-Flores, Rodríguez-Buenfil, & Gallegos-Tintoré, 2011). The function of these alkaloid forms vary. For instance, carpaine present in papaya leaves native to Indonesia and Central and South America was believed to be harmful (Head & Lauter, 1956). In other reports, carpaine present in *C. papaya* leaves in general was found not to be microbicidal (Nkuo-Akenji, Ndip, McThomas, & Fru, 2001; Dawkins, Hewitt, Wint, Obiefuna, & Wint, 2003; Leite, Nardi, Nicoli, Chartone-Souza, & Nascimento, 2005; Nayak, Pereira, & Maharaj, 2007). Moreover, many studies have reported that antimicrobial and antifungal properties of papaya leaf extract were dependable on the type of solvent being used. For instance, papaya leaf extract dissolved in alcohol-based solvents such as ethanol and methanol has been known to show positive antibacterial and antifungal properties. However, papaya leaf extract dissolved in hot water was unable to inhibit microbe and fungi activity (Baskaran, Bai, Velu, & Kumaran, 2012).

The papaya plant yields a lot of by-products after its fruit is harvested. These by-products include the stem and leaves, which are usually disposed of in open areas and left to rot. Eventually, phytopathogens will inhabit and grow on the waste that will subsequently cause ecological problems and health risks for human (Thomas et al., 2009). A potential solution to this problem of papaya waste is to extract the bioactive compounds and exploit their usage. To the best of our knowledge, comprehensive information on the efficacy of crude alkaloids dissolved in organic solvents such as dichloromethane on bacteria and fungi has not been elucidated so far. Hence, this study was carried out a) to profile phytochemical components of papaya leaf extract and b) to test the antimicrobial and antifungal efficacy of crude alkaloids dissolved in dichloromethane against selected strains of bacteria and fungi.

MATERIALS AND METHOD

Plant Sample Collection

Fresh leaves of papaya (*Carica papaya* L. var. Eksotika) were randomly sampled from Merlimau, Malacca, Malaysia. All the

leaf samples were dried in an incubator at 55°C and finely crushed with a mortar and pestle prior to use.

Extracts and Phytochemicals Screening to Identify Active Compounds

Phytochemical tests for alkaloids, flavonoids, saponins and tannins were carried out according to protocol described by Harborne (1973) with some modification. Initial detection of alkaloids was tested by moistening approximately 20 g of dried leaves with 10% ammonia solution. On two separate occasions, the crushed leaves were soaked completely with dichloromethane or methanol until a uniform pulp was formed. The leaf mixture soaked in dichloromethane or methanol was heated for 30 min followed by normal filtration. Hydrochloric acid (5%) was added to the collected filtrate and the mixture was tested with 2 to 3 drops of Mayer's reagent. A positive reading for alkaloids is indicated by the appearance of a turbid or milky solution.

Flavonoids were tested for by soaking 20 g of crushed dried leaves with dichloromethane or methanol on two separate occasions and filtered. Next, 2 to 3 drops of concentrated hydrochloric acid was added together with a strip of magnesium ribbon into the filtrate. The appearance of the colours red or blue green after a few seconds was considered a positive reading.

Saponins were tested for using the froth test. Briefly, approximately 20 g of crushed dried leaves was soaked in either dichloromethane or methanol on two separate occasions and were filtered. Next, 3 ml of boiled distilled water was added to the filtrate and the mixture was shaken vigorously. The mixture was left at room temperature for 30 min. The formation of froth was considered a positive reading.

Tannins were determined by dissolving 20 g of crushed dried leaves soaked in either dichloromethane or methanol in potassium hydroxide (10%). The formation of a white precipitation indicated the presence of tannins.

Extraction of Crude Alkaloids

Extraction of alkaloids was carried out according to protocols described by Head and Lauter (1956) with modification. The leaves of *C. papaya* were dried and finely ground, then de-fattened, which involved soaking in hexane for 3 days at room temperature in a Soxhlet extractor and eventually being filtered. The filtrate was dried at room temperature for 24 h and was subsequently moistened by ammonium (25%). Next, dichloromethane was added and the mixture was left at room temperature for 17 h and filtered. The filtrate was concentrated to approximately 500 ml in a rotary evaporator. The solution was filtered and 10 ml of hydrochloric acid (5%) was added to the filtrate. The mixture was left to settle until two layers of solution were formed. The top layer of solution was removed and made basic by adding ammonia (10%) continuously until pH 11 was achieved. Next, 50 ml of dichloromethane was added to the solution and it was left to settle. The bottom

aqueous layer was removed, rinsed with water and precipitated using anhydrous sodium sulfate. Subsequently, the mixture was filtered and the collected filtrate was air-dried to obtain crude alkaloids.

An alkaloid stock solution was prepared by dissolving 50 g of crude alkaloids in 10 ml of dichloromethane. Finally, the stock solution was diluted with dichloromethane into a series of different amounts of solution i.e. 10, 20 and 30 mg/ml. The different amounts of crude alkaloid were achieved by performing a serial dilution from the stock solution. Intermediary dilution was carried out if the amount of stock was too small to be pipetted.

Identification of Crude Alkaloids

The crude alkaloid extract was assayed using Dragendroff's reagent. Briefly, the crude alkaloid was first separated using the thin liquid chromatography (TLC) method, where it was soaked in dichloromethane and then the alkaloids were identified by spraying the TLC plate with Dragendorff's reagent. The presence of orange spots was considered a positive reading.

Nuclear Magnetic Resonance Spectrometry ^{1}H-NMR

An amount of 3 mg of crude alkaloids was dissolved in deuterated chloroform ($CDCl_3$) and a ^{1}H-NMR spectrum was taken on a nuclear magnetic resonance (JOEL 500 MHz JNM-FX-100) spectrometer with tetramethylsilane (TMS) as the internal standard. The chemical shift, δ, was expressed in parts per million (ppm).

In Vitro Antimicrobial and Antifungal Assay

Several bacterial strains (two Gram negative, one Gram positive and one antibiotic resistant Gram positive) and a fungal strain were obtained from the Microbiology Laboratory, Universiti Pendidikan Sultan Idris (UPSI). The strains used were: *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus* (MRSA) and *Candida albicans*. The strains were kept in LB agar and the cultures were stored at 37°C in an incubator.

Bacterial and fungal growth inhibition was carried out using the standard Kirby Bauer test on crude alkaloids only. The zone of inhibition was measured using a ruler in millimetres in three replicates per set-up. The results were expressed as the average zone of inhibition diameter of different alkaloid amounts (10, 20 and 30 mg/ml) and dichloromethane solvent was used as a negative control for the different bacterial and fungal strains.

RESULTS

Phytochemical Screening

Phytochemical screening detected the presence of alkaloids in the leaf extract soaked in dichloromethane only while the test for saponins and tannins was positive only for extracts soaked in methanol. Flavonoids were not detected in the leaf extract for both the dichloromethane and methanol treatment (Table 1). On the other hand, identification of crude alkaloids using Dragendroff's reagent was positive.

Table 1
Qualitative identification of phytochemical compounds of papaya leaves soaked in dichloromethane and methanol

By-product	Solvent	Alkaloids	Flavonoids	Saponins	Tannins
Leaves	Dichloromethane	+	-	-	-
	Methanol	-	-	+	+

Positive (+), negative (-)

Alkaloid Characterisation via Nuclear Magnetic Resonance Spectrometry ^{1}H-NMR

The ^{1}H-NMR spectrum revealed the following peaks for extracted crude alkaloids: δ ^{1}H (500 MHz, $CDCl_3$): 1.0 – 2.0, as shown in Figure 1. The ^{1}H-NMR spectrum of extracted alkaloids corresponded with aliphatic carbon compounds. The presence of halogens, aromatics, carboxyl and aldehyde functional groups revealed by ^{1}H-NMR spectrum was highly unlikely.

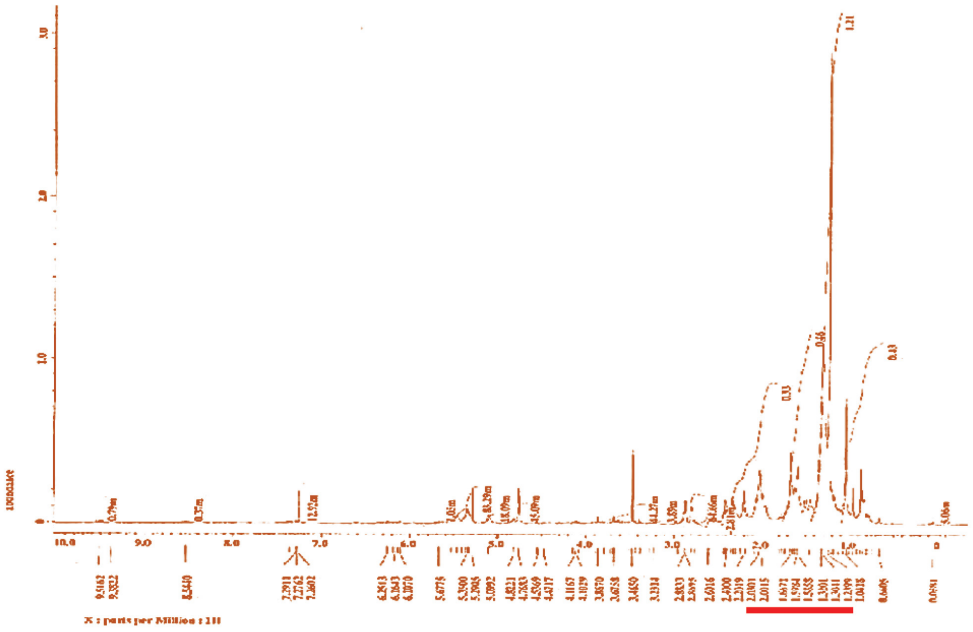

Figure 1. The ^{1}H-NMR spectrum of crude alkaloid extract from papaya leaves. The peak of chemical shift range, δ ^{1}H (500 MHz, $CDCl_3$), 1.0 – 2.0, is highlighted by the red line

In Vitro Antimicrobial and Antifungal Property

The antifungal assay results obtained in this study showed no inhibiting properties of alkaloids towards the only fungal strain tested, *C. albicans.* Interestingly, antimicrobial assay inhibition of alkaloids towards strains of Gram positive and Gram negative bacteria was selective. Inhibition was negative for both *S. aureus* (Gram positive) and *S. typhi* (Gram negative). However, the Gram positive antibiotic resistant bacteria, MRSA, and the Gram negative bacteria, *E. coli*, were susceptible to alkaloid treatment (Table 2).

Inhibition efficacy as measured by the zone of inhibition diameter showed increment in the diameter when the amount of alkaloid increased. In other words, the zone of inhibition increased with the amount of alkaloids used (Table 2).

Table 2
Mean and standard deviation (*means ± STDEV*) *of inhibition zone on fungal and bacterial strains*

Amount (mg/ml)	Zone of Inhibition Diameter (mm)				
	Candida albicans	*Escherichia coli*	*Salmonella typhi*	*Staphylococcus aureus*	*Methicillin-Resistant Staphylococcus aureus* (MRSA)
Control	-	-	-	-	-
10	-	6.2 ± 0.06	-	-	7.1 ± 0.11
20	-	6.7 ± 0.06	-	-	8.0 ± 0.11
30	-	7.2 ± 0.10	-	-	9.3 ± 0.20

No inhibition (-)

DISCUSSION

Alkaloids are a secondary metabolite compound that are found abundantly in the leaves of plants. The presence of alkaloids is believed to serve as a plant natural's defence system against plant pathogens (Oliva, Meepagala, & Wedge, 2003) and as a repellent to herbivores. Besides that, humans have been using secondary metabolites from plants as medicine, flavouring and recreational drugs for centuries. However, several reports have shown that alkaloid ingestion by humans resulted in physiological defects such as inhibition of enzymes, neurotransmission interference and loss of coordination and caused hallucinations, convulsions, vomiting and even death (Agosta, 1997; Karban & Baldwin, 1997; Bidlack, Omaye, Meskin, & Topham, 2000; Rosenthal & Berenbaum, 2012).

The function of alkaloids in papaya leaf cannot be overlooked as many researchers have identified alkaloids as having antibacterial and antifungal properties (Chávez-Quintal et al., 2011;

Krishna, Paridhavi, & Patel, 2008). For instance, Krishna et al. (2008) reported that herbal formulation of papaya leaves alone showed antibacterial activity towards *S. typhi*. However, our study discovered that crude alkaloid extract did not show any antibacterial inhibition towards *S. typhi*. This might have been due to the different types of solvent used in the two studies. Perhaps, antibacterial properties of papaya leaves are less effective when dissolved in dichloromethane rather than a herbal formulation. On the other hand, Chávez-Quintal et al. (2011) discovered that leaf extracts dissolved in an organic solvent was a better antifungal inhibitor compared to when dissolved in an aqueous solvent. However, in our study, no antifungal inhibition of *C. albicans* was detected when the alkaloid leaf extract was dissolved in an organic solvent, dichloromethane. Perhaps, alkaloids from the leaves alone are insufficient to inhibit fungal growth. Possibly, fungal inhibition might have been effective on *C. albicans* if the treatment had involved alkaloids present together with other phytochemical compounds. Indeed, studies by Giordani, Gachon, Moulin-Traffort and Regli (1997) had shown that papaya latex together with Fluconazole, an antifungal medication, had synergistic action against *C. albicans* inhibition.

All the three different amounts of crude alkaloid extract used during antibacterial treatment was effective as MRSA and *E.coli* inhibitors. Furthermore, this study also showed a significant increment in inhibition efficacy with the amount of alkaloids used. The Gram positive bacteria, MRSA, was believed to have been more prone towards treatment using antibiotics due to its lack of an outer membrane that could impede entry of drugs. On the other hand, Gram negative bacteria such as *E. coli* was expected to be more resistant towards antibiotics because of the presence of an outer membrane in its cell wall, a common feature in Gram-negative bacteria. However, in this study crude alkaloid extract from papaya leaves showed positive inhibition towards *E. coli*. Further to this, empirical results from other researchers had likewise shown that papaya seed and pulp extract was bacteriostatic against several enteropathogens including *E. coli* (Osato, Santiago, Remo, Cuadra, & Mori, 1993). MRSA is very often associated with healthcare due to the prevalence of MRSA-caused illness among health caregivers. This strain of bacteria, once prone to methicillin treatment, developed resistance by modifying penicillin-binding protein on its cell wall (Van Bambeke, Mingeot-Leclercq, Struelens, & Tulkens, 2008). The present study, which showed positive inhibition of MRSA growth by crude alkaloid extract, proved the potential of crude alkaloid extract as an alternative natural antibiotic. At this stage, *in vivo* experiments need to be undertaken simultaneously to test consistency between *in vivo* and *in vitro* results. *In vivo* conditions can be complex and might cause susceptibility of treatment, causing results to differ from those observed in *in vitro* conditions (Ekanem, Obiekezie, Kloas, & Knopf, 2004).

The ^{1}H-NMR spectrum on crude alkaloid extract obtained in this study revealed that δ ^{1}H (500 MHz, $CDCl_3$) was 1.0-2.0, corresponding with aliphatic carbon compounds. Conventionally, many researchers have associated alkaloids present in papaya with carpaine (Burdick, 1971; Fhaizal et al., 2014), but in this study, the exact chemical structure classification based on the ^{1}H-NMR spectrum results obtained was only partial and imprecise, making it difficult to make any conclusive predictions. In fact, Govindachari (2002) and Tang (1979) revealed that carpaine from papaya leaves inadvertently contained other impurities such as carpane, carpamic acid and dehydrocarpaine 1 and 2, making chemical structure identification difficult.

Papaya by-products such as leaves contain bio-active phytochemicals that can serve as a potential source of natural antibacterial agents. This study showed that papaya leaf extract contains secondary metabolites such as saponins, tannins and alkaloids. The crude alkaloid extract soaked in dichloromethane exhibited selective antibacterial properties towards Gram-positive (MRSA) and Gram-negative (*E. coli*) bacteria. The present study supports the potential use of papaya by-products as alternative natural antibiotics for both the community and healthcare personnel.

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REFERENCES

Agosta, W. C. (1997). *Bombardier Beetles and Fever Trees: A Close-up look at chemical warfare and signals in animals and plants*. Retrieved August 26, 2016, from http://go.galegroup.com/ps/i.do?id=GALE%7CA19344881&sid=googleScholar&v=2.1&it=r&linkaccess=fulltext&issn=00063568&p=AONE&sw=w

Baskaran, C., Bai, V. R., Velu, S., & Kumaran, K. (2012). The efficacy of *Carica papaya* leaf extract on some bacterial and a fungal strain by well diffusion method. *Asian Pacific Journal of Tropical Disease*, *2*(SUPPL2), S658–S662. http://doi.org/10.1016/S2222-1808(12)60239-4

Bidlack, W. R., Omaye, S. T., Meskin, M. S., & Topham, D. K. W. (2000). *Phytochemicals as bioactive agents*. Lancester, PA: Technomic Publishing Company Inc. http://doi.org/10.1016/S0144-8617(01)00296-X

Burdick, E. M. (1971). Carpaine: An alkaloid of *Carica papaya* – Its chemistry and pharmacology. *Economic Botany*, *25*(4), 363–365. http://doi.org/10.1007/BF02985202

Chávez-Quintal, P., González-Flores, T., Rodríguez-Buenfil, I., & Gallegos-Tintoré, S. (2011). Antifungal activity in ethanolic extracts of *Carica papaya* L. cv. maradol leaves and seeds. *Indian Journal of Microbiology*, *51*(1), 54–60. http://doi.org/10.1007/s12088-011-0086-5

Dawkins, G., Hewitt, H. H., Wint, Y., Obiefuna, P. C. M., & Wint, B. (2003). Antibacterial effects of *Carica papaya* fruit on common wound organisms. *West Indian Medical Journal*, *52*(4), 290–292. Retrieved from http://europepmc.org/abstract/med/15040064

Ekanem, A. P., Obiekezie, A., Kloas, W., & Knopf, K. (2004). Effects of crude extracts of Mucuna pruriens (Fabaceae) and *Carica papaya* (Caricaceae) against the protozoan fish parasite Ichthyophthirius multifiliis. *Parasitology Research*, *92*(5), 361–366. http://doi.org/10.1007/s00436-003-1038-8

Fhaizal, M., Bukhori, M., Rahman, N. A., Khalid, N., Rashid, A. H., & Diah, M. M. (2014). The supercritical fluid extraction of alkaloids from papaya (*Carica papaya* L. var. Eksotika) leaves. *Borneo Jurnal of Resource Science and Technology*, *4*(2), 35–49.

Giordani, R., Gachon, C., Moulin-Traffort, J., & Regli, P. (1997). A synergistic effect of *Carica papaya* latex sap and fluconazole on Candida albicans growth. *Mycoses, 40*(11-12), 429-437. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1439-0507.1997.tb00180.x/abstract

Govindachari, T. (2002). Five decades in the study of natural products. *Journal of Chemical Sciences, 114*(3), 175-195. Retrieved from http://link.springer.com/article/10.1007/BF02704262

Harborne, J. B. (1973). Phenolic compounds. In *Phytochemical methods* (pp. 33–88). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-94-009-5921-7_2

Head, W. F., & Lauter, W. M. (1956). Phytochemical examination of the leaves of *carica papaya* L. *Economic Botany*, *10*(3), 258–260. http://doi.org/10.1007/BF02899003

Karban, R., & Baldwin, I. T. (1997). *Induced responses to herbivory*. London: The University of Chicago Press. Retrieved from https://books.google.com.my/books?hl=en&lr=&id=S5rPc_VNVMoC&oi=fnd&pg=PR7&dq=Induced+Responses+to+Herbivory.+&ots=NlrA0bX7Zj&sig=x64c-WD1BaIADCKrqPYJMGy2d1s&redir_esc=y#v=onepage&q=Induced Responses to Herbivory.&f=false

Krishna, K. L., Paridhavi, M., & Patel, J. A. (2008). Review on nutritional, medicinal and pharmacological properties of papaya (*Carica papaya* linn.). *Indian Journal of Natural Products and Resources*, *7*(4), 364–373. http://doi.org/0975-6299

Leite, A. a M., Nardi, R. M. D., Nicoli, J. R., Chartone-Souza, E., & Nascimento, A. M. a. (2005). *Carica papaya* seed macerate as inhibitor of conjugative R plasmid transfer from Salmonella typhimurium to Escherichia coli in vitro and in the digestive tract of gnotobiotic mice. *The Journal of General and Applied Microbiology*, *51*(1), 21–6. http://doi.org/10.2323/jgam.51.21

Nayak, B. S., Pereira, L. P., & Maharaj, D. (2007). Wound healing activity of *Carica papaya* L. in experimentally-induced diabetic rats. *Indian Journal of Experimental Biology*, *45*(August), 739–743.

Nkuo-Akenji, T., Ndip, R., McThomas, A., & Fru, E. C. (2001). Anti-Salmonella activity of medicinal plants from Cameroon. *The Central African Journal of Medicine*, *47*(6), 155–8. Retrieved from http://www.academia.edu/download/30967153/Bioconversion-of-Renewable-12-2004.pdf#page=229

Oliva, A., Meepagala, K., & Wedge, D. (2003). Natural fungicides from Ruta graveolens L. leaves, including a new quinolone alkaloid. *Journal of Agricultural, 51*(4), 890-896. Retrieved from http://pubs.acs.org/doi/abs/10.1021/jf0259361

Osato, J., Santiago, L., Remo, G., Cuadra, M., & Mori, A. (1993). Antimicrobial and antioxidant activities of unripe papaya. *Life Sciences, 53*(17), 1383-1389. Retrieved from http://www.sciencedirect.com/science/article/pii/002432059390599X

Rosenthal, G. A., & Berenbaum, M. R. (2012). *Herbivores: Their interactions with secondary plant metabolites: Volume II: Ecological and Evolutionary Processes* (Vol. 2). Retrieved from https://books.google.com/books?hl=en&lr=&id=g6BlbAfDveQC&oi=fnd&pg=PP1&dq=Herbivores,+Their+Interactions+with+Secondary+Plant+Metabolites.&ots=jPjcpRVr3x&sig=uVD-dm02ZxYt5o_nzLnr1M-wFJE

Tang, C. (1979). New macrocyclic, Δ1-piperideine alkaloids from papaya leaves: dehydrocarpaine I and II. *Phytochemistry, 18*(4), 651-652. Retrieved from http://www.sciencedirect.com/science/article/pii/S003194220084279X

Teixeira, Rashid, Z., Nhut, D. T., Sivakumar, D., Gera, A., Teixeira, M., & Tennant, P. F. (2007). Papaya (*Carica papaya* L.) Biology and Biotechnology. *Tree and Forestry Science and Biotechnology, 1*(1), 47-73. Retrieved from https://www.researchgate.net/profile/Jaime_Teixeira_Da_Silva/publication/283224907_Papaya_Carica_papaya_L_Biology_and_Biotechnology/links/5643f12108ae9f9c13e39a93.pdf

Thomas, G. E., Rodolfo, H. G., Juan, M. D., Georgina, S. F., Luis, C. G., Ingrid, R. B., & Santiago, G. T. (2009). Proteolytic activity in enzymatic extracts from *Carica papaya* L. cv. Maradol harvest by-products. *Process Biochemistry*, *44*(1), 77–82. http://doi.org/10.1016/j.procbio.2008.09.013

Van Bambeke, F., Mingeot-Leclercq, M. P., Struelens, M. J., & Tulkens, P. M. (2008). The bacterial envelope as a target for novel anti-MRSA antibiotics. *Trends in Pharmacological Sciences*, *29*(3), 124–134. http://doi.org/10.1016/j.tips.2007.12.004

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Isolation and Characterization of Genotype VII Newcastle Disease Virus from NDV Vaccinated Farms in Malaysia

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ABSTRACT

Molecular analysis, particularly sub-genotype classification, and study on the relationship of recent Malaysian NDVs with other isolates from around the world are lacking. Therefore, in the present study, a molecular epidemiological investigation was conducted to characterise six Newcastle disease viruses (NDV) isolated between 2014 and 2015 from vaccinated commercial poultry flocks. Partial Fusion (F) and Hemagglutinin-neuraminidase (HN) genes were amplified from IBS046/2014, IBS060/2014, IBS061/2014, IBS074/2014, IBS160/2015, and IBS162A/2015 isolates using one-step reverse transcription polymerase chain reaction (RT-PCR), sequenced and phylogenetically analysed. Sequence and phylogenetic analysis revealed that all the recently isolated strains of NDV belonged to sub-genotype VIIa and lineage 5a. Moreover, deduced amino acid sequence at the F protein cleavage site of the isolates revealed either ^{112}RRQKRF^{117} or ^{112}KRRKRF^{117} consistent with the motif found in velogenic pathotypes. The study concluded that the genotype VIIa was the causative agent of recent ND outbreaks in vaccinated broiler flocks from Malaysia. Interestingly, five out of the six isolates characterised in this study had a unique F0 protein cleavage site (^{112}KRRKRF^{117}). Further studies are required to determine the role of these motifs on the virulent potential of the isolates.

Keywords: Genotype VII Newcastle disease virus, F protein cleavage site, F and HN phylogenetic analysis

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INTRODUCTION

Newcastle Disease Virus (NDV) is similar to avian paramyxovirus-1(APMV 1), which is a member of the Avulavirus genus in the Paramyxoviridae family, including a different collection of single-stranded, non-segmented, negative-sense enveloped

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RNA viruses that are about 15.2 kb. NDV has a wide range of hosts and is known for its ability to infect more than 200 different species of bird (Fauquet & Fargette, 2005). The genome of NDV encodes for six main essential proteins: phosphoprotein (P), hemagglutinin-neuraminidase (HN), matrix (M), nucleocapsid (NP), fusion (F), the RNA dependent RNA polymerase (large structural protein) (L), and also for a seventh protein (V), which is resulted through frame shifts that are bordered by the P coding region (Chambers & Samson, 1982; Collins et al., 1982). Viruses with low virulence are habitually exploited as vaccines and characteristically cause mild diseases that are associated with respiratory organs or the digestive system. The clinical signs of Newcastle Disease (ND) are moderate and sometimes, the disease may present only mild respiratory symptoms with sporadic nervous signs and sometimes, death may occur. Severe forms of ND are classical in animal organs that are affected by NDV. The viscerotropic form of ND causes widespread hemorrhaging in several organs of the gastrointestinal tract with slight nervous signs, while the neurotropic form principally affects the central nervous system with little or no additional gross injuries or lesions (Alexander, 2000).

NDV of low virulence has monobasic fusion cleavage location motifs at amino acid (aa) positions 112-113 and 115-116 and a leucine (L) at position 117 of the F protein (Glickman et al., 1988) that will only cleave through trypsin-like enzymes that are within the intestinal and respiratory tracts, limiting their duplication in these systems (Aldous & Alexander, 2001). The virulent ND viruses have various basic amino acids in the fusion cleavage site namely, ^{112}R-K/R-Q-K/R-R-F^{117}. Additionally, the length of HN genes has often been used to classify NDV into virulent and avirulent strains. In the case of the virulent strains, the length of the HN protein is 571aa (Munir et al., 2012; Wang et al., 2013) while most lentogenic strains, including the conventional LaSota vaccine strains and other genotype 1 strains have either 577 or 616 amino acids (Tirumurugaan et al., 2011; Yuan et al., 2012). Analysis of the HN gene sequence shows several enteric NDV strains having low virulence possess an open-reading frame (ORF) that is large (616 amino acids) with extra 45 aa at the C-terminus comparing to some virulent and some low virulent NDV strains (571 and 577 aa). Presently, molecular epidemiological studies are not available on the outbreak causes of ND among commercial poultry farms vaccinated against ND in Malaysia. Molecular investigation of the sub-genotype of NDVs and also analysis of the relationship between recent Malaysian NDVs and other isolates from different parts of the world, in particular, are not available. In view of the unavailability of data showing the relationship between the recent Malaysian NDV strain and other isolates from different region of the globe, the aim of this present study was to determine the molecular epidemiology and to characterise six Newcastle Disease

Viruses (NDV) isolated between 2014 and 2015 from vaccinated commercial poultry flocks in Malaysia.

MATERIALS AND METHOD

Isolation of NDV

Viral RNA extraction. The Laboratory of Vaccine and Immunotherapeutics, Institute of Bioscience (IBS)/UPM received tissue samples of suspected cases of NDV outbreaks between 2014 and 2015 for diagnosis of NDV. The isolates were from different parts of Malaysia: IBS 160/2015 and IBS 074/2014 were from Johor; IBS 162A/2015 was from Melaka; IBS 060/2014 and IBS 046/2014 were from Perak; and IBS 061/2014 was from Penang. All the isolates were obtained from vaccinated flocks. Viral RNA was extracted using TRIzol LS® (Life Technologies, USA) via phenol and guanidine thiocyanate system with some modification. The processed sample (250 µL) was mixed with 750 µL TRIzol LS® in a 1.5-mL micro-centrifuge tube and incubated at room temperature for 5 min. Chloroform (200 µL) was added and the tube was vigorously shaken to mix the contents well and then incubated at room temperature for another 10 min. After that, the tubes were centrifuged at 12,000 xg for 20 min at 4°C. The colourless aqueous top layer was collected and mixed with 800 µL isopropanol. After 15 min of incubation at room temperature, all the samples were centrifuged at 12,000 xg for 20 min at 4°C. Then, the supernatants were discarded and the pellet was washed twice with 75% of ethanol (800 µL) and absolute ethanol (1000 µL), respectively. Finally, the RNA pellets were air-dried in laminar flow, dissolved with RNase-free water (Promega, USA) and stored in a -70°C freezer for future use. RNA concentration and purity were measured using a UV/Visible spectrophotometer (Ultraspect 3000 Pro-Biochorm, UK) based on the method described by Wilkinson (Martin et al., 2001). The optical density of each sample was measured at both 260 nm and 280 nm wavelength. Concentration and 260/280 absorbance ratio were recorded. Only RNA with the ratio of 1.8 to 2.0 was used in the following study.

F and HN gene RT-PCR amplification

Reverse transcriptase-polymerase chain reaction (RT-PCR) PCR amplification, primers and sequencing were achieved through the use of degenerative primers 5'-ATGGGC(C/T) CCAGA(C/T) CTTCTAC-3' (sense) and 5'-CTGCCACTGCTAGTTGTGATAATC C-3' (antisense), which are precise to fusion (F) protein gene (Yang et al., 1999)1984, and 1995. The sequences 5'-ATATCCCGCAGTCGCATAAC-3'(sense) and 5'-TTTTTCTTAATCA AGTGACT-3' (antisense) were specific to the HN protein gene (Peroulis-Kourtis et al., 2002) (Table 3). This primer produced an estimated size of band of the amplicon of 535 bp (nt 47-535) fragment covering from nucleotides 47 to 581 of the fusion protein that comprised the F0 cleavage position and 320 bp products, demonstrating fragmentd

inside HN protein gene, separately. Standard RT-PCR was implemented by means of the SuperScript®III One-Step RT-PCR kit (Invitrogen, USA) in 25 µl reaction volume. The cycling parameters of F and HN genes' specific primers were 50°C for 30 min at reverse transcription (RT), followed by an initial denaturation of 2 min at 94°C; 40 cycles of 15 s of denaturation at 94°C; 30 s of annealing at 59°C (48°C for HN); and 1 min (30 s for HN) of extension at 68°C and finally, extension at 68°C for 5 min using C1000 Touch™ thermo-cycler (Bio-Rad, USA). A percentage of 1.5% agarose gel was used to separate the amplicons by electrophoresis, and the gel eas then stained in ethidium bromide before final visualisation under ultraviolet light using gel doc (Bio-Rad, USA).

Partial NDV F and HN Gene Sequencing

Samples that were positive for NDV amplification were analysed by sequence analysis (ABI PRISM® 377 DNA Sequencer). Gel purified RT-PCR products for both partial F and HN genes from each of the samples were sent to 1st Base™ Sdn Bhd (Kuala Lumpur, Malaysia).

Sequence Alignment, Analysis and Phylogenetic Study

The Basic Local Alignment Search Tool (BLAST) was used to analyse the raw sequence data and the sequence were compared with other sequences in GenBank NCBI (Johnson et al., 2008). The evolutionary relationship of F and HN genes of NDV isolates in our study was compared with other NDV isolates of different genotypes recorded in other studies (39 isolates for F gene and 31 isolates for HN gene) (Table 1).

Analysis of sequences and phylogeny of the partial F and HN genes was done using the ClustalW multiple alignment method of the Molecular Evolutionary Genetics Analysis Version 6 (MEGA 6) software (Tamura et al., 2011) that employed 1000 bootstrap replications. Construction of the phylogenetic trees was done using the maximum likelihood method (Zhang & Sun, 2008).

Table 1
Newcastle disease virus (ndv) strains used in this study for phylogenetic analysis of the fusion protein gene and Hemagglutinin-Neuraminidase Gene

F Gene	Genotype	HN Gene	Genotype
HM125898_China_I_2004	I	HM063422 isolate D3 I	I
DQ097394__Hungary_I	I	JX401404 isolate CBU2179 I	I
JX401404_Korea_I_2007	I	FJ939313 NDV/Chicken/Egypt/1/2005 II	II
JX524203_Australia_V4_I_1966	I	AF07761 LaSota 578aa II	II
HM063422_D_3_China_I	I	JX193082 duck/China/Guangxi21/2010 II	II
AF077761 B1_USA__II_1947	II	HQ902590 NDV2K17/Quail/Chennai/ India/1998 II	II
Y18898_Clone_30_USA__II	II	Y18898 clone 30 II	II

HQ902590_India_II_1998	II	FJ430160 isolate JS/9/05/Go III	III
JF950510_LaSota_USA_ II_1946	II	FJ430159 isolate JS/7/05/Ch III	III
FJ939313_Egypt_II_2005	II	KM056353 isolate NDV55/TN/Namakwa IV	IV
JX193082_China_II_2010	II	KF915807 strain NDV-BJ IX	IX
JF950509_Mukteswar_China_III	III	FJ436302 strain F48E8 IX	IX
FJ430159 isolate JS/7/05/Ch III	III	KC246549 HBNU/LSRC/F3 IX	IX
FJ430160 isolate JS/9/05/Go III	III	HM117720 isolate NDV-P05 V	V
EU293914_Italiano_italy_IV_1944	IV	KJ577136 strain Chimalhuacan V	V
FJ986192 isolate 2K3/Chennai/Tamil Nadu IV	IV	AY562990 isolate mixed species/U.S./ Largo/71 V	V
HM117720_Mexico_2005_V	V	HQ839733 Chicken_Sweden_95 VI	VI
AY562990_Largo_USA_V_1971	V	FJ766527 isolate JS/07/16/Pi VI	VI
AY562988_Fontana_USA_VI_1972	VI	AY562988 isolate chicken/ U.S.(CA)/1083(Fontana)/72 VI	VI
FJ766529 isolate ZhJ-3/97 VI	VI	JN618349 strain JS-3-05-Ch VII 571aa	VII
HQ839733 strain Chicken_Sweden_95 VI	VI	KC542893 isolate Chicken/China/ Liaoning/02/2005VIId 571aa	VII
AY562985_cockato_Indonesia_VII 1990	VII	KF188408 UPM-IBS/002/2011 VII 571aa	VII
JN986837_Netherlands_VII_1993	VII	KM670337strain chicken/Pak/Quality Operations Lab/SFR-611/13 VIIi 571aa	VII
GU585905_Sweden_97_VIIb	VII	HQ697254 strain chicken/Banjarmasin/010/10 VII	VII
JN618348_VII_China_1997	VII	AB605247 strain: NDV/Bali-1/07 VIIa 571aa	VII
GQ901895 strain MB047/05 VII	VII	KC542892 isolate Chicken/China/ Liaoning/01/2005 VIId 571aa	VII
JN800306_Peru_VII_2008	VII	HQ697256 strain chicken/Makassar/003/09 VII 571aa	VII
JX390609_Togo_Peru_VII_2009	VII	HQ697260 strain chicken/Kudus/018/10 VII 571aa	VII
HQ697254_benjamasin_Indonesia_VII_2011	VII	KP776462 strain chicken/NDV/Pak/AW-14 VIIi 571aa	VII
KF026013 IBS_002 Malaysia 2011	VII	FJ751918 isolate QH1 VIII	VIII
HQ697255_sukarjo_Indonesia_VII_2011	VII	FJ751919 isolate QH4 VIII	VIII
JQ823260 IBS005 Malaysia 2011	VII		
JX532092_Pakistan_VII_2012	VII		
FJ751918_west_China_VIII	VIII		
JX012096_AF2240__malaysia_VIII_2010	VIII		
FJ751919_West China_VIII	VIII		
KF915807_China_IX_2013	IX		
FJ436302_F48E9_China_IX	IX		
KC246549_China_IX_2012	IX		

RESULTS AND DISCUSSION

From the phylogenetic analysis using the maximum likelihood method, a phylogenetic tree was constructed based on the partial length F and HN genes nucleotide sequences of six isolates and 39 previously published NDVs. Genotypes I to IX of Class II of NDV were used for the analysis. Furthermore, the phylogenetic relationship of the partial F gene nucleotide sequence of NDV represented by sub-genotype VII for nine sub-genotypes (a b, c, d, e, f, g, h and i) was studied. The results of the analysis revealed that six NDV isolates were grouped as genotype VII (Figure 1) and sub-genotype (VIIa) (Figure 2) and the partial HN gene sequence for the same six NDV isolates was grouped as genotype VII (Figure 3). They were phylogenetically close to previous NDV isolates from Malaysia and Indonesia.

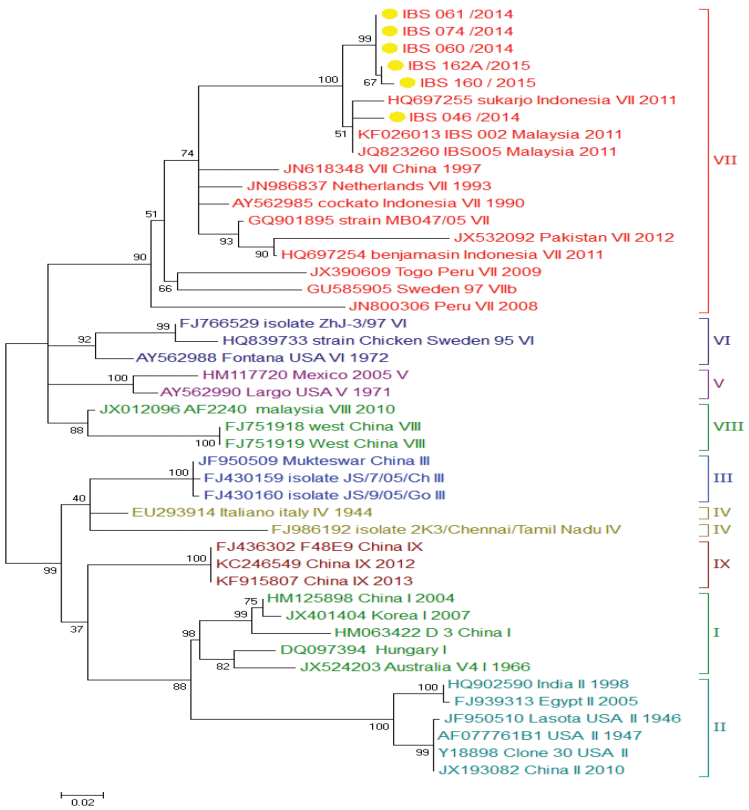

Figure 1. The phylogenetic analysis of Malaysian NDV isolates based on partial sequence of the F protein gene is given here. Viruses highlighted with the coloured circle (●) were characterised in this study. The phylogenetic tree was constructed by the maximum likelihood method as implemented in MEGA 6. Numbers at the nodes indicate a confidence level of bootstrap analysis with 1000 replications as a percentage value. The isolates were grouped under genotype VII

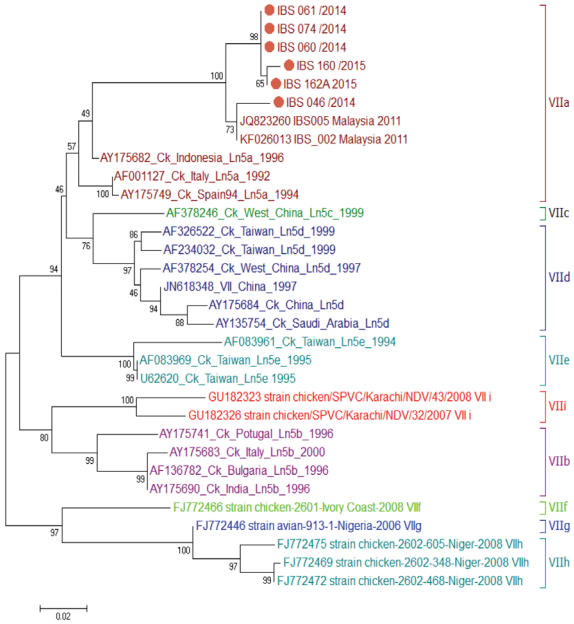

Figure 2. The phylogenetic analysis of Malaysian NDV isolates based on partial sequence of the F protein gene is given here. Viruses highlighted with the coloured circle (●) were characterised in this study. The phylogenetic tree was constructed using the maximum likelihood method as implemented in MEGA 6. Numbers at the nodes indicate a confidence level of bootstrap analysis with 1000 replications as a percentage value. The isolates were grouped under sub-genotype VIIa

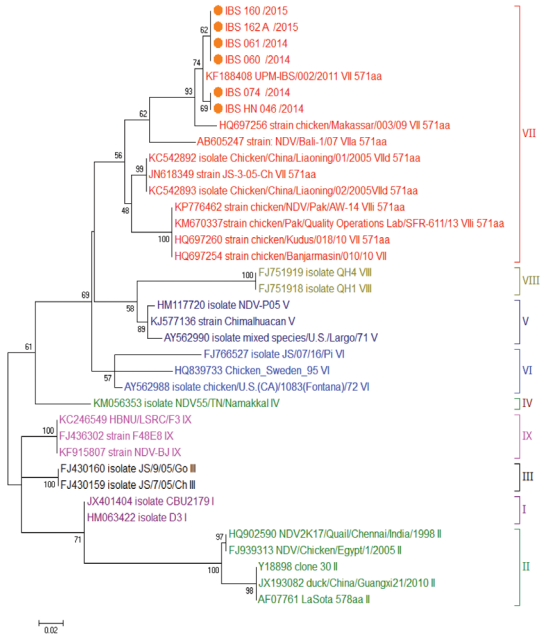

Figure 3. The phylogenetic analysis of Malaysian NDV isolates based on partial sequence of the HN protein gene of NDV isolates is given here. Viruses highlighted with the coloured circle (●) were characterized in this study. The phylogenetic tree was constructed by maximum likelihood method as implemented in MEGA 6. Numbers at the nodes indicate a confidence level of bootstrap analysis with 1000 replications as a percentage value

Detection of NDV and Virus Isolation

A total of six suspected cases had positive RT-PCR. These isolates were designated as IBS 046/2014, IBS 060/2014, IBS 061/2014, IBS 074 /2014, IBS 160/2015 and IBS 162A/2015. All the isolates possessed a multiple basic amino acid motif at the F cleavage site of either ^{112}RRQKRF^{117} or ^{112}KRRKRF^{117} (Table 2).

Table 2
Cleavage site of F Gene of Malaysian NDV isolates

Isolate	Cleavage site
IBS 160 /2015	^{112}KRRKR↓F^{117}
IBS 162A /2015	^{112}KRRKR↓F^{117}
IBS 060 /2014	^{112}KRRKR↓F^{117}
IBS 046 /2014	^{112}RRQKR↓F^{117}
IBS 061/ 2014	^{112}KRRKR↓F^{117}
IBS 074 /2014	^{112}KRRKR↓F^{117}

It is believed that the HN gene can recognise the genotypes of NDV and may expect the pathogenicity of the isolates in light of the fact that the length of the HN protein differs and the cleavage site is not the single criterion for pathogenicity. Along these lines, the phylogenetic examination was directed to utilise the complete coding locale of the HN gene. As a rule, a similar topology of the tree was observed with the Bayesian tree of F quality investigation. IBS 046/2014, IBS 060/2014, IBS061/2014, IBS 074/2014, IBS 160/2015 and IBS 162A/2015 were grouped together under genotype VII in connection to its HN protein length of 571 aa (Table 3).

Table 3
Analysis of C-terminus extension length of HN gene protein

Isolate	Deduce Amino Acid Sequence	C-Terminus Amino Acid Extension Length	HN Length **
IBS046/2014	KDDRV*	0	571
IBS074/2014	KDDRV*	0	571
IBS060/2014	KDDRV*	0	571
IBS061/2014	KDDRV*	0	571
IBS2A/2015	KDDRV*	0	571
IBS 160/2015	KDDRV*	0	571
HQ697256 strain chicken/ Makassar/003/09	KDDRV*	0	571
Y18898 clone 30 II	KDDGVREARSG*	6	577
AF07761 LaSota 578aa II	KDDGVREARSG*	6	577
AY562989 isolate dove/Italy/2736/00	KDDGVREARSG*	6	577
JX524203 strain NDV V4	KDDGVREARSSRLSQLR EGWKDDIVSPIFCDAKN QTEYRRELESYAASWP*	45	616
HM125898 WDK/ JX/7793/2004	KDDGVREARSGRLSQLQ EGWKDDIVSPIFCDIKNQ TEYRRGLESYAASWP*S	45	616
GQ922501 strain MB091/05	KDDRV*	0	571
JX193082 duck/China/ Guangxi21/2010	KDDGVREARSG*	6	577
HQ902590 NDV2K17/ Quail/Chennai/ India/1998	KNDGVREARSG*	6	577
AY562985 isolate cockatoo/ Indonesia/14698/90	KDDRV*	0	571
HM063422 isolate D3 I	KDDGVRKARSG*	6	577
DQ097394 strain PHY-LMV42	KDDGVREARSGRLSQLR EGWKDDIVLPIFCDAKN QTEYRSMLESYAASWP*	45	616
GQ922500 strain MB043/06	KDDRV*	0	571

* Indicates the stop codon
** Predicted number of amino acid based on ORF analysis of the gene nucleotide sequence

The distance matrix analysis results of the F and HN genes compared to other isolates (from genotype I to genotype IX) are shown in (Table 4). All the isolates showed a higher genetic variation with genotype II and lowest distance to genotype VII. A maximum distance of 24.1% nucleotide variation was observed between the F genes of IBS/046/2014 and genotype II. The same pattern was observed for the HN gene in which the maximum distance was detected between the group, IBS/061/2014, IBS 060/2014 and IBS 160/2015, and genotype II, with 20.7% nucleotide variation (Table 5).

Table 4
Distance matrix analysis of Malaysian NDV isolates based on F Gene

Isolates	Genotypes								
	I	II	III	IV	V	VI	VII	VIII	IX
IBS 160 /2015	23.7%	24.0%	21.5%	18.5%	17.8%	16.5%	10.7%	16.1%	19.8%
IBS 162A /2015	22.8%	23.1%	20.7%	17.7%	17.0%	15.7%	10.0%	15.4%	19.0%
IBS 061 /2014	23.2%	23.5%	21.1%	18.1%	17.4%	16.1%	9.9%	15.2%	19.4%
IBS 046 /2014	23.2%	24.1%	20.3%	19.9%	16.6%	16.1%	9.8%	15.5%	21.0%
IBS 060 /2014	23.2%	23.5%	21.1%	18.1%	17.4%	16.1%	9.9%	15.2%	19.4%
IBS 074 /2014	23.2%	23.5%	21.1%	18.1%	17.4%	16.1%	9.9%	15.2%	19.4%

Table 5
Distance Matrix Analysis of Malaysian NDV Isolates Based on HN Gene

Isolates	Genotypes								
	I	II	III	IV	V	VI	VII	VIII	IX
IBS 160 /2015	12.3%	20.7%	14.8%	14.8%	8.5%	13.8%	7.1%	14.8%	15.6%
IBS 162A /2015	11.7%	20.0%	14.1%	14.1%	8.5%	13.4%	6.5%	14.1%	14.9%
IBS 061 /2014	12.3%	20.7%	14.8%	14.8%	8.5%	13.8%	7.1%	14.8%	15.6%
IBS 046 /2014	11.6%	19.8%	14.0%	14.0%	10.6%	14.1%	6.7%	15.6%	14.8%
IBS 060 /2014	12.3%	20.7%	14.8%	14.8%	8.5%	13.8%	7.1%	14.8%	15.6%
IBS 074 /2014	11.6%	19.8%	14.0%	14.0%	10.6%	14.1%	6.7%	15.6%	14.8%

Similarly, the distance analysis results of sub-genotype VII (from a to i) based on F gene sequencing showed that all the isolates had the highest genetic distance to the sub-genotype VIIh and the lowest genetic distance to sub-genotype VIIa. A maximum distance of 20% nucleotide variation was observed between the F genes of IBS/160/2015 and the sub-genotype VIIh (Table 6).

Table 6
Distance matrix analysis of sub-genotype VII of Malaysian NDV isolates

Isolates	Genotypes								
	VIIa	VIIb	VIIc	VIId	VIIe	VIIf	VIIg	VIIh	VIIi
IBS 160 /2015	6.9	16.3	11.2	12.0	13.6	17.6	16.8	20.0	14.9
IBS 162A /2015	6.3	15.6	10.5	11.3	12.9	16.8	16.8	20.0	14.1
IBS 061 /2014	6.0	15.2	10.8	11.7	13.3	17.2	16.4	19.5	14.1
IBS 046 /2014	5.6	15.0	10.8	11.7	12.5	17.2	16.4	19.0	14.9
IBS 060 /2014	6.0	15.2	10.8	11.7	13.3	17.2	16.4	19.5	14.1
IBS 074 /2014	6.0	15.2	10.8	11.7	13.3	17.2	16.4	19.5	14.1

The causative agent of ND outbreaks in vaccinated broiler flocks from Malaysia between 2014 and 2015 was isolated and characterised. Sequencing of the F cleavage site of the isolated viruses showed the presence of the polybasic amino acid motif ^{112}KRRKRF^{117} and ^{112}RRQKRF^{117}, indicating that all the NDV isolates analysed in this study (IBS 046/2014, IBS 060/2014, IBS 061/2014, IBS 074 /2014, IBS 160/2015 and IBS 162A/2015) were classified as a velogenic NDV. It is widely accepted that the number of basic amino acids immediately upstream to the F0 protein cleavage site determines viral pathogenicity, which is clearly described by the World Organisation for Animal Health (OIE, 2013). The presence of these characteristic patterns of amino acid demonstrated that the isolates could be considered as virulent. It has been observed that F proteins of virulent NDV strains contain lysine (K) and arginine (R) at their cleavage site (^{112}R-R-Q-R/K-R^{116}) and a phenylalanine at position 117 of F_1. It is of utmost importance to note that the F0 cleavage site of isolates (IBS 060/2014, IBS 061/2014, IBS 074/2014, IBS 160/2015 and IBS 162A/2015) isolated from Malaysia, was unusual, containing a lysine (K) arginine (R) substitution at residue 112-114, unlike the results of a previous study by Roohani et al (2015) that indicated that genotype VII viruses isolated from Malaysia in 2011 had different motifs at the F cleavage site. However, there is no major sequence difference between our five isolates and the NDV isolates of 2011 other than in the cleavage site. The IBS 046/2014 isolate had motifs similar to the MB047/05 isolate at the F cleavage site as described by Berhanu et al. (2010). However, in recent years, similar results have been reported in South African genotype VIII viruses (Abolnik et al., 2004) and in Taiwan (Tsai et al., 2004) as well as in Eurasian collard dove and pigeon isolates containing a ^{112}R-R-K-K-R^{116}, ^{112}R-R-Q-K-R^{116} and ^{112}R-R-R-K-R^{116} motif (Huovilainen et al., 2001; Terregino et al., 2003). Even though the contribution of arginine (R) at amino acid 114 in our isolates needs further study, other studies have indicated that arginine residue at different positions 113, 115 and

116 contribute to intracellular cleavage of virulent NDV fusion proteins (Fujii et al., 1999).

Based on the regions we characterised, there were no changes in the neutralising epitopes, the cysteine residues and the N-linked glycosylation sites of the F0 protein. The changes were only in the cleavage site of five isolates as mentioned in the manuscript. It is, however, possible that other epitopes not located in the regions we studied might have mutated, thereby further facilitating the emergence of these virulence isolates. Further study is needed to confirm this.

Virulence was confirmed by the length of the HN protein. Analysis of the C-terminus extension length of the HN protein gene revealed that the six virulent NDV isolates shared 0 amino acid extension length with a total HN length of 571 amino acids regardless of their cleavage site sequence profile (terminating in the sequence KDDRV). Most lentogenic strains, including the conventional LaSota vaccine strains and other genotype I strains have either 577 or 616 amino acids and share either six or 45 amino acid extension length. Moreover, phylogenetic studies on the partial F and HN gene revealed that six NDV isolates were classified as a genotype VII NDV and clustered together with other genotype VII isolates from Indonesia (Xiao et al., 2012), Cambodia (Choi et al., 2013) and China (Xie et al., 2012). A phylogenetic relationship between the partial F gene nucleotide sequence of NDV in the genotype VII group for nine sub-genotypes (a, b, c, d, e, f, g, h, and i) revealed that six NDV isolates were classified under the sub-genotype, VIIa, contrary to what was reported by Berhanu et al. (2010), who indicated that genotype VII viruses isolated from Malaysia between 2004 and 2007 belonged to the sub-genotype, VIId. The distance matrix analysis of the length F and HN genes demonstrated that all six isolates had the highest amino acid variation compared to the genotype II (23.5%-24.1%). The same pattern was observed for the HN gene in which the maximum distance was detected between six NDV isolates and genotype II (19.8%-20.7%). It was expected that the F and HN amino acid sequence of the six NDV isolates would share a close similarity (6.7%-10.7%) with the genotype VII strains.

CONCLUSION

In conclusion, the causative agent of recent ND outbreaks in vaccinated broiler flocks from Malaysia was found to belong to the velogenic genotype VIIa. This strain is genetically close to other Malaysian genotype VII isolates obtained in the last decade. The deduced amino acid sequence of the F0 protein cleavage site showed a unique amino acid motif in five of the isolates incriminated for sporadic cases that occurred in different parts of the country.

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REFERENCES

Abolnik, C., Horner, R. F., Bisschop, S. P. R., Parker, M. E., Romito, M., & Viljoen, G. J. (2004). A phylogenetic study of South African Newcastle disease virus strains isolated between 1990 and 2002 suggests epidemiological origins in the Far East. *Archives of Virology*, *149*(3), 603–619.

Aldous, E. W., & Alexander, D. J. (2001). Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). *Avian Pathology, 30*, 117–128.

Alexander, D. J. (2000). Newcastle disease and other avian paramyxoviruses. *Revue scientifique et technique (International Office of Epizootics), 19*(2), 443-462.

Berhanu, A., Ideris, A., Omar, A. R., & Bejo, M. (2010). Molecular characterization of partial fusion gene and C-terminus extension length of haemagglutinin-neuraminidase gene of recently isolated Newcastle disease virus isolates in Malaysia. *Virology Journal*, *7*(1), 183-192.

Chambers, P., & Samson, A. C. R. (1982). Non-structural proteins in Newcastle disease virus-infected cells. *Journal General Virology, 58*(1), 1–12.

Choi, K. S., Kye, S. J., Kim, J. Y., Damasco, V. R., Sorn, S., Lee,Y. J.,… Lee, H. S. (2013). Molecular epidemiological investigation of velogenic Newcastle disease viruses from village chickens in Cambodia. *Virus Genes, 47*(2), 244–9.

Collins, P. L., Wertz, G. W., Ball, L. A., & Hightower, L. E. (1982). Coding assignments of the five smaller mRNAs of Newcastle disease virus. *Journal of Virology*, *43*(3), 1024–1031.

Fauquet, C. M., & Fargette, D. (2005). International Committee on Taxonomy of Viruses and the 3,142 unassigned species. *Virology Journal*, *2*(1), 64.

Fujii, Y., Sakaguchi, T., Kiyotani, K., & Yoshida, T. (1999). Comparison of substrate specificities against the fusion glycoprotein of virulent Newcastle disease virus between a chick embryo fibroblast processing protease and mammalian subtilisin-like proteases. *Microbiology and Immunology*, *43*(2), 133–140.

Glickman, R. L., Syddall, R. J., Iorio, R. M., Sheehan, J. P., & Bratt, M. A. (1988). Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. *Journal of Virology*, *62*(1), 354–6.

Huovilainen, A., Ek-Kommonen, C., Manvell, R., & Kinnunen, L. (2001). Phylogenetic analysis of avian paramyxovirus 1 strains isolated in Finland. *Archives of Virology*, *146*(9), 1775–85.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., & Madden, T. L. (2008). NCBI BLAST: A better web interface. *Nucleic Acids Research*, *36*(Web Server issue), W5–9. https://doi.org/10.1093/nar/gkn201

Martin, L. A., Smith, T. J., Obermoeller, D., Bruner, B., Kracklauer, M., & Dharmaraj, S. (2001). RNA purification. *Molecular Biology Problem Solver: A Laboratory Guide*, *7*, 197–224.

Munir, M., Cortey, M., Abbas, M., Afzal, F., Shabbir, M. Z., Khan, M. T., ... & Zohari, S. (2012). Biological characterization and phylogenetic analysis of a novel genetic group of Newcastle disease virus isolated from outbreaks in commercial poultry and from backyard poultry flocks in Pakistan. *Infection, Genetics and Evolution, 12*(5), 1010–1019.

OIE. (2013). Manual of diagnostic tests and vaccines for terrestrial animals. *World Organisation for Animal Health*, 1185–91.

Peroulis-Kourtis, I., O'Riley, K., Grix, D., Condron, R. J., & Ainsworth, C. (2002). Molecular characterisation of Victorian Newcastle disease virus isolates from 1976 to 1999. *Australian Veterinary Journal*, *80*(7), 422–4.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutioanry distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*(10), 2731–9.

Terregino, C., Cattoli, G., Grossele, B., Bertoli, E., Tisato, E., & Capua, I. (2003). Characterization of Newcastle disease virus isolates obtained from Eurasian collared doves (Streptopelia decaocto) in Italy. *Avian Pathology*, *32*(1), 63–8.

Tirumurugaan, K. G., Vinupriya, M. K., Vijayarani, K., & Kumanan, K. (2011). Analysis of the fusion protein cleavage site of newcastle disease virus isolates from India reveals preliminary evidence for the existence of II, VI and VII genotypes. *Indian Journal of Virology*, *22*(2), 131–7.

Tsai, H. J., Chang, K. H., Tseng, C. H., Frost, K. M., Manvell, R. J., & Alexander, D. J. (2004). Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996. *Veterinary Microbiology*, *104*(1–2), 19–30.

Wang, J., Liu, W., Ren, J., Tang, P., Wu, N., & Liu, H. (2013). Complete genome sequence of a newly emerging Newcastle disease virus. *Genome Announcements*, *1*(3), 3–4.

Xiao, S., Paldurai, A., Nayak, B., Samuel, A., Bharoto, E. E., Prajitno, T. Y., … & Samal, S. (2012). Complete genome sequences of Newcastle disease virus strains circulating in chicken populations of Indonesia. *Journal of Virology, 86*(10), 5969–70.

Xie, Z., Xie, L., Chen, A., Liu, J., Pang, Y., Deng, X., …. & Fan, Q. (2012). Complete genome sequence analysis of a Newcastle disease virus isolated from a wild egret. *Journal of Virology, 86*(24), 13854–5.

Yang, C., Shieh, H. K., Lin, Y. L., & Chang, P. (1999). Newcastle disease virus isolated from recent outbreaks in Taiwan phylogenetically related to viruses (genotype VII) from recent outbreaks in Western Europe. *Avian Diseases*, *43*(1), 125–30.

Yuan, P., Paterson, R. G., Leser, G. P., Lamb, R. A., & Jardetzky, T. S. (2012). Structure of the Ulster strain Newcastle disease virus hemagglutinin-neuraminidase reveals auto-inhibitory interactions associated with low virulence. *PLoS Pathogens*, *8*(8), 1–15.

Zhang, W., & Sun, Z. (2008). Random local neighbor joining: A new method for reconstructing phylogenetic trees. *Molecular Phylogenetics and Evolution*, *47*(1), 117–28.

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Short Communication

An Outbreak of *Vibrio alginolyticus* Infection in Juvenile Sea Cucumbers *Holothuria scabra* in Sabah, Malaysia

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ABSTRACT

Sea cucumber breeding was carried out in Universiti Malaysia Sabah Hatchery Facilities in April 2015. Approximately 690,000 eggs were fertilised with a hatching rate of 9%, an estimated 60,000 larvae were produced. In June, the surviving 359 sea cucumber juveniles were sorted according to size. In late July, it was found that only 30 juvenile sea cucumbers had survived, and the remaining were dead, with multiple ulcers on the skin and excessive mucus covering the entire body. In December 2015, a total of 20 juvenile sea cucumbers were sampled for bacterial isolation and histopathological examinations. *Vibrio alginolyticus* was isolated and histopathological examinations revealed peracute inflammation and accumulation of extensive mucus surrounding the body.

Keywords: *Vibrio alginolyticus*, outbreak, sea cucumbers, *Holothuria scabra*

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INTRODUCTION

Sea cucumber, *Holothuria scabra*, is a marine organism that is popular in Malaysia for its medicinal values (Choo, 2004). One study reported that sea cucumbers contain high levels of protein and certain bioactive components such as mucopolysaccharides, chondroitin sulfate and antioxidant compounds (Bordbar, Anwar, & Saari,

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2011). A total of 52 morphospecies of sea cucumber from four orders comprising 12 genera have been identified in Malaysia; 38 species were reported in Sabah, 24 species in Peninsular Malaysia and 10 species in both regions (Kamarudin et al., 2015). Sea cucumber fisheries are exploited off the coastal waters of Sabah in East Malaysia and Langkawi Island in West Malaysia. In Sabah, annual landings of sea cucumber in 1980s were between 400 and 500 tonnes but this declined in the 1990s to approximately 100 tonnes. Sea cucumber is an endangered species and until now, sea cucumbers in Malaysia are harvested from the wild (Choo, 2012), thus severe overfishing is leading to a significant decrease in the natural sea cucumber population (Conand, 2004). Therefore, breeding and seeding of sea cucumber in Malaysia was initiated in 2012 (Mazlan & Hashim, 2015).

Most studies on sea cucumber involve the Japanese sea cucumber, *Apostichopus japonicus* (Yang, Sun, & Xu, 2015), and infection by *Vibrio splendidus* (Zhao et al., 2011). The infection leads to skin ulceration syndrome, which limits the development of the sea cucumber culture industry (Gao et al., 2015). This paper reports the first outbreak of *V. alginolyticus* infection in juvenile sea cucumber, *H. scabra*, in Malaysia. It describes the isolation and pathological changes in cultured juvenile sea cucumber naturally infected by *V. alginolyticus*.

First breeding of sea cucumber was carried out in the Universiti Malaysia Sabah hatchery facilities in April 2015. Large (250-300 g) adult and healthy sea cucumber brood-stocks were brought to the hatchery and stocked in one-tonne tanks with a flow-through water system. The bottom of the tank was covered with sand of about 10 cm in thickness from the specimens' natural habitat and the sand was changed twice a month. The seawater supply for the brood-stocks was direct from the nearby seabed but was filtered using a coral filter. The brood-stocks' stocking density was 20 individuals per 1000 L. During spawning induction, 30 sea cucumbers from the brood-stocks were placed in a flat-bottomed spawning tank filled with filtered seawater and subjected to thermal stimulation (Kubota & Tomari, 1998). The eggs of the sea cucumber were placed in a separate larval rearing tank containing seawater that was filtered using a 1-µm filter and treated with UV light. Egg development, fertilisation and hatching rates were recorded.

Approximately 690,000 eggs were fertilised and approximately 60,000 larvae were harvested. By late June, the surviving 359 sea cucumber juveniles were sorted according to size; a total of 113 juveniles were of the size $\geq$1 cm while 246 juveniles were <1 cm. By late July, only 30 juvenile sea cucumbers had survived. The juveniles were dead after showing inactivity with multiple small ulcers on the skin that started as a white spot on the skin followed by excessive mucus covering the entire body (Figure 1). Eventually, by December 2015 the remaining 15 surviving juvenile sea cucumbers were showing similar clinical signs.

Figure 1. Gross lesions that started as small white spot on the skin (left picture; arrow) that eventually led to mucus surrounding almost the entire body (right picture)

A total of 20 dead and moribund juvenile sea cucumbers were sampled for bacterial isolation. Swab samples were collected from the body ulcer and the mucus, and hemolymph and organs were streaked directly onto Tryptic Soy Agar (Merck, Germany) and incubated at 30^0C for 24 to 48 h. Pure cultures were obtained before the colonies were subjected to Gram staining to determine morphology. Bacterial cultures were further identified using biochemical characterisation of API-20E (bioMérieux, France) and were confirmed using PCR (Nehlah, Ina-Salwany, & Zulperi, 2016). The entire body of juvenile sea cucumbers was then fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (HE) for histopathological examinations.

Pure bacterial cultures were observed after 24 h of incubation. They were Gram-negative short rods that were confirmed as *V. alginolyticus,* and were successfully isolated from all 20 (100%) sampled *H. scabra* juveniles. The biochemical characterisation of API-20E identified as *V. alginolyticus* (Gonzalez-Escalano, Blackstone, & DePaola, 2006) and PCR (Nehlah, Ina-Salwany & Zulperi, 2016) revealed the amplification of the 846 bp band (Figure 2). Histopathological examinations revealed that peracute inflammation of the blood vessels

consisted of polymorphonuclear cells and accumulation of extensive mucus surrounding the body with total destruction of the epidermis that exposed the connective tissue. The blood vessels of the organs were hyperaemic, while the internal organs were severely congested.

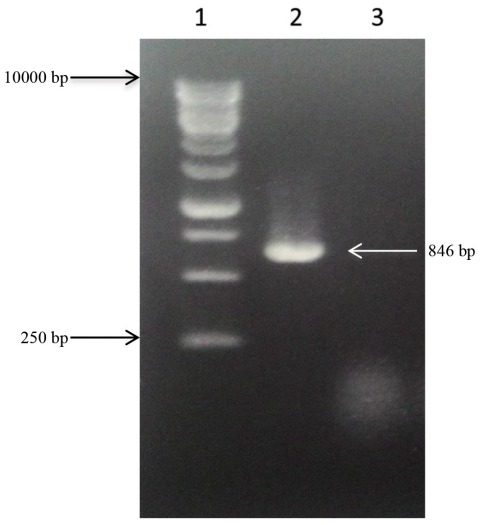

Figure 2. Polymerase chain reaction of the outer membrane protein, ompK, gene of *Vibrio* isolate RFZ-IPMB-16. Amplification targetted the DNA at 846-bp. Lane 1. DNA GeneRuler, 1kb DNA Ladder (Thermo Scientific, USA); Lane 2. ompK gene of *Vibrio* isolate RFZ-IPMB-2016; Lane 3. Negative control

It was concluded that the mortalities of the juvenile sea cucumber, *H. scabra*, in a sea cucumber hatchery facility at UMS Sabah, Malaysia were due to *V. alginolyticus* infection, which affected 100% of the juveniles between the ages of 2 and 8 months old. The source of *V. alginolyticus* is believed to have been the sea water (Lavilla-Pitogo et al., 1990; Becker et al., 2004) and ultraviolet treatment of the sea water seemed to have been ineffective (Shikongo-Nambabi, Kachigunda, & Venter, 2010). Affected sea cucumbers showed typical lesions of vibriosis, particularly skin ulcerations. Yang, Hamel and Mercier (2015) identified infection by *V. splendidus* and *V. alginolyticus* in Japanese sea cucumber as a skin ulcerative syndrome that frequently affects and kills young rather than mature *A. japonicus* sea cucumber. This hampers attempts to artificially breed and cultivate sea cucumber. Similarly, Becker et al. (2004) reported a skin ulceration disease of juvenile *H. scabra*, the widely marketed edible sea cucumber in Madagascar. The first sign of the infection is a white spot on the integument close to the cloacal aperture that spreads quickly to the whole integument, leading to the death of individual sea cucumbers within three days. Infection by *V. alginolyticus* in juvenile *A.*

japonicus sea cucumber has been reported by Wang et al. (2005) as producing skin ulceration syndrome.

This is believed to be the first report of the outbreak of *V. alginolyticus* infection in juvenile *H. scabra.* The infection killed all the juveniles quickly within three days, producing typical peracute lesions of skin ulceration syndrome. However, the excessive mucus production that covered the affected individuals observed in this outbreak has not been previously reported.

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REFERENCES

Becker, P., Gillan, D., Lanterbecq, D., Jangoux, M., Rasolofonirina, R., Rakatorao, J., & Eeckhaut, I. (2004). The skin ulceration disease in cultivated juvenile of *Holuthuria scabra* (Holothurioidea, Echinodermata). *Aquaculture, 242*(1), 13–30.

Bordbar, S., Anwar, F., & Saari, N. (2011). High-value components and bioactive from sea cucumbers for functional foods – A review. *Marine Drugs, 9*(10), 1761–1805.

Choo, P. S. (2004). Fisheries, trade and utilization of sea cucumbers in Malaysia. In A. Lovatelli, C. Conand, S. Purcell, S. Uthicke, J. F Hamel, & A. Mercier (Eds.), *Advances in sea cucumber aquaculture and management* (pp. 81–118). Rome: Food and Agriculture Organization of the United Nations.

Choo, P. S. (2012). The sea cucumber fishery in Semporna, Sabah, Malaysia. *SPC Beche-de-mer Information Bulletin, 32,* 43–48.

Conand, C. (2004). Present status of sea cucumber resources and utilization: An international overview. In A. Lovatelli, C. Conand, S. Purcell, S. Uthicke, J. F Hamel, & A. Mercier (Eds.), *Advances in sea cucumber aquaculture and management* (pp. 13–23). Rome: Food and Agriculture Organization of the United Nations.

Gao, Q., Liao, M., Wang, Y., Li, B., Zhang, Z., Rong, X., Chen, G., & Wang, L. (2015). Transcriptase analysis and discovery of genes involved in immune pathways from coclomocytes of sea cucumber (*Apostichopus japanicus*) after *Vibrio splendidus* challenge. *International Journal of Molecular Science, 16*(7), 16347–16377.

Gonzalez-Escalona, N., Blackstone, G. M., & De Paola, A. (2006). Characterization of a *Vibrio alginolyticus* strain, isolated from Alaskan oysters, carrying a hemolysin gene similar to the thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus. Applied and Environmental Microbiology, 72*(12), 7925–7929.

Kamarudin K. R., Usup G., Hashim R., & Rehan M. M. (2015). Sea cucumber (Echinodermata: Holothuroidea) species richness at selected localities in Malaysia. *Pertanika Journal of Tropical Agricultural Science, 38*(1), 7–32.

Kubota, T., & Tomari, M. (1998). Reproduction in the apodid sea cucumber *Polycheira rufescens: Semilunar spawning* rhythm and sex change. *Journal of the Marine Biological Association of the United Kingdom, 78*(1), 249–267.

Lavilla-Pitogo, C. R., Baticados, M. C. L., Cruz-Lacierda, E. R., & de la Pena, L. D. (1990). Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Aquaculture, 91*(1), 1–13.

Mazlan N., & Hashim R. (2015). Spawning induction and larval rearing of the sea cucumber *Holuthuria scabra* in Malaysia. *SPC Beche-de-mer Information Bulletin, 35,* 32–36.

Nehlah R., Ina-Salwany M. Y., & Zulperi Z. (2016). Antigenicity analysis and molecular characterization of two outer membrane proteins of *Vibrio alginolyticus* strain VA2 as vaccine candidates in tiger grouper culture. *Journal of Biological Sciences, 16*(1), 1–11.

Shikongo-Nambabi, M. N. N. N., Kachigunda, B., & Venter, S. N. (2010). Evaluation of oxidizing disinfectants to control *Vibrio* biofilms treated seawater used for fish processing. *Water SA, 36*(3), 215–220.

Wang, Y. G., Zhang, C. Y., Rong, X. J., Chen, J. J., & Shi, C. Y. (2005). Diseases of cultured sea cucumber *Apostichopus japonicus* in China. *FAO Fisheries Technical Paper, 463,* 297–310.

Yang, H., Hamel, J-F., & Mercier, A. (2015). Immunology and diseases: Skin ulceration syndrome. In The sea cucumber *Apostichopus japanicus*. History, biology and aquaculture (pp. 280–281). Academic Press.

Yang, Z., Sun, J., & Xu, Z. (2015). Beneficial effects of *Rhodotorula* sp. C11 on growth and disease resistance of juvenile japanes spiky sea cucumber *Apostichopus japanicus. Journal of Aquatic Animal Health, 27*(2), 71–76.

Zhao, Y., Mai, K., Xu, W., Zhang, W., Ai, Q., Zhang, Y. … & Luifu, Z. (2011). Influence of dietary probiotic *Bacillus* TC22 and prebiotic fructooligoc-saccharide on growth, immune response and disease resistance against *Vibrio spendidus* infection in sea cucumber *Apostichopus japanicus. Journal of the Ocean University of China, 10*(3), 293–300.

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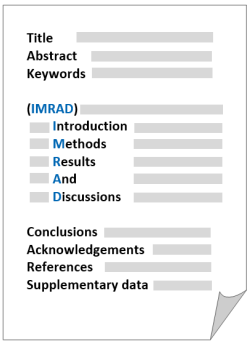

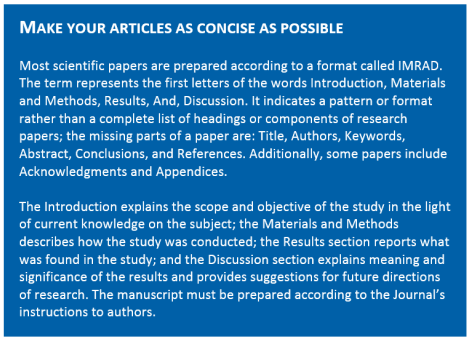

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