Journal of Tropical Agricultural Science

About the Journal

Overview

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

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

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

The Journal is available world-wide.

Aims and scope

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.

History

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

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

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

Future vision

We are continuously improving access to our journal archives, content, and research services. We have the drive to realise exciting new horizons that will benefit not only the academic community, but society itself.

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

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International Standard Serial Number (ISSN)

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

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Lag time

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

Authorship

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

Manuscript preparation

Refer to Pertanika's **Instructions to Authors** at the back of this journal.

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

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

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

Pertanika follows a **double-blind peer-review** process. Manuscripts deemed suitable for publication are usually sent to reviewers. Authors are encouraged to suggest names of at least three potential reviewers at the time of submission of their manuscript to Pertanika, but the editors will make the final choice. The editors are not, however, bound by these suggestions.

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.

The Journal's peer-review

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.

Operating and review process

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

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

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

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

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

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

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

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

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

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

ABSTRACTING/INDEXING

Pertanika is now over 35 years old; this accumulated knowledge has resulted the journals being indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge [BIOSIS & CAB Abstracts], EBSCO, DOAJ, Google Scholar, AGRICOLA, ISC, Citefactor, Rubriq and MyAIS.

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Foreword

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

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

This issue contains **ten articles**, out of which one is a short communication and nine are regular papers. The authors of these articles come from different countries, namely, **Malaysia**, **Pakistan**, **Kazakhstan**, **Bangladesh**, **Thailand** and **Iran**.

The short communication paper briefly reports on the effectiveness of various botanical traps against apple snail, *Pomacea maculata.* (Gastropoda: Ampullariidae) in a rice field. In the findings, all the traps studied have the potential to attract juvenile and adult snails. However, jackfruit skin and damaged pomelo were the most attractive botanical traps (*Syamsul, R. B., Muhamad, R., Arfan, A. G.* and *Manjeri, G.*).

The nine articles cover a wide range of topics. In the first research paper, the accumulation of Potato Virus Y- in *Nicotiana tabacum* callus culture was studied. In the findings, virus showed the highest optical density (OD) on the 25th day of inoculation (*Khassanov, V. T., Beisembina, B.* and *Fida, M. A.*). The next paper discusses on the Arsenic management in contaminated irrigation water for rice cultivation. The experiment was conducted at the Bangladesh Rice Research Institute (BRRI) farm Bhanga, Faridpur (*A. L. Shah, U. A. Naher, Z. Hasan, S. M. M. Islam, M. S. Rahman, Q. A. Panhwar* and *J. Shamshuddin*). The other papers are consist of the study on the influence of salt and water stress on growth and yield of soybean genotypes (*M. S. A. Khan, M. A. Karim, M. M. Haque, M. M. Islam, A. J. M. S. Karim* and *M. A. K. Mian*); discrimination between cave and house-farmed edible bird's nest based on major mineral profiles (*Seow, E. K., Ibrahim, B., Muhammad, S. A., Lee, L. H., Lalung, J.* and *Cheng, L. H.*); path analysis of agronomic traits of Thai cassava for high root yield and low cyanogenic glycoside (*Kongsil, P., Kittipadakul, P., Phumichai, C., Lertsuchatavanich, U.* and *Petchpoung, K.*); nutritional compositions and antioxidant activities of non-polar and polar extracts of germinated brown rice (*Lim, S. M., Goh, Y. M.* and *Loh, S. P.*); effect of elemental sulphur timing and application rates on soil p release and concentration in maize (*Karimizarchi, M., Aminuddin, H., Khanif, M. Y.* and *Radziah, O.*); natural product compounds from *Calophyllum depressinervosum* (*Nor Hisam Zamakshshari, Gwendoline Cheng Lian Ee, Soek Sin Teh, Shaari Daud, Thiruventhan*

Karunakaran and *Intan Safinar*); and finally, a seroprevalence and detection of Contagious Bovine Pleuropneumonia (CBPP) in northeast states of peninsular Malaysia (*Zarina, M., Zamri-Saad, M., Latiffah, H., Shahrom, M. S.* and *Norlida, O.*).

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

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

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

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Short Communication

Effectiveness of Various Botanical Traps against Apple Snail, *Pomacea maculata* (Gastropoda: Ampullariidae) in a Rice Field

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ABSTRACT

The adverse effects of molluscicides applied for the control of the invasive apple snails, *Pomacea* spp., have led to the search for eco-based cultural, mechanical and biological control techniques. Therefore, a field study on the relative effectiveness of locally available and cost effective plant-based traps against *Pomacea* spp. was conducted. Results showed jackfruit skin (9.03 ± 0.60 / m² and 6.03 ± 0.60 / m²) and damaged pomelo (9.00 ± 0.61 / m² and 5.78 ± 0.74 / m²) were relatively more effective than tapioca leaves, water spinach leaves and old newspaper. Snails also displayed preference for fresh materials as compared to rotten materials. Thus, incorporating these findings in rice fields during early susceptible growth will ease the collection and destruction of snails.

Keywords: Apple snail, Pomacea, rice, botanical trap

INTRODUCTION

Invasive apple snails, *Pomacea maculata* Perry, 1810 and *Pomacea canaliculata* Lamarck, 1822 (Gastropoda; Ampullariidae)

are serious pests of many aquatic macrophytes including rice (Hayes et al., 2008; Horgan et al., 2014). These invasive snails were introduced into Malaysia around 1991 and spread to all rice growing areas of the country, causing heavy losses to rice yields (Yahaya et al., 2006; Arfan et al., 2014). Snails mostly feed on young rice seedlings and their severe damage could result in complete loss of rice crop (Teo, 2003). In Malaysia, growers often spend

approximately RM 425 per hectare to control snails (Yahaya et al., 2006), whereas global cost of apple snail infestation could reach billion of US$ (Horgan et al., 2014). In an attempt to control snails, growers mostly apply chemicals which are often not specific molluscicides. These random chemicals are more preferred due to their easy application and fast action (Schnorbach et al., 2006). However, the adverse effect of chemicals on men and their environment always necessitate for alternative cultural, mechanical and biological control measures to manage apple snails (Yusa, 2006). To date, the effectiveness of botanical traps as an alternative control measure in the collection and destruction of snails have been evaluated in different countries with varying success (Joshi et al., 2001; Teo, 2003). However, such studies are still lacking in Peninsular Malaysia, with the only available work done by Amzah and Yahya (2014). Lettuce, jackfruit, papaya fruit and leaves, cassava leaves, sweet potato, tapioca, taro, water melon and aubergines are some of the botanical materials used as baits against apple snails, which are mainly to ease in their collection and killing so as to manage their population below the threshold levels (Glover & Campbell, 1994; Fukushima et al., 2001; Cagauan, 2003; Teo, 2003). Therefore, considering the potential of botanical traps in managing apple snail populations, the relative effectiveness of various locally available and cost effective botanical traps was thoroughly evaluated against *Pomacea* spp. in a rice field. The results of the study could be utilised in the management of apple snails in rice field by facilitating their easy picking and destruction, thus reducing damages to rice.

MATERIALS AND METHODS

Study site

The study was conducted at a 0.405 hectare farmer managed rice field in Kodiang, Kedah (6° 21' 55.19" N, 100° 20' 21.31"E) during the months of August-September, 2014. The field is under the management of MUDA Agricultural Development Authority (MADA). Rice variety MR220CL1 was cultivated by direct seeding.

Botanical Traps

The attractants used in this study were jackfruit skin (*Artocarpus heterophyllus* L.), tapioca leaves (*Manihot esculenta* Crantz), water spinach leaves (*Ipomoea aquatic* Forssk.), damaged pomelo (*Citrus maxima* Merr.) and old newspapers (Figure 1). The attractants were selected according to their local availability throughout the rice growing season and cost effectiveness. All the attractant materials were filled into individual containers of 17 cm x 12 cm x 5.5 cm size and covered with wire mesh to prevent attractant materials from floating into the field. All traps set up were fully submerged in the rice field to enhance their attractiveness to snails (Fukushima et al., 2001).

Experimental Design, Data Collection and Analysis

The experiment was conducted in a Randomised Complete Block Design (RCBD). The rice field was divided into four blocks depending on the source of irrigation in the field. In each block, all five attractants were randomly placed at four locations (replicates) with a one meter distance between the individual attractants to avoid their interference in attracting the snails. The observations were started one week after sowing of rice and continued up to week four as snails are more destructive to young rice seedlings than older seedlings (Sanico et al., 2002). The observations were taken twice in a week i.e., day one (fresh attractant) and day four (rotten attractant). Materials were deemed as rotten when it deteriorated in shape after being set as traps. The attractants were replaced with fresh materials every week. On each observation, the total number of apple snails attracted were counted, identified and classified as juveniles and adults according to their life stages. The identification was done according to Cowie et al. (2006), Hayes

et al. (2012) and Marwato and Nur (2012) based on the external morphology of the apple snails.

Data collected for the level of attractiveness of different traps were analysed using two-way analysis of variance. The means, with significant differences, were separated using Least Square Difference (LSD). All the analyses were done using SAS 9.3 statistical package (SAS Institute Inc. 2009).

RESULTS AND DISCUSSION

Species Identification

All the snails collected during the study were identified as *P. maculata* according to their shell morphology Cowie et al. (2006), Hayes et al. (2012) and Marwato and Nur (2012). The presence of at least four apple snail species (*P. canaliculata, P. maculata, P. scalaris* and *P. diffusa*) has been reported in Southeast Asia, with former two being most abundant and widely distributed (Rawlings et al., 2007; Hayes et al., 2008). Higher abundance and wider distribution of *P. canaliculata* in comparison to *P. maculata*
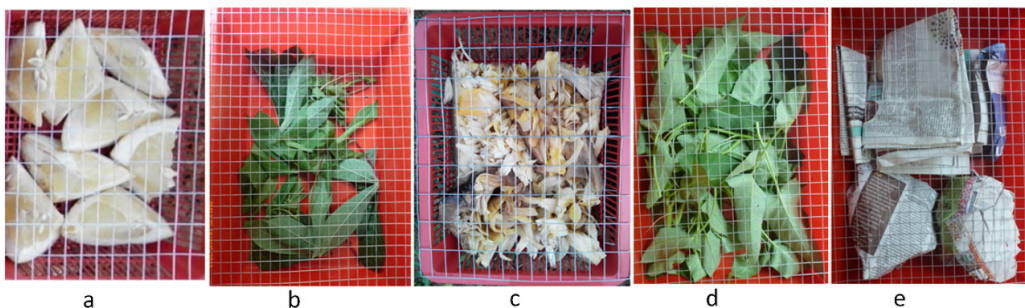
Figure 1. Different attractants used in the study
(a) damaged pomelo; (b) tapioca leaves; (c) jackfruit skin; (d) water spinach (e) old newspaper

in invaded areas including Malaysia have also been reported (Yahaya et al., 2006; Rawlings et al., 2007; Hayes et al., 2008). However, recent studies in Peninsular Malaysia confirmed the abundance and wide scale distribution of *P. maculata* as compared to *P. canaliculata* (Arfan et al*.,* 2014).

Level of Attractiveness of Different Botanical Traps Against Juveniles and Adult P. maculata

Results showed that all the materials used have the potential to attract juvenile and adult snails (Figure 2). However, jackfruit skin (9.03 ± 0.60 / m^2 and 6.03 ± 0.60 / m^2) and damaged pomelo (9.00 ± 0.61 / m^2 and 5.78 ± 0.74 / m^2) showed significantly higher attractiveness for both juvenile and adult snails, respectively ($P < 0.05$), although no difference was recorded between them ($P > 0.05$). Similarly, no significant difference was also recorded in the relative attractiveness of tapioca leaves, water spinach and old newspapers ($P < 0.05$). Moreover, juveniles showed significantly more preference for fresh jackfruit skin (10.06 ± 0.60 / m^2), damaged pomelo (9.81 ± 0.71 / m^2) and tapioca leaves (7.69 ± 0.10 / m^2) as compared to rotten traps of respective materials ($P < 0.05$; Figure 3). Moreover, no significant difference was recorded between fresh and rotten traps of water spinach and newspapers in attracting juveniles ($P > 0.05$; Figure 3). However, the adult snails only showed significant difference between fresh (6.50 ± 0.61 / m^2) and rotten (5.06 ± 0.50 / m^2) jackfruit skin ($P < 0.05$; Figure 4). Previous studies also highlighted the potential of various botanical materials such as lettuce, cassava, sweet potato, taro, tapioca, giliricidia and papaya to attract apple snails with varying success (Glover & Campbell, 1994; Cowie, 2002; Teo, 2003). Comparatively higher preferences for water melon, lettuce, aubergines and tomato in
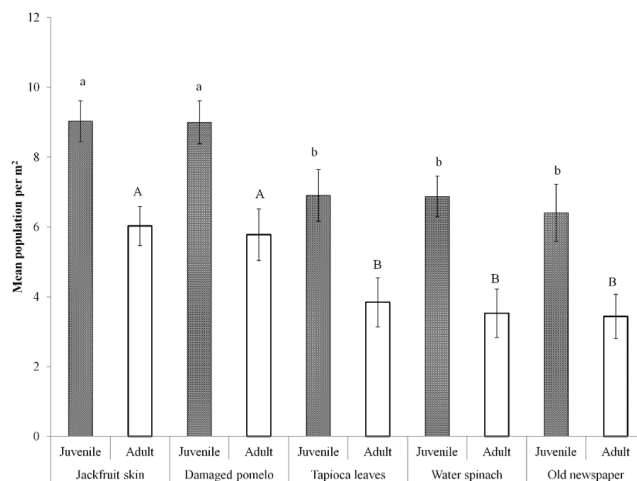
Figure 2. Relative effectiveness of different attractants against juvenile and adult *P. maculata*
*Means followed by the same letters (small letters = juveniles; capital letters = adult) are not significantly different ($P < 0.05$)

comparison to rice have been reported for apple snails and have been suggested to be exploited for easy hand picking of snails (Fukushima et al., 2001; Cagauan, 2003). Jackfruit, papaya fruit and leaves, cassava leaves, water spinach, banana leaves and old newspapers have also been evaluated as potential attractants of apple snails, where jackfruit was found to show the highest attractiveness for the apple snails (Amzah & Yahya, 2014). As observed in this study, relatively higher attractiveness of jackfruit skin and damaged pomelo for apple snails could be due to their strong fragrance as
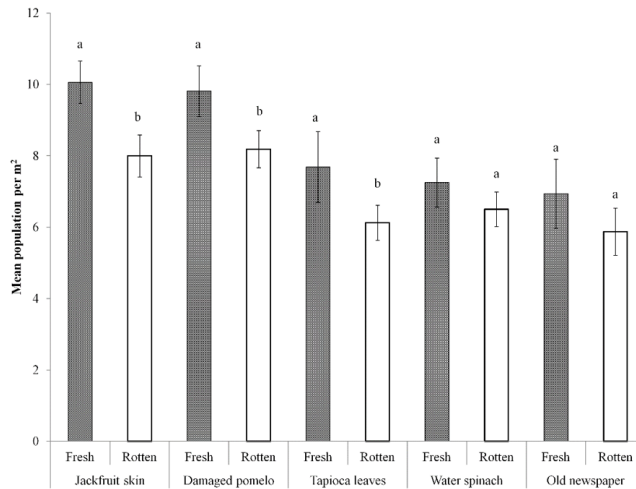
Figure 3. Relative effectiveness of fresh and rotten attractants against juvenile *P. maculata*
*Means followed by different letters against individual traps are not significantly different
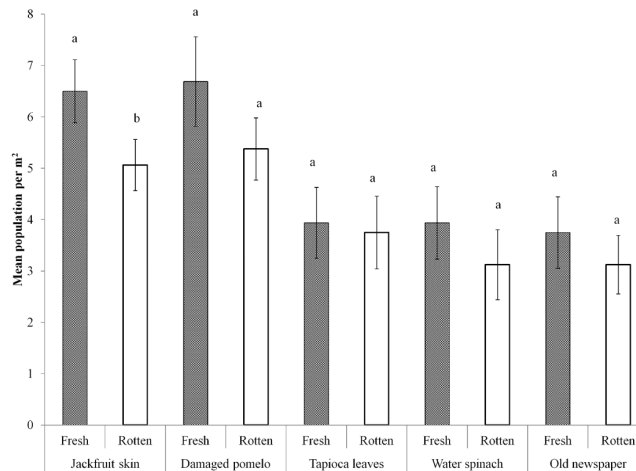
Figure 4. Relative effectiveness of fresh and rotten attractants against adult *P. maculata*
*Means followed by different letters against individual traps are not significantly different

compared to other attractants. Estebenet (1995) has also observed significant role of odour towards damage potential of snails to different macrophytes. The snails are highly dependent on their chemoreceptive ability in detecting macrophytes with strong odour that also supports their faster growth (Cowie, 2002; Van Dyke et al., 2013). In this study, fresh botanical trap materials showed higher attractiveness for adult and juvenile snails. Therefore for better attractiveness of traps, their freshness should be maintained as rotten traps may repel snails away from traps towards rice. It is also important that botanical traps used should be more attractive than rice to divert the snails towards traps (Cowie, 2002).

In conclusion, all the traps studied have the potential to attract juvenile and adult snails. However, jackfruit skin and damaged pomelo were the most attractive botanical traps. In addition, fresh traps of individual materials were more attractive than their rotten traps. This study successfully highlights a variety of potential and effective botanical traps that can be incorporated into the management of *Pomacea* spp. to ease their collection and destruction. All the materials studied are easily available and cost effective for farmers. Fresh traps can be set up on a weekly basis for a better management of snails considering their higher attractiveness. Overall, the findings of this study can serve as an effective option against the commonly applied hazardous chemical control techniques.

ACKNOWLEDGEMENTS

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TROPICAL AGRICULTURAL SCIENCE

Accumulation of Potato Virus Y- in *Nicotiana tabacum* Callus Culture to Obtain a Virus Preparation

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ABSTRACT

The accumulation of potato virus Y- (PVY) in tissue culture of *Nicotiana tabacum* (*N. tabacum*) was studied. Plants of *N. tabacum* variety, Samsun, inoculated with PVY-infected sap of potato variety, Cherie, were used for the virus accumulation. According to enzyme-linked immunosorbent assay (ELISA) results, virus showed the highest optical density (OD) on the 25th day of inoculation. Murashige and Skoog medium containing kinetin 2 mg/l, 2.4-D 0.5 mg/l, indole-acetic acid 1 mg/l sucrose 2% agar 0.7%, in addition to the standard components, was used to induce callus culture from *N. tabacum* leave explants. ELISA results showed that the callus culture was able to maintain viral infection during four transplantations. Slightly and highly purified (Y-Cherie) virus preparations were obtained from the PVY-infected tissue culture. The slightly-purified antigens showed an OD approximately equal to the positive control in sandwich ELISA. The Y-Cherie antigen was detected as PVY necrotic strain. Specific to the virus polyclonal antibodies that reacted with a maximum 1/3200 titer of antigen in indirect ELISA were obtained in the result of the laboratory mouse immunisation.

Keywords: Potato virus Y- (PVY), callus tissue, ELISA, antigen, polyclonal antibodies

INTRODUCTION

Potato is one of the major crops in Kazakhstan; it takes the second place in terms of its importance, after wheat. Potato productivity mainly depends on the stability and resistance of its varieties to their fungal, viral and bacterial diseases (Hooks et al., 2007; Karasev et al., 2012). Viruses, as

intracellular pathogens, are able to change plant's metabolism and cause degeneration, as characterised by a big decline in potato productivity and significant decrease in tubers nutritional and raw values (Whitworth et al., 2006; Rusevski et al., 2013).

Potato virus Y is one of the pathogenic viruses, which causes mostly wrinkled and banding mosaic on leaves in potato (Pallas et al., 2011). PVY virions have thread-like form, with 750 x 12 nm size, and their inactivation temperature is around 55-65ºC and infected-sample sap can be diluted up to 10^{-2} - 10^{-4}. The virus is transmitted to host plants via mechanical contacts and insect-vectors. Diseases caused by PVY are the most harmful as they can dramatically reduce potato production efficiency (Gray et al., 2010). In order to obtain high yield for potatoes, application of sensitive diagnostic methods in potato seed production is very important. The key element in the development of modern diagnostic methods and their implementation in agricultural practice is the availability of qualitative antigen. It is known that highly purified viral antigens are used to produce specific antibodies (Gnutova, 1993). At the same time, specific antibodies are indispensable components of immunoassay diagnostic tests. In vitro, technology allows for production of environmentally clean raw materials throughout the year to increase the content of biologically active substances and regulate virus mass and accumulation in tissue culture (Simakov et al., 2000; Hooks et al., 2007; Islam et al., 2014). Individual authors have used callus tissue

as the source of virus antigens (Kogovšek et al., 2011; Rusevski et al., 2013). Growing potato callus tissue with simultaneous virus accumulation has paved way for *in vitro* PVY as mono-infection for a long time (Ding et al., 1998). Callus tissue allows us to obtain highly purified virus antigen as it lacks many specific proteins. The callus culture can be used to extract sufficient antigen and get homogeneous infectious material that is free from contamination by other viruses and pigments all year round (Gnutova, 1993).

The purpose of this study was to analyse PVY accumulation dynamic in *N. tabacum* tissue culture for its further purification and obtain specific antibodies.

MATERIALS AND METHODS

Plants Samsun a variety of *N. tabacum* were used as the accumulators of potato virus Y. Potato banded mosaic is caused by PVY ordinary strain (PVY [O-F]) and manifested in the form of necrotic veins and dark-brown necrosis in leafstalk. Necrosis is clearly visible from the lower side of the leaf. Severely infected leaves have dark-brown necrosis on their petioles and stems. By the end of the growing season, almost all leaves, firstly the lower ones, dry up and hang on potato stems. Under natural conditions, banded leaf mosaic is accompanied by wrinkliness (Sohair et al., 2007). Test plants were grown from seeds in bio humus "Terra Vita" and soils at a ratio of 1:1. Plants were grown under constant lighting, with 1000 luxes intensity of light and 24-25°C of temperature. Plants were inoculated

using standard methods (Kotzampigikis et al., 2009). PVY-infected potato variety Cherie plants were used to inoculate *N. tabacum* test-plants. The inoculated plants were shaded for 24 hours a day. After that, they were contained in diffused light After 20-25 days of inoculation, and the upper young leaves containing viral antigen were separated, washed with distilled water and disinfected, before they were sequentially incubated in 20% C_2H_5OH (1 min), 7% Ca(Cl)OCl (15 min), and 5% NaOCl (20 min) (Gnutova, 1993). Then, the leaves were washed 3 times with sterile water and cut into square shaped segments of 0.5-0.7 mm size. Explants were planted in petri dishes on agar nutrient medium based on mineral Murashige and Skoog containing in addition to the standard components: kinetin 2 mg/l, 2.4-D 0.5 mg/l, indole-acetic acid 1 mg/l, sucrose 2%, agar 0.7%. Incubation of callus over the entire period was performed under constant illumination (1500 lux) at the temperature of 25-26°C. Accumulation of viral antigen in the test plants and callus tissue was monitored by enzyme-linked immune-sorbent assay (Malyshenko et al., 1993). "Sandwich" ELISA commercial diagnostic kits were used to detect potato virus in test-plants (Salim Khan, 2003). The presence of the virus in the test-plants samples was registered using a spectrophotometer of wavelength 490 nm and light vertical flow (ASYS Expert-96, Austria).

Virus antigen was obtained from the non-pigment tissue culture of *N. tabacum* (grown in darkness) after the 4th transplantation.

Virus purification was conducted using two different methods. Using the first method, slightly-purified PVY preparation was obtained after homogenizing tissue culture, squashing through two layers of gauze and centrifuging at 3000 rotations per minute for 10-15 minutes. The immunogenic supernatant was used to immunise mice for polyclonal antibodies production. PVY-Cherie antigen received from *N. tabacum* using the second method was obtained in the Russian Academy of Agricultural Sciences (RAAS named after Lorkh A.G.). Purification of the virus was carried out according to the procedure adopted at the Department of Biotechnology and Immunodiagnostic of the Institute (Atabekov, 2002). In this method, *N. tabacum* callus tissue was homogenised by adding 0,1 M buffer of K_2PO_4, 1% 2-mercaptoethanol and 0,01 M Na_2EDTA into the sample in a ratio of 1:5. The prepared sap was clarified by centrifuging it at 12000 rotations per minute for 20 minutes. After centrifugation, 0.5% of nonionic-detergent triton-X-100 was added into the solution, followed by precipitation of the virus by PEG-6000 and low-speed centrifugation. The final purification was carried out by ultracentrifugation through a 25% sucrose pad, followed by suspending the virus in solution and low-speed centrifugation at 10000 rotations per minute for 15 min. Concentration of the virus was determined using the spectrophotometer (SmartSpec plus BioRad), 260 nm, USA, extinction coefficient 2.35 (Schubert et al., 2004). To study immunogenic property of the slightly-purified-PVY and produce antibodies, the

following scheme of mice immunisation was used: 100 µL of a 1 µg/ml the virus was injected intraperitonealy. Then on the 7th and 19th days of immunisation, the same patterns were repeated with buffered solution pH 7,2-7,4 (Gnutova, 1993; Čeřovská et al., 2003).

RESULTS AND DISCUSSION

In total, 47 plants of *N. tabacum* variety Samsun were inoculated in the juvenile phase of growth. After 14 days of inoculation, the first symptoms of viral infection were observed in the form of veins lightening, leaves deformation and mottling. Injured leaves were transparent and shrivelled (Christopher, 2001). Results presented in Table 1 show that a positive reaction was found in six lines of *N. tabacum*.

ELISA optical density for the samples № 38, 42, 43 exceeded the commercial positive control on the 15th day of inoculation, which was earlier than expected. It should be noted that OD markedly decreased on the 25th day of inoculation.

This decline in OD corresponded to the literature data, according to which, PVY could be defined only 15 days after inoculation. The high concentration of virus in the culture was short (15 day) and after

Table 1
PVY infected Nicotiana tabacum *plants optical density in ELISA*

Lines №	Plant, variety	OD values in ELISA, units		
		7th day	15th day	25th day
10	*N. tabacum,* Samsun	0,008	0,826	1,335
38	*N. tabacum,* Samsun	0,003	1,313	0,767
42	*N. tabacum,* Samsun	0,571	1,211	0,936
43	*N. tabacum,* Samsun	0,497	1,070	0,977
44	*N. tabacum,* Samsun	0,102	0,571	0,232
1000	*N. tabacum,* Samsun	0,180	0,412	0,343
-	Positive	0,865	1,021	0,912
-	Negative	0,019	0,023	0,021

Table 2
Test results for the callus of N. tabacum *Samsun variety in ELISA*

№	PVY- infected plant samples	ELISA, OD, units		
		Sample	Positive	Negative
1	callus tissue obtained from the line *N. tabacum* №10, №1	0.709	0.375	0.014
2	callus, (*N. tabacum* № 10), the sample №2	0.256	0.375	0.014
3	callus, (*N. tabacum* № 10), the sample №3	0.291	0.375	0.014
4	callus, (*N. tabacum* № 10), the sample №4	0.578	0.317	0.015
5	callus, (*N. tabacum* № 10), the sample №5	0.371	0.375	0.014

a few weeks, it decreased dramatically, whereas on the 60th day of inoculation, no virus was captured by specific antibodies in ELISA (Gnutova, 1993).

However, *N. tabacum* line № 10 showed a maximum accumulation of PVY exactly on the 25th day of inoculation. Primary callus was transplanted onto fresh nutrient medium of the same composition. Callus transplantation was repeated four times (once in 3-4 weeks) during the experiment (Figure 1).

N. tabacum callus average growth-rate throughout the research was 107%. ELISA results showed PVY presence in all the tested callus cultures derived from the infected plant of *N. tabacum*, variety Samsun № 10 (Singh et al., 1983; Salim Khan et al., 2003) (Table 2).

Samsun № 10 optical density always remained at the level of positive control, though sometimes exceeded it. In the next step of our research, PVY was purified from non-pigment tissue culture of *N.*

tabacum. Supernatant obtained as the result of callus homogenisation and low-speed centrifugation was used in the research without any further dilution. The purified antigen was comparatively studied in sandwich-ELISA with commercial PVY antigen. Table 3 presents results of the test.

Table 3
Sandwich-ELISA results of PVY antigens

ELISA, OD, units		
Sample	Positive	Negative
0,922	1, 087	0,033

Table 3 presents the ELISA results of slightly-purified virus antigen. Optical density of the virus in the sample was at the same level with positive control. Purification of PVY from *N. tabacum* callus tissue was done using the second method that included multiple steps (Table 4).

From Table 4, it is evident that after each step of purification, virus concentration reduced significantly in the extract. The greatest loss occurred after clarification
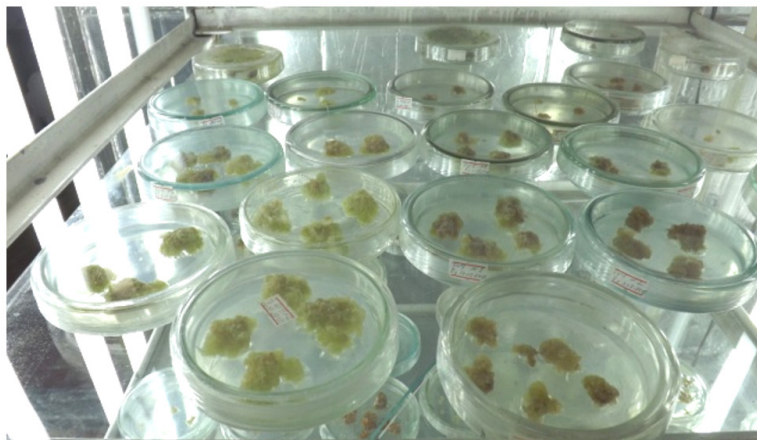
Figure 1. PVY-infected *in vitro* callus of *Nicotiana tabacum*

and extraction of the virus, precipitated with PEG. At the same time, the virus concentration increased significantly as the volume of virus containing-extract reduced multiple times (Syller, 2014). In order to study optical density of the virus, PVY-Cherie was scanned in spectrophotometer SmartSpec Plus, wavelength 240-360 nm (Atabekov I.G.). The results showed that the virus has the same absorption specter (minimum 240 and maximum 278 nm) as threadlike RNA potyvirus.

Potato virus Y is characterised by emergence of new strains, which causes tubers necrosis and reduces their quality (Friemel, 1987; Al-Ani et al., 2011). Taking into account strains diversity, the next step of our research was to study PVY-Cherie for strains identification. For this purpose, ELISA "sandwich" was conducted to test PVY-Cherie in comparison with commercial-collection strains PVY^o-F and PVY^N-L (Tribodet et al., 2005; Nasir et al., 2012) (Table 5).

The PVY-Cherie showed to be of necrotic strain as it reacted weakly with antibodies specific to the PVY "ordinary" strain. Nowadays, polyclonal antibodies as specific immunological reaction component are used widely for potato viral diseases diagnosis (Cojocaru et al., 2009). Based on this, white mice were immunised with PVY antigens (slightly-purified and highly-purified Y-Cherie) to produce specific polyclonal antibodies and determine viral preparations antigenicity (Fridlyanskaya, 1987). Titre of specific antibodies is main indicator of antivirus-diagnostic sera

efficiency (Clark et al., 1977). In our research, indirect ELISA was conducted to test the antibodies (Table 6).

The data presented in Table 6 show that the obtained antibody reacted with a 1/3200 titre of PVY-Cherie (the same as commercial PVY) and with a 1/200 titre of slightly-purified antigen.

Polyclonal antiserum, specific to PVY with a maximum titre of 1/3200 in indirect ELISA, was received from the laboratory white mice (Zulaykha et al., 2014). Thus, PVY accumulation and maintenance in plants and tissue culture of *N. tabacum* indicate the possibility of obtaining viral antigens suitable for mice immunisation and PVY-specific antibodies production.

CONCLUSION

PVY was accumulated and maintained in the test-plants of *N. tabacum*, growing in environmental chamber. Plant of *N. tabacum* variety Samsun, line №10 showed maximum optical density in sandwich-ELISA on the 25th day of inoculation. *N. tabacum* callus tissue average growth-rate was 107%. Slightly-purified and highly-purified (PVY-Cherie) antigens were obtained from the callus culture. PVY-Cherie was detected as PVY "necrotic" strain. Antibody received in answer to PVY-Cherie antigen, showed a titre 16 times higher than in answer to slightly-purified antigen.

Table 5
Highly-purified virus Y-Cherie strains identification-test results

Virus concentration ng/ml	ELISA, OD, units, A_{450}					
	Group of ordinary strains			Group of necrotic strains		
	PVY^0-F	PVY^N-L	Y-Cherie	PVY^0-F	PVY^N-L	Y-Cherie
500	1,936	1,317	0,737	0,679	1,113	0,959

Table 6
Mouse antibody test in indirect ELISA

Serum specific PVY antigens	Titers						
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Slightly-purified viral antigen	+	+	-	-	-	-	-
Highly-purified Y-Cherie antigen	+	+	+	+	+	+	-
Commercial PVY (positive control)	+	+	+	+	+	+	-

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Arsenic Management in Contaminated Irrigation Water for Rice Cultivation

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ABSTRACT

Arsenic (As) contaminated irrigation water (groundwater) is a threat to irrigated rice cultivation. Studies were conducted during three consecutive Boro seasons (fully dependent on irrigation) at highly As contaminated areas in Bangladesh to determine a suitable water management practice to reduce As accumulation in rice. In this study, two water management techniques were evaluated: 1) alternate wetting and drying (AWD) and continuous standing water (CSW) with surface (25 µg L^{-1} As); and 2) groundwater (419 µg L^{-1} As). A high yielding rice variety, BRRI dhan28, was grown. Results showed that the yield obtained by two management techniques were almost similar, except in CSW with groundwater application where significant yield reduction was observed. Significantly lower As content was found in the straw (77.23%) and rice grain (38.14%) of AWD with groundwater and CSW (straw 70.41% and 26.36%) with surface water application compared to CSW with ground water application. Among the water management practices, AWD with groundwater application showed similar benefit to CSW with surface water irrigation. Thus, alternate wetting and drying (AWD) with groundwater or surface water irrigation with CSW can be advocated as an appropriate agronomic practice for rice cultivated in As contaminated soils of Bangladesh.

Keywords: Arsenic mitigation, water management, alternate wetting and drying, continuous standing water

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INTRODUCTION

Rice is the main food crop in Bangladesh. Some of this rice is cultivated on areas contaminated with As. This As comes from irrigation water extracted from underground. Arsenic contaminated irrigation water remains a threat to rice cultivation in Bangladesh and such water adds As to rice soils in Bangladesh, India and some other countries in the South and Southeast Asia (Smith et al., 2000). The added As through irrigation water for 10-20 years or more accumulates in the topsoil gradually, and the amounts now appear to be reaching levels that are toxic (>10.0 mg kg^{-1}) to rice growth (Brammer, 2007). It is reported that As level has gone up to 1.8 mg kg^{-1} in rice grain in some parts of the highly As contaminated areas (Mehrag & Rahman, 2003).

In many countries including Bangladesh, the statutory permissible limit for rice grain is only 1.0 mg of As kg^{-1} dry weight (Khan et al., 2010), but as in grains obtained from As contaminated areas is above the permissible level. A recent study showed that As concentration in the rice straw has reached the level of 92 mg kg^{-1} in Bangladesh (Abedin et al., 2002), whereas the statutory level for As in rice straw is only 0.2 mg kg^{-1} (Nicholson et al., 1999). Arsenic contamination in rice is not only affecting human health, but also drastically reduces grain yield. It is proven that As significantly reduces plant height, effective tiller number, straw weight and results in less rice grain yield (Wang, 2006). Arsenic is one of the most global toxicants that is transferred to human body via the food chain.

Arsenic speciation in the soil environment is dynamic. Inorganic species of arsenite (As-III) and arsenate (As-V) are mostly taken up by the rice plant. However, oxidised condition arsenate and waterlogged condition arsenite are the dominant forms of As. It is proven that As uptake by rice root is influenced by the presence of dominant species in the soil solution, root morphology and the presence of Fe, P and Si (Abedin et al., 2002; Lauren et al., 2003). Arsenite appears to be the main As species that is transported from the root cortical cells to the xylem vessels and its accounts for 60-100% of the total As. It was also found that arsenite accumulation is higher under flooded conditions than non-flooded areas (Li et al., 2009).

The application of As contaminated water increases the level of As in the soils when anaerobic condition occurs. From the previous studies, it has been reported that 85–95% of total As in rice was found under flooded conditions such as wetland rice. Moreover, it was observed that As concentration in soils, groundwater and plants is above the acceptable limits (>1.0 mg of As kg^{-1}). This circumstance poses a severe threat to human and livestock health, and highlights the need for more studies (Hossain, 2006).

There are 150 species of As bearing minerals in nature, namely, As sulfide or realgar (As_2S_2), As tri-sulfide or orpiment (As_2S_3) and arsenopyrite. According to Fazal et al. (2001), in Bangladesh, arsenopyrite has been recognised as the major source of As pollution. The original sources of As

exists as both sulfide and oxide minerals. Furthermore, the oxidation of pyrite from these sources during sediment transport would have released soluble arsenic and sulfate (Karim et al., 1997; BGS, 2000).

Application of irrigation water from different sources having different levels of As and different water management techniques may affect its uptake from soil by the rice plant. Rice grown under flooded conditions is found to accumulate much more As than that grown under aerobic conditions. Roberts et al. (2011) found at the early growth stages, As porewater concentrations reached up to 500 $\mu g\, L^{-1}$ and were dominated by arsenite, while in the later part of the season, soil conditions became toxic and porewater concentration was only 150 $\mu g\, L^{-1}$ of arsenate. Based on these findings, two water management techniques [alternate wetting and drying (AWD) and continuous standing water (CSW)] with two sources of water (groundwater 419 $\mu g\, L^{-1}$ As and less contaminated surface/pond water, 25 $\mu g\, L^{-1}$ As) were tested in this study to mitigate As contamination in soils as well as in rice plant.

MATERIALS AND METHODS

Location

The experiment was conducted at the Bangladesh Rice Research Institute (BRRI) farm Bhanga, Faridpur, during three consecutive Boro seasons (Boro 2011, 2012, 2013). The experimental soil contained OC (%) 1.74, pH 6.5, total N (%) 0.17, available P 9 mg kg^{-1}, K 0.56 $cmol_{(+)}$

kg^{-1} soil, S 29 mg kg^{-1}, Zn= 0.9 mg kg^{-1} and 12.7 mg kg^{-1} of As with sandy clay loam in texture. In Bangladesh, Boro season is considered as a dry season (from November to April) and rice is cultivated mainly with ground irrigation water. The experimental location was at BRRI regional station at Bhanga, Faridpur (23°23'20.49'' N and 89°59'27.07''E).

Planting

The popular high yielding rice variety BRRI dhan28 was grown in this study. Thirty-day-old rice seedlings were transplanted (20 cm × 20 cm) in the research plots. The fertilisers applied were N, P, K, S and Zn at 115-20-60-12-3 kg ha^{-1} from urea, triple super phosphate (TSP), muriate of potash (MOP), gypsum and zinc sulfate, respectively. Each plot size was 4 m × 4 m and the distance between plants was one meter. There were two water sources and two cultivation methods. The experimental design was Randomized Complete Block Design (RCBD) with three replications. Rice plant was harvested at maturity. The experiment was repeated for three successive seasons.

Water Management

Two water management techniques (AWD and CSW) were maintained with two sources of water, namely, groundwater and surface water containing 419 $\mu g\, L^{-1}$ and 25 $\mu g\, L^{-1}$ As, respectively. Irrigation water was continuously applied during the first 2 weeks in all the research plots. However, after 2 weeks, AWD plots received irrigation water

and kept up to 15 cm depth from the soil surface for the period of panicle initiation to maturity in all the plots.

Determination of As in Soil and Water

Samples were dried and processed before digestion and total As content was determined by digesting the soil, paddy and straw samples with tri-acid mixture (HNO_3:$HClO_4$:H_2SO_4, 5:2:1) until it became whitish or clear (Rahman et al*.,* 2007).

Sample Preparation and Digestion

Rice samples were sun-dried soil sampled and ground. About 1.0 g ground samples were taken separately into digestion tube and 10 mL of 69% concentrated nitric acid and 70% of perchloric acid mixture at the ratio of 5:3 were added. The samples were left to react overnight in a chemical hood, and then heated in a block digester (M-24 plazas/ samples, JP Selecta, Spain) at 160ºC for two hours, and later it was increased to 300ºC for about 4-5 hours until colourless clear watery fluid appeared. Tubes were gently shaken several times to facilitate destruction of all the carbonaceous material. This digestion converts all arsenicals to inorganic arsenic for FI-HG-AAS determination. Tubes were removed from the digestion block, cooled, diluted to 50 mL adding Millipore water, filtered through filter paper and stored in 50 mL plastic bottles.

Clean and oven-dried rice straw samples were ground and digested as described by Wang et al. (2006) with some modifications. About 0.45 - 0.50g ground rice straw sample was taken after further drying at 60ºC to

constant weight. It was taken separately into digestion tube and 7 mL of 69% concentrated nitric acid was then added. Similar procedures were followed as before.

Detection of Arsenic

The digest was cooled and then filtered, and the volume was finally made up to 50 mL. Concentrations of As in digested samples were determined using atomic absorption spectrophotometer (AAS), model PG – 990, equipped with a computer with atomic absorption (AA) Win software (PG Instruments Ltd., UK), as described by Samanta et al. (1999). Briefly, the samples were spiked with standards at different concentrations. Arsenic in the sample was calculated using the following formula:

$$\text{As concentration} = \frac{\text{As in sample solution (μL)} \times \text{mL of sample}}{\text{Sample weight (g)} \times 1000}$$

Statistical Analysis

The analysis of variance (ANOVA) was done and Duncan's multiple range test (DMRT) was used for mean comparisons of the treatment at 5% level of probability. Pearson correlation coefficients among the parameters were also determined.

RESULTS

As Concentration in the Paddy Soils

The concentration of As in the soils varied due to different water management practices (Table 1). It was observed that higher As concentration (11.6-13.9 mg kg^{-1}) was recorded in the first Boro (2011) compared to that of the following season for both water

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management practices. In the CSW system, comparatively high As values were obtained in the first season of Boro 2011 compared to AWD, whereas similar values were observed for the remaining seasons. Among the water sources, irrigation with surface water resulted in lower As concentrations than the groundwater. This was due to the surface water containing lower As compared to that of the groundwater. In Bangladesh, it is known that arsenopyrite ($FeAsS_2$) occurs in some of the sediments in its floodplains, especially a few meters below the surface.

When wells are dug in those areas, mineral is disintegrated and oxidised, releasing As into the water.

As Uptake in Straw and Grain of Paddy

The present study showed that significantly higher As content was found in the straw and rice grain of the CSW compared to that of the AWD technique (Table 2). Additionally, higher concentrations of As were found in the rice straw for both of the water management practices. However,

Table 1
Arsenic contents in the soils at harvest

Methods of irrigation	Source	Soil As ($\mu g\ kg^{-1}$) (post-harvest)		
		Boro 2011	Boro 2012	Boro 2013
CSW	surface water	13.9	9.91	10.38
	ground water	13.9	10.47	10.70
AWD	surface water	11.6	9.2	9.28
	ground water	12.9	11.22	10.80
	LSD $_{(5\%)}$	3.18	1.49	2.89
	CV %	12.2	8.5	14.1

AWD = alternate wetting and drying, CSW = continuous standing water
Initial soil As =12.7 mg kg^{-1}, STW water As=419 $\mu g\ kg^{-1}$, Pond water As =25 $\mu g\ kg^{-1}$

Table 2
Arsenic contents (mg kg^{-1}) in the straw and paddy

Methods of irrigation	Source	Boro 2011		Boro 2012		Boro 2013	
		Straw	Paddy	Straw	Paddy	Straw	Paddy
		---------------------------- mg kg^{-1} ----------------------------					
CSW	surface water	1.79	0.48	0.52	0.35	1.07	0.46
	ground water	3.43	0.52	0.85	0.46	1.48	0.65
AWD	surface water	1.55	0.34	0.39	0.29	0.70	0.45
	ground water	1.73	0.40	0.67	0.37	0.85	0.41
	LSD $_{(5\%)}$	0.61	0.61	0.35	0.08	1.17	0.14
	CV %	14.4	14.4	33.2	12.7	57.4	14.1

AWD = alternate wetting and drying, CSW = continuous standing water

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the concentrations of As were higher in the first (Boro 2011) compared to that of the following two seasons.

Among the water sources, groundwater showed higher As values during the planting period compared to those of surface water. The trend of the As uptake by the rice plant was found to follow the order of straw > grain. Higher As concentration observed in the CSW system might be due to rice plant growing under water saturated soil conditions through the entire life period in which more As was mobilised and absorbed into the rice plant. On the other hand, in the AWD system, part of the growth phase was under dry condition with plenty of oxygen around the root system that helps to form Fe plaque. The actual As in bulk was locked in the soils and did not get into the plants to such an extent as for rice cultivated under CSW condition.

In the present study, CSW agronomic practice using surface water showed less As accumulation in the paddy and post-harvest soils. Approximately 77.23 and 38.14% of straw and grain As reduction, respectively, were found in AWD with groundwater irrigation which was closer to CSW with surface water treatment, where 70.41% in straw and 26.36% in grain As reductions were observed compared to CSW with groundwater irrigation.

The addition of As with irrigation water severely affected the rice tillers number, panicle length and grain yield significantly ($p \leq 0.05$). The maximum plant height and straw yield was observed at lower As concentrations. However, the lowest tillers number, panicles number, panicle length and grain yield were found in control treatment.

Grain Yield

The effects of different water management practices on the grain yield during the three consecutive years differed (Figure 1). For Boro 2011, the grain yield of BRRI dhan28 differed significantly among the treatments. Maximum grain yield was observed in the AWD treatment irrigated with groundwater (8.28 t ha^{-1}), followed by (8.21 t ha^{-1}) continuous standing water irrigated with surface water treatments (Figure 1a). Unlike grain yield, grain As content was not affected by the water management practices. Grain As content of BRRI dhan28 ranged from 0.17 to 0.26 mg kg^{-1} and was much lower than the permissible limit of 1 mg kg^{-1}.

The study showed that the grain yield was significantly decreased by the As concentration present in the groundwater used for the CSW, although it was not the case for surface water used in the AWD irrigation system. It was observed that Boro 2012 showed lower grain yield than that of the other seasons (Figure 1b and Figure 1c). Among all seasons, a decrease in yield was found in the CWS planting seasons irrigated with groundwater (Boro 2011-2013). Post-harvest soils As concentration was increased by the continuous standing water condition. In the AWD system, such an increase was not observed in soil whereas surface water irrigation system showed a decreasing tendency of As in the soil. However, more As content in both straw and grain was found in the CSW system than the AWD
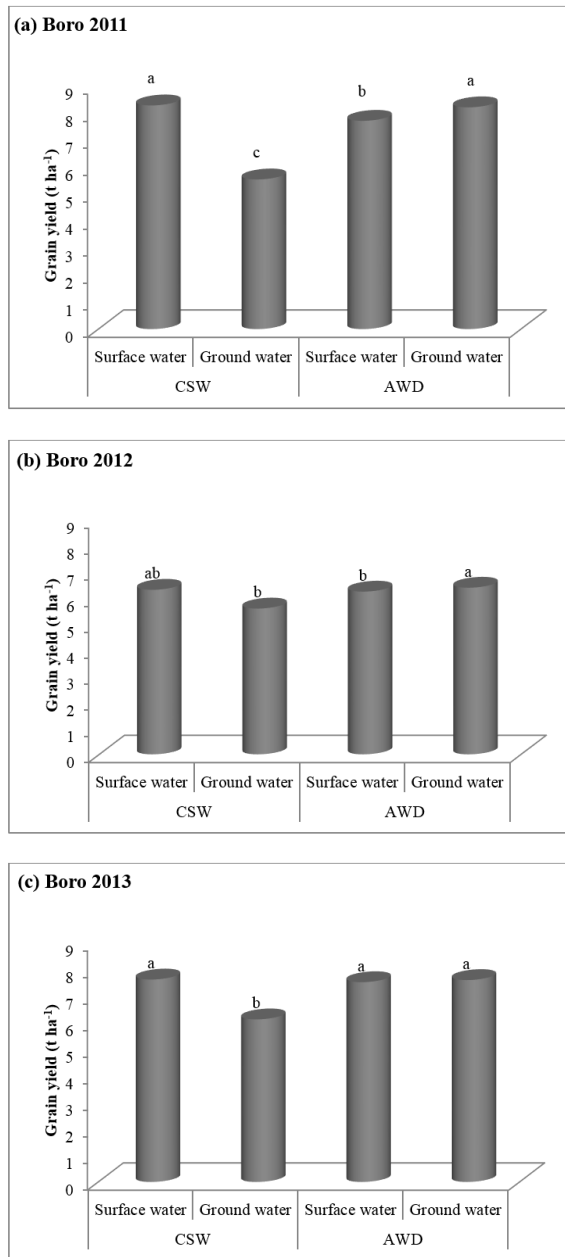
Figure 1. Effects of arsenic contaminated irrigation water on the rice yield (a) Boro 2011, (b) Boro 2012 & (c) Boro 2013

(AWD = alternate wetting and drying, CSW = continuous standing water)

system, yet these values were lower in both irrigation systems irrespective of different As contents in the irrigation water. The grain yield of BRRI dhan28 significantly decreased under continuous standing water system (CSW) irrigated with groundwater which might be due to continuous standing of arsenic contaminated groundwater. Furthermore, the As concentrations in either irrigation water or as soil-applied rice reduced yield significantly.

DISCUSSION

Arsenic contaminated irrigation water used for the paddy cultivation increased As concentration in the cropland. Simultaneously, the As accumulated in the soil becomes hazardous to the plants. A similar finding has also been reported by Roberts et al. (2007). Flooded conditions in the paddy soils lead to As mobilisation that enhances its bioavailability to the rice plant (Takahashi et al., 2004; Xu et al., 2008). Similar findings were also reported by Ahmed et al. (2011) that As in grain was higher in the Boro than the Aman season, which was nearly twice as high. Hence, the seasonal differences in grain As concentration could be caused by irrigation of Boro rice with As contaminated groundwater. On the other hand, reduced condition in the soils releases iron oxides that increase inorganic arsenic content. Furthermore, even the flooding (monsoon) is unable to prevent the As accumulation in rice soils. Farmers usually practice intermittent irrigation which temporally limits As release into porewater during rice growth (Roberts

et al., 2012). Li et al. (2009) proved that there was a rapid rise in As concentration in water as Eh dropped below 200 mV. Rice plant mostly takes arsenite, which is the dominant species under flooded conditions. Arsenate, rather than arsenite, is the main species that could adsorb on Fe-oxide root precipitates (Liu et al., 2006) and that is a positive factor as arsenate adsorption will be higher than that of arsenite in lower pH environment of rice rhizosphere (Dixit & Hering, 2003). Under oxidised state and also due to root respiration, iron plaque forms in the rhizosphere result in the oxidation of ferrous to ferric ion that precipitates as Fe hydroxides on the root surface. Fe hydroxides have a strong adsorptive capacity for arsenate making it unavailable for rice plant uptake (Chen et al., 2005). The presence of P also influences As uptake by rice plant and As concentration in rice shoot has been found to significantly reduce under low P condition (Sun, 2008).

Furthermore, it is clearly shown that soil becomes highly contaminated with As due to the excessive use of arsenic rich groundwater for irrigation (Bhattacharya et al., 2009). According to the findings of BGS (2000), groundwater As problem occurs because of three factors: 1) a source of As (As is present in the aquifer sediments); 2) mobilisation (As is released from the sediments to the groundwater); and 3) transport (As is flushed in the natural groundwater circulation).

The mobilisation of As into groundwater is mainly due to arsenopyrite oxidation and oxy-hydroxide reduction (Zheng et al., 2004). These As contaminants in the vadose

zone in to the soil may be due to long-term source of groundwater contamination and these require remedy evaluations. Most remediation for the vadose zone needs to be made in part based on projected impacts to groundwater of the soil (Truex & Carroll, 2013). Due to the lowering of water table, the oxidation of arsenopyrite in the vadose zone releases As and this As can be adsorbed on Fe hydroxide during the subsequent recharge period; the reduction of Fe hydroxide releases As into groundwater. Moreover, the extent of formation of Fe-oxide that may precipitate on rice roots and in soil rhizosphere is one of the important factors leading to adsorption of As from soil solution (Hossain et al., 2009) and can reduce As uptake by rice (Mei et al., 2009).

Lauren and Duxbury (2005) and Li et al. (2009) suggested cultivating non-flooded cereal crops in Boro season in As contaminated areas. It is known that As contamination in soil hampers rice plant growth and yield, although trace amount is required for plant growth (Khan et al., 2010). Rice grown on As contaminated soils suffers from a disease called stripped head that decreases crop yield. Similar findings were reported by Dilday (2000), whereby the presence of soil contamination with As caused yield reduction in rice. There is no doubt that irrigating rice fields with groundwater enhances food security in Bangladesh. Nonetheless, the presence of high As concentrations in the groundwater limits its use for rice cultivation. To a certain extent, the hazard of using groundwater for irrigating rice fields can be reduced via special management practice proposed by this study. The accumulation of As and Cd in rice grains in opposite trends was affected by both water management and rice cultivar. Hence, the safe rice in relation to As and Cd may be possible through balancing water management and rice cultivar based on the severity of soil pollution (Hu et al., 2013).

CONCLUSION

Contamination of As in groundwater is a threat for rice cultivation in Bangladesh. In this study, different water management techniques were evaluated for As uptake and accumulation in soil and plant. The results of the three years of field study demonstrated that AWD with groundwater irrigation system or irrigation with surface water could reduce As accumulation in the rice plant and soil. Hence, alternate wetting and drying (AWD) with groundwater or surface water irrigation with CSW can be advocated as an appropriate agronomic practice for rice cultivated in As contaminated soils of Bangladesh.

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Influence of Salt and Water Stress on Growth and Yield of Soybean Genotypes

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ABSTRACT

Water uptake by the plant is difficult in saline and water stress condition due to decreasing osmotic potential of soil. Soybean yield is highly affected by the soil water scarcity. An experiment was conducted to evaluate the growth and yield of three selected soybean genotypes in salt and water stress conditions at a vinyl house. Three soybean genotypes, namely, Galarsum, BD 2331 and BARI Soybean 6, were tested in six different treatments. The six different treatments were: T1 (Control, no salt or water stress), T2 (Water shortage, WS), T3 (50 mM NaCl), T4 (50 mM NaCl + WS), T5 (75 mM NaCl) and T6 (75 mM NaCl + WS). Plant height, shoot dry weight and dry matter distribution in different parts were sharply decreased when the plants were exposed to the combined salt and water stress conditions. The combined effect of salt and water stress was more severe in yield reduction than the single effect. The seed yield of soybean was decreased with increase in salinity. Among the genotypes, dry matter reduction was the lowest in Galarsum. In addition, Galarsum also showed the highest filled pods and 100 seed weight. The maximum seed yield was found in Galarsum. Galarsum was found to be suitable to grow in saline and water stress condition. Therefore, this genotype can be recommended for saline and water shortage zones.

Keywords: Soybean, water stress, combined salinity and water stress, yield

INTRODUCTION

Soybean has become an important crop in Bangladesh for its increasing demand as an ingredient of poultry and fish meal. Therefore, soybean is now one of the stable and economic crops in Bangladesh and it mostly concentrates in the coastal area of the southern part of the country, more specifically in greater Noakhali district. Its production area is increasing day by day and in the year 2013 it reached above 61000 ha (Chowdhury, 2014). In Bangladesh, thirty percent of the net cultivable areas are in the coast. Out of 2.85 million hectares of the coastal and off-shore areas, about 0.83 million hectares of the arable lands are affected by varying degrees of soil salinity (Karim et al., 1990). High levels of salt in the soil of the coastal region often cause serious limitations to crop production.

High levels of salt in soil can often cause serious limitations to crop production. Soybean is classified as a moderately salt-tolerant crop and the final yield of soybean is reduced when soil salinity exceeds 5 dS/m (Ashraf, 1994). It is common in the arid and semi-arid regions that when the crop growth season progresses, precipitation decreases, and temperature and evapotranspiration increase, resulting in rising salt concentration in the soil solution (Abdulrahman & Williams, 1981). Thus, salt and water stress prevails at the same time in the dry seasons, which very often adds extra harm on plant growth (Karim et al., 1993). Water shortage decreases matric potential that increases the resistance of water flow to the roots (Homaee et al., 2002), whereas soil salinity reduces the soil water potential but does not reduce water flow to the roots. Plant roots contain varying concentrations of ions that create a natural flow of water from the soil into the plant roots and maintain the root growth (Everard et al., 1994). Salinity has damaging effect on plant growth mostly due to the toxicity of specific ions or as a result of osmotic stress (Munns, 2002). However, the adverse effects of both salt and water stress are primarily due to the restriction of water uptake by the roots (Karim et al., 1993). Therefore, plants are unable to maintain metabolic activities or turgidity for normal growth because of the low osmotic potential in soil.

Raptan et al. (2001) reported that salinity decreased dry matter production in different plant parts of mungbean. Salinity also caused drastic reduction in grain yield of many crops including soybean (Ghassemi-Golezani et al., 2009), mungbean (Aziz et al., 2006) and peas (Duzdemir et al., 2009). Aziz et al. (2006) observed a relationship in salt tolerance between vegetative and maturity stages in mungbean.

Blum (1985) reported that salt tolerance at the seedling stage was highly predictive to that at the reproductive stage of crop plants. However, Ashraf and McNeilly (1988) and Kingsbury and Epstein (1986) did not find such relationship in salt tolerance between early and mature stages of growth in wheat. Soybean germplasms displayed a broad spectrum of salt tolerance capability and the degree of their salt tolerance varied with the developmental stages and environmental factors (Abel & MacKenzie, 1964; Maas & Hoffman, 1977; Blum, 1988; Chang et

al., 1994). Moreover, soybean genotypes also varied in their growth responses to combined salt and water stress (Khan et al., 2014). At the terminal stage of the soybean crop, due to high evaporative demand of the air in the dry seasons, salt and water stress prevails at the same time, which very often adds extra harm on plant growth. Therefore, selection of soybean genotype for salt and water stress tolerance would be helpful for increasing total production of soybean that ultimately improve the income level of the soybean growers.

However, the yield response of the soybean genotypes to salt and water stress has not been reported. Therefore, this experiment was initiated with the objective to find out suitable soybean genotypes grown under salt and water stress conditions based on their growth and yield.

MATERIALS AND METHODS

Study Site

The experiment was conducted in a vinyl house of the Department of Agronomy at Banghabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Salna, Gazipur, Bangladesh, during January to May, 2012. The location is situated at about $24^0 23'$ north latitude, $90^0 08'$ east longitude and at an altitude of 8.4m above sea level and adjacent to the capital city, Dhaka.

Cropping Season

The experiment was set up in winter (*Robi*) and carried out up to summer (*Kharif-1*) with crop duration.

The Test Crop

Three genotypes of soybean (*Glycine max* L.) viz. Galarsum, BD 2331 and BARI Soybean 6 were used to observe dry matter production and seed yield performance under salt and water stress condition. These genotypes were selected based on their performance at vegetative stage in a previous study (Khan et al., 2014).

Potting Preparation and Seed Sowing

The experiment was carried out in plastic pots having 30 cm in height and 24 cm inner diameter. Each pot contained 12 kg of air dried sandy loam soil. The soils of each pot were fertilised uniformly with 0.30, 0.90, 0.60 and 0.60 g pot^{-1} of urea, triple super phosphate, muriate of potash and gypsum, respectively, before sowing. Soybean seeds of the respective genotypes were washed several times in the tap water for surface cleaning, and then sown in the soil medium on January 20, 2012, in plastic pots. Five seeds were dibbled in soil at a depth of 1 cm.

Experimental Design and Treatments

The experiment was laid out in two factors Completely Randomized Design (CRD) with four replications. The factors were three genotypes (Galarsum, BD 2331 and BARI Soybean 6) of soybean, with six treatments of salinity and water stress. The environmental conditions were T1 (Control), T2 (Water shortage, irrigation with 70% depletion of available soil water when wilting sign developed), T3 (50 mM NaCl irrigation), T4 (50 mM NaCl irrigation + Water shortage), T5 (75 mM NaCl

irrigation), and T6 (75 mM NaCl irrigation+ Water shortage). Treatments were imposed after three weeks of seedling emergence. In salt water irrigation and water shortage treatments, all pots were initially irrigated with salt water for a week, followed by water shortage and salt water irrigations thereafter. The control plants were irrigated with tap water only with maintained field capacity. Treatments were applied up to maturity.

Intercultural Operation

After the emergence and establishment, two uniform healthy seedlings per pot were allowed to grow for three weeks in equal environment. Admire 200SL @ 1 ml L^{-1} of water was sprayed at 10 and 25 days after emergence to control Jassids and white flies. Ripcord 10 EC @ 1 ml L^{-1} of water was sprayed at 45 and 60 days after emergence to control leaf roller and pod borer.

Harvesting and Data Recording

The crop was harvested at maturity from April 1-25, 2012. The plant samples were collected 56 days after emergence. Plant height was measured and different plant parts were separated before oven drying them at 70ºC for 4 days to measure the dry weight of shoot. After maturity, yield contributing characters like total pods per plant, filled pods per plant, 100-seed weight and seed yield per plant were recorded. Electrical conductivity of the pot soil was also recorded using EC meter at different times.

Statistical Analysis

The analysis of variance for growth parameters and crop yield were done following the ANOVA test and the mean values were compared by LSD (Least significant difference). Computation and preparation of graphs were done using Microsoft EXCEL 2003.

RESULTS AND DISCUSSION

Changes of Electrical Conductivity

The electrical conductivity was influenced by the different treatments (Table 1). It ranged from 1.05 to 14.4 dSm^{-1}. The highest electrical conductivity (14.4 dSm^{-1}) was found in 75 mM NaCl, followed by 50 mM NaCl (11.3 dSm^{-1}) at harvest. Among the days after emergence, 63 DAE showed the maximum electrical conductivity (9.30 dSm^{-1}) in 75 mM NaCl, followed by 56 DAE in the same salinity level. The minimum electrical conductivity (1.05 dSm^{-1}) was in 42 DAE at water stress. It was observed that electrical conductivity was increased with the increase in days after emergence. The electrical conductivity also increased with the increase in the salinity levels. Water stress + NaCl have decreased electrical conductivity compared to only NaCl due to maintaining 70% depletion of salt water irrigation.

Plant Height

The plant height of soybean genotypes was significantly influenced by the combined effects of genotypes and different treatments (salinity and water stress). Except for the

control, BD 2331 showed the highest plant height (57.7 cm) which was identical to BARI soybean 6 and Galarsum in 50mM NaCl (Figure 1). The lowest plant height (33.5cm) was found in BARI Soybean 6 at 75 mM NaCl along with water stress. Among the treatment combinations, T_3 (50 mM NaCl) showed the highest plant height (average 56.3cm), followed by T_5 (75mMNaCl) and the lowest was in T_6 (75

mM NaCl + water stress) (Figure 1). It was observed that the plant height of soybean decreased with the increase in the salinity level. Similar results were also reported by Ozturk et al (2004) and Sari and Ceylan (2002). Osmotic potential and matric potential were decreased due to salt water and water shortage, respectively, in soil. The phenomena interrupted in water uptake, resulting in reduction of shoot growth.

Table 1
Changes in electrical conductivity in the growth media as influenced by the treatment and days after emergence (DAE)

Treatment	Electrical conductivity (dSm⁻¹)				
	42 days after emergence	49 days after emergence	56 days after emergence	63 days after emergence	At harvest (70-95 DAS)*
Control	1.17	1.36	1.38	1.38	1.38
Water stress (WS)	1.05	1.17	1.21	1.21	1.21
50 mM NaCl	3.60	3.63	5.90	6.00	11.3
50 mM NaCl + WS	2.77	3.50	4.50	5.25	7.80
75 mM NaCl	5.50	5.83	8.75	9.30	14.4
75 mM NaCl + WS	4.51	4.90	4.90	7.20	9.55

* Maturity varied due to treatment effect
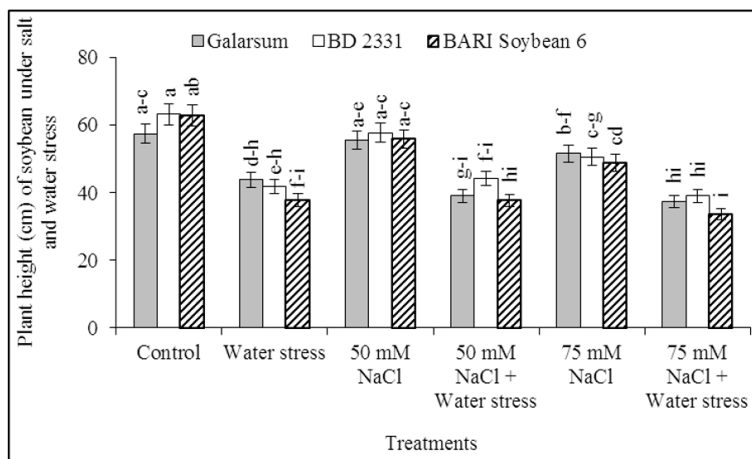
Figure 1. Plant height of soybean genotypes as influenced by the salinity and water stress at 56 days after emergence. Figure(s) in bars having common letter (s) do not differ significantly (*p*=0.05*)*

Among the genotypes, BD 2331 showed the maximum height (average 49.4 cm), followed by Galarsum (47.4 cm) and the minimum (46.1 cm) was in RARI soybean 6. Genotypic difference in reduction of plant height due to salinity was reported by Mannan et al. (2012) in soybean. Sultana et al. (2009) and Podder et al. (2012) also observed reduction of shoot growth of mungbean at different salinity levels, the result which is in agreement with the findings of our results.

Shoot Dry Weight

Shoot dry weight of soybean was significantly variable among the interaction effects of treatment and genotype. Except for the control, the shoot weight ranged from 2.37 to 6.63 g plant[-1]. The highest shoot weight (6.63gplant[-1]) was found in BD 2331 at 50

mM NaCl, which was significantly higher than the other treatment combinations. The lowest shoot weight (2.37g plant[-1]) was recorded in BARI soybean6 at water stress condition (T_2) Table 2). Galarsun in 50 and 75 mM NaCl showed statistically similar shoot weight. It was observed that the shoot weight of soybean decreased with the increase in salinity levels. It was also found that NaCl, alone or in combination with water stress, produced more shoot biomass than the only water stress; this might be due to salt being added along with water. Here, plant received water which helped it to grow. Under water stress, the resistance of water flow into the root increased with the decrease in matric potential (Homaee et al., 2002). At given salt water content reduces the soil water potential but does not reduce water flow to the roots. Root cortical cells can osmotically adjust to some

Table 2

Shoot dry weight of soybean (g/plant) as influenced by the genotypes and treatment (salinity and water stress) (percentages to control in parenthesis) at 56 days after emergence

Treatment	Genotypes			
	Galarsum	BD 2331	BARI Soybean 6	Mean
Control	9.07 ± 0.29a (100)	8.67 ± 0.26ab (100)	8.49 ± 0.31b (100)	8.74
Water stress (WS)	3.26 ± 0.21gh (35.94)	2.68 ± 0.46ij (30.91)	2.37 ± 0.37j (27.92)	2.77
50 mM NaCl	5.86 ± 0.21d (64.61)	6.63 ± 0.18c (76.47)	5.30 ± 0.09e (62.43)	5.93
50 mM NaCl + WS	3.57 ± 0.10gh (39.36)	3.59 ± 0.18g (41.41)	3.25 ± 0.55gh (38.28)	3.49
75 mM NaCl	5.38 ± 0.08de (59.32)	5.20 ± 0.20e (59.98)	4.69 ± 0.68f (55.27)	5.09
75 mM NaCl + WS	3.41 ± 0.10gh (37.60)	3.10 ± 0.21hi (35.76)	3.18 ± 0.11gh (37.46)	3.23
Mean (Except control)	4.30	4.20	3.76	

Figure(s) in columns having common letter (s) do not differ significantly (*p*=0.05*)*. CV % = 6.20

extent allowing water to move into the root. Therefore, the shoot dry weight of soybean was affected more in only water stress than the salt and water stress combined. This result corroborates with the findings of Meiri (1984), where the matric potential preferentially affected the shoot growth of bean more than the osmotic potential. Wang et al. (2011) also reported that shoot biomass was decreased significantly in tamarisk seedlings due to water scarcity under salt and water stress. The reduction in shoot dry weight due to salinity was observed by Jamil et al. (2007) in sugarbeet, Sultana et al. (2009) in mungbean, Chookhampaeng (2011) in pepper plant, and Mannan et al. (2013) in soybean. Among the genotypes, Galarsum showed the highest biomass (average 5.09 g plant^{-1}), followed by BD 2331 (4.98 g plant^{-1}) and the lowest was in BARI soybean 6.

Dry Matter Distribution

Dry matter distribution in different plant parts of soybean genotypes, as affected by water stress, salt stress and combination of salt and water stress at 56 days after emergence, is given in Figure 2. All plant parts were reduced by stresses in all genotypes and the reduction was more in the combined salt and water stress than the only salt stress in both the salinity levels (50 mM and 75 mM NaCl). It was observed that the dry matter reduction was increased with the increase in salinity in combination with water stress. At 75 mM NaCl salt and water stress, the highest dry matter of stem (1.38 g), petole (0.43 g) and leaves (1.55 g)

was recorded in Galarsum and the lowest dry matter (1.27, 0.40 and 1.38 g for stem, petiole and leaves, respectively) was in BD 2331 genotype.

The dry matter reduction was more in the leaf, but it varied from one genotype to another. At 75 mM, NaCl along with water stress, the highest leaf weight (1.55 g) was found in Galarsum, followed by BARI Soybean 6 (1.42 g) and the lowest (1.38 g) was in BD 2331. Wang et al. (2011) also reported that the leaf biomass decreased significantly in tamarisk (*Tamarix chinensis* Lour) seedlings due to water scarcity under salt and water stress.

Pods per Plant

The number of pods per plant was significantly affected by the salinity and water stress (Table 3). The lowest number of pods of 12.22 per plant (21.7% to control) was recorded in Galarsum at 75 mM NaCl with water stress, followed by BD2331 (12.67) at the same salt concentration. All the three genotypes also produced identical number of pods in 50 and 75mM NaCl in combination with water stress. It was also observed that soybean genotypes showed significantly lower number of pods plant^{-1} in salt, along with water stress than salt stress only. It is difficult for the plant to uptake water in either saline or water stress condition due to the decreasing osmotic potential of soil. Water stress was caused by the salinity or water shortage as a result of decreased pod number in peas (Duzdemir et al., 2009). Westgate and Peterson (1993) reported that soybean

yield is more sensitive to drought stress during its early reproductive stage (i.e., flowering to early pod expansion) than the other developmental stages. Drought stress during this period increases the rate of pod dropping (Westgate & Peterson, 1993), leads to a lesser number of pods per plant (Desclaux et al., 2000) and ultimately decreases seed yield (Kokubun et al., 2001).

Filled Pods per Plant

The number of filled pods of soybean was significantly variable among the interaction effects of genotypes and treatment (Table
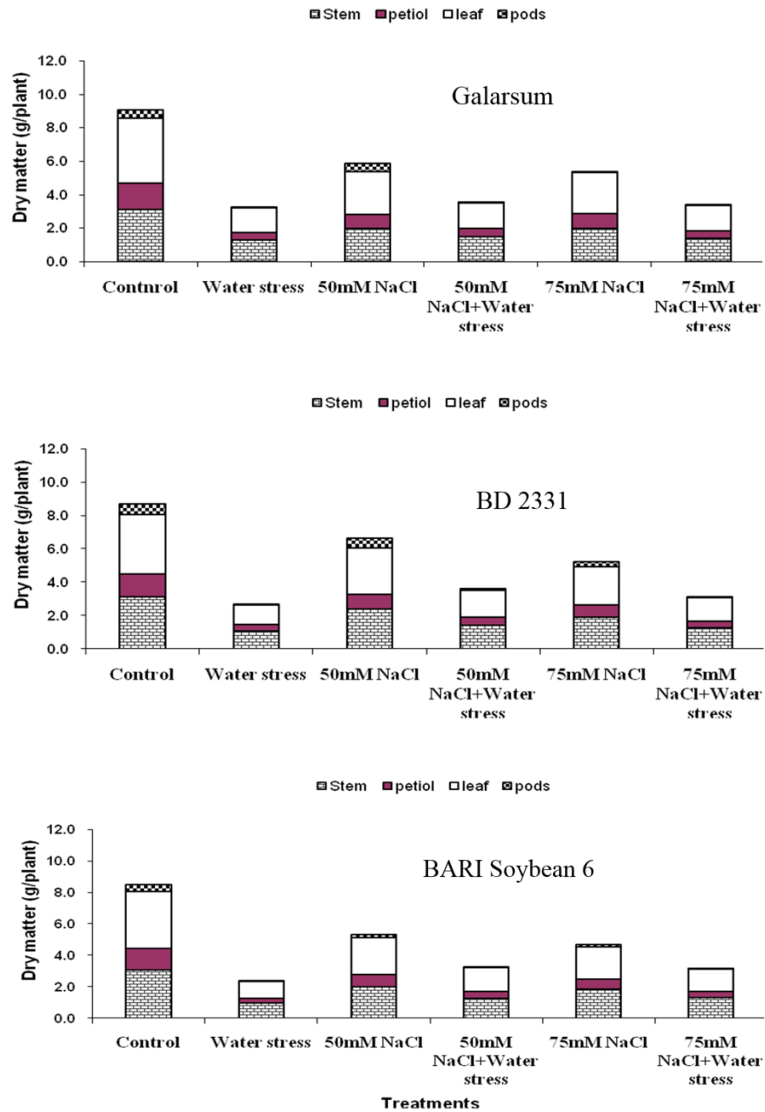
Figure 2. Dry matter accumulation in different plant parts of soybean as influenced by the genotypes and treatment (salinity and water stress at 56 days after emergence

4). The least number of filled pods of 7.91 per plant (16.59% to control) was found in BARI Soybean 6 at 75 mM NaCl salt combined with water stress. In 50 mM NaCl, BARI sotbean 6 also showed the highest filled pods (17.11), which was followed by Galarsum (15.89 pods plant^{-1}) in the same salt concentration. Among the genotypes, Galarsum showed the highest filled pods (average: 13.0 pods plant^{-1}), followed by BD 2331(average: 13.0 pods plant^{-1}) and the minimum was in BARI Soybean 6 (average 11.32 pods plant^{-1}). The numbers of filled pods decreased to 41.04, 26.42 and 18.23% of the control under 75 mM NaCl salinity in Galarsum, BD 2331 and BARI Soybean 6, respectively. Under water stress, the number of filled pods decreased to 41.07, 29.87 and 30.06% of the control in Galarsum, BD 2331 and BARI Soybean 6, respectively. The reduction in the filled pods might be due to

the reduction of pollen fertility caused by water stress and/or salt stress. Duzdemir et al. (2009) reported that the number of seeds in peas decreased by water shortage was caused by salinity or water stress. Salinity induced reduction in the number of seed in soybean was also reported by Ghassemi-Golezani et al. (2009).

100-Seed Weight

The 100-seed weight ranged from 4.44 to 9.86 g (Table 5). The lowest 100-seed weight of 4.44 g (48.37% to control) was recorded in BARI Soybean 6 at 75 mM NaCl salt stress, which was statistically similar with BD 2331 (4.98 g) at the same treatment, and this was also identical with all genotypes at 75 mM NaCl along with water stress. However, Galarsum showed the highest 100-seed weight than the other genotypes at 50 and 75 mM NaCl salt stress

Table 3

Number of pods in soybean (number plant^{-1}) as influenced by the genotypes and treatment (salinity and water stress) (percentages to control in parenthesis)

Treatment	Genotypes			
	Galarsum	BD 2331	BARI Soybean 6	Mean
Control	42.33 ± 6.11b (100)	61.00 ± 9.17a (100)	56.33 ± 6.66a (100)	53.2
Water stress (WS)	16.67 ± 3.06e (39.38)	17.67 ± 4.16de (28.97)	17.00 ± 3.61e (30.18)	17.1
50 mM NaCl	23.78 ± 1.39cd (56.18)	23.89 ± 4.55cd (39.16)	27.33 ± 3.18c (48.52)	25.0
50 mM NaCl + WS	12.89 ± 0.51e (30.45)	14.56 ± 1.58e (23.87)	13.44 ± 2.27e (23.86)	13.6
75 mM NaCl	24.00 ± 1.20cd (56.70)	23.44 ± 1.07cd (38.43)	24.33 ± 2.08c (43.19)	23.9
75 mM NaCl + WS	13.11 ± 0.51e (30.97)	12.67 ± 3.53e (20.77)	12.22 ± 0.84e (21.69)	12.7
Mean (except control)	18.1	18.4	18.9	

Figure(s) in columns having common letter (s) do not differ significantly (*p*=0.05*)*.CV % = 15.8

alone or in combination with water stress. It was also observed that salinity badly affected the 100-seed weight of soybean, while the reduction of 100-seed weight was not significant in water stress in comparison to the control. Yield components like pod number and individual seed weight are genetically determined and subjected

Table 4
Filled pods of soybean (number plant^{-1}) as influenced by the genotypes and treatment (salinity and water stress) (percentages to control in parenthesis)

Treatment	Genotypes			
	Galarsum	BD 2331	BARI Soybean 6	Mean
Control	37.33 ± 3.21b (100)	51.33 ± 3.51a (100)	47.67 ± 4.51a (100)	45.4
Water stress (WS)	15.33 ± 3.06c (41.07)	15.33 ± 3.06c (29.87)	14.33 ± 3.06cd (30.06)	15.0
50 mM NaCl	15.89 ± 1.84c (42.57)	15.67 ± 3.33c (30.53)	17.11 ± 1.92c (35.89)	16.2
50 mM NaCl + WS	10.33 ± 0.33de (27.67)	8.50 ± 1.50e (16.56)	8.56 ± 2.59e (17.96)	9.13
75 mM NaCl	15.32 ± 1.22c (41.04)	13.56 ± 1.71cd (26.42)	8.69 ± 1.02e (18.23)	12.5
75 mM NaCl + WS	8.11 ± 1.26e (21.73)	8.44 ± 2.59e (16.44)	7.91 ± 1.33e (16.59)	8.15
Mean (except control)	13.0	12.3	11.3	

Figure(s) in columns having common letter (s) do not differ significantly (*p*=0.05*)*.CV (%) = 14.2

Table 5
100-seed weight (g) of soybean as influenced by the genotypes and treatment (salinity and water stress) (percentages to control in parenthesis)

Treatment	Genotypes			
	Galarsum	BD 2331	BARI Soybean 6	Mean
Control	9.86 ± 0.37a (100)	9.06 ± 0.27ab (100)	9.18 ± 0.27ab (100)	9.37
Water stress (WS)	8.67 ± 0.46b (87.93)	8.99 ± 0.51b (99.23)	9.13 ± 0.41ab (99.46)	8.93
50 mM NaCl	6.11 ± 0.60cd (61.97)	5.79 ± 0.06cdef (63.91)	6.07 ± 0.30cd (66.12)	5.99
50 mM NaCl + WS	6.53 ± 0.49c (66.23)	6.01 ± 0.29cd (66.34)	5.84 ± 0.66cde (63.62)	6.13
75 mM NaCl	5.40 ± 1.39defg (54.77)	4.98 ± 0.37fgh (54.97)	4.44 ± 0.39h (48.37)	4.94
75 mM NaCl + WS	5.02 ± 0.52efgh (50.91)	4.80 ± 0.26gh (52.98)	4.99 ± 0.30efgh (54.36)	4.94
Mean (except control)	6.35	6.11	6.09	

Figure(s) in columns having common letter (s) do not differ significantly (*p*=0.05*)*. CV (%) = 7.67

to environmental conditions that prevail during reproductive development. Salinity and water stress delayed flowering and enhanced pod maturity that shortened the pod development period. Short period of maturity ultimately affected grain growth making the grain shriveled under saline conditions (Ghassemi-Golezani et al., 2009; Mannan et al., 2013).

Seed Yield

Seed yield of soybean genotypes was significantly influenced by salinity and water stress. The seed yield varied from 0.55 to 8.36 g plant^{-1} (Table 6). The lowest seed yield of 0.55 g plant^{-1} (7.11% to control) was found in BARI Soybean 6 at 75 mM NaCl salt combined with water stress which was followed by BD 2331 (0.59 g plant^{-1}) in the same salt concentration. Among the salt and water stress treatments, Galarsum showed the highest seed yield (1.92 g plant^{-1}) in only water stress, followed by BARI soybean 6 in 50 mM NaCl. All genotypes of soybean produced statistically similar seed yield in only water stress and in 50 mM NaCl. It was observed that the combined effect of salt and water stress was more severe in yield reduction than the single effect of salt or water stress. Moreover, the seed yield of soybean was decreased with the increase in salinity level. Among the genotypes, Galarsum showed the highest seed yield (average 1.34 g plant^{-1}), followed by BD2331 (average 1.20 g plant^{-1}) and the lowest was in BARI Soybean 6 (Table 6). The water uptake by the plant is difficult under saline and water stress conditions

due to decreasing osmotic potential of soil. Soybean yield is highly affected by soil water scarcity (Doss et al., 1974). Water shortage during the early reproductive stage increases the rate of pod dropping (Westgate & Peterson, 1993), leads to a less number of pods per plant (Desclaux et al., 2000) and ultimately decreases seed yield (Kokubun et al., 2001). Meanwhile, salinity leads to many metabolic changes that are identical to those caused by water stress, and there are still salt specific effects. Accumulation of the toxic level of Na$^+$ in leaves results in necrosis and premature leaf senescence (Munns, 2002). During seed development, sucrose is delivered via the phloem from source leaves to the developing seeds to support the growth (Patrick, 1988). Leaf senescence or defoliation leads to short supply of current photosynthates (Grodzinski et al., 1998; Komor, 2000) that ultimately reduces seed yield under salinity and water stress. Duzdemir et al. (2009) reported that seed yield of pea is very sensitive to water stress or salinity. The reduction in seed yield of mungbean and soybean due to salinity has also been reported by several authors (Raptan et al., 2001; Aziz et al., 2006; Sultana et al., 2009; Ghassemi-Golezani et al., 2009; Mannan et al., 2013).

CONCLUSION

Plant height, shoot dry weight and dry matter distribution in different parts were sharply decreased when the plants were exposed to the combined salt and water stress conditions. The combined effect

of salt and water stress was more severe in yield reduction than the single effect alone. The seed yield of soybean was decreased with the increase in salinity. Among the genotypes, dry matter reduction was the lowest in Galarsum. Galarsum also showed the highest filled pods, seed yield and 100 seed weight. This means that Galarsum was found to be suitable to grow in saline (50 mM NaCl) and water stress (70% depletion of available soil moisture) conditions. Therefore, this genotype can be recommended for saline and water shortage zones.

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Discrimination between Cave and House-Farmed Edible Bird's Nest Based on Major Mineral Profiles

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ABSTRACT

The high priced cave edible bird's nest (EBN) has attracted unscrupulous EBN producers to adulterate EBN with lower priced house-farmed EBN due to the fact that both are almost indistinguishable by visual inspection. In the present study, major mineral contents such as calcium, sodium, magnesium and potassium of both EBN types were analysed using inductively coupled plasma-optical emission spectrometry (ICP-OES). Three pattern recognition techniques namely hierarchical cluster analysis (HCA), principal component analysis (PCA) and linear discriminant analysis (LDA) were employed to determine the influence of harvesting origins on mineral profiles. With the use of HCA and PCA, EBN samples have successfully been grouped into two distinct clusters. From the PCA score plot, principal component 1 (49.53 %) and principal component 2 (41.11%) accounted for 90.64% of the total variability. In addition, LDA presented excellent performance in discriminating and predicting membership of the two EBN sample types with classification rate of 100%.

Keywords: Edible bird's nest, hierarchical cluster analysis, inductively coupled plasma-optical emission spectrometry, linear discriminant analysis, mineral content, principal component analysis

INTRODUCTION

Edible bird's nest (EBN) is highly consumed by the Chinese community because they uphold the belief handed down based on anecdotal evidences that EBN is beneficial to relief respiratory ailments and enhance body energy. The work by Kong et al. (1987), which suggests the presence of epidermal growth factor (EGF)-like substance in EBN, has drawn the attention of consumers as well as researchers. Since then, extensive research activities have been conducted to confirm the presence of EGF-like substance in EBN and its potential use in medical field and cosmetic industry for cell proliferative effect. This idea was substantiated by positive results reported in studies using human adipose-derived stem cells (Roh et al., 2012), corneal keratocytes (Zainal Abidin et al., 2011) and Caco-2 cells (Aswir & Wan Nazaimoon, 2010). Apart from that, EBN extract has been found effective in curing erectile dysfunction (Ma et al., 2012), improving bone strength and dermal thickness (Matsukawa et al., 2011) and inhibiting influenza virus infection (Guo et al., 2006).

Generally, EBN is built by gelatinous strand of nest cement secreted by swiftlets, namely, White nest swiftlet (*Aerodramus fuchipagus*) and Black nest swiftlet (*Aerodramus maximus*) during breeding seasons (Koon & Cranbrook, 2002). These swiftlets are found in the South-East Asia region and inherently inhabit the caves (Chantler & Driessen, 1999). Comparatively, EBN produced by the White nest swiftlet is of higher economic value as it is entirely made of pure salivary nest cement with only traces of impurities. On the other hand, though the nest of Black nest swiftlet is full with feathers and requires tedious cleaning process, it is still heavily harvested as the exploitation is worthwhile due to the fact that the price of the nest is extremely high.

With the increasing demand for EBN, the price of this product is expected to increase as the stock available in the market could not fulfil the growing needs. A recent survey reported by Manan and Othman (2012) revealed that the raw pre-processed EBN was sold at RM 3000/kg to RM 4500/kg in the market in year 2010 to 2011. The market price of EBN is always doubled after the laborious and time consuming cleaning process (Lim, 2006). Therefore, many investors are lured by the lucrative revenue and venture into EBN house-farming. Efforts have been done by the house farmers to ensure that only the pure breed of White nest swiftlet, which could produce EBN of high commercial value, would inhabit and breed in the farm (Lim, 2006). Unfortunately, EBN harvested from the house farm is much lower priced in the market than those harvested from the cave.

Driven by the unscrupulous desire, unethical EBN manufacturers tend to adulterate cave EBN with lower priced house EBN; some even make intentional false claims by selling house nest as cave nest. Besides, adulteration of EBN with addition or substitution with less expensive materials such as egg white, *Tremella* fungus, gelatin, karaya gum, fried porcine skin, starch, soybean and red seaweed

(Marcone, 2005; Ma & Liu, 2012), is commonplace.

Authentication methods at molecular level using Taqman-based real-time PCR (Guo et al., 2014), combination of DNA based PCR and protein based two dimensional gel electrophoresis methods (Wu et al., 2010), DNA sequencing-based method (Lin et al., 2009) and SDS-PAGE electrophoresis (Marcone, 2005) have been proposed. However, these techniques are rather tedious, time-consuming and costly.

EBN was built by swiftlets inhabiting in the caves and house farms and it was hypothesised that the minerals profile of EBN would be affected by the environments, as well as the supporting materials it attached to. The objective of this study is to distinguish EBN samples harvested from the cave and the house farm based on simple minerals profile analysed using inductively coupled plasma-optical emission spectrometry (ICP-OES). Correlation of mineral pairs within each group of sample was analysed using Pearson correlation analysis and pattern recognition techniques, namely, hierarchical cluster analysis (HCA), principal component analysis (PCA) and linear discriminant analysis (LDA) were employed to investigate the relationship between elemental concentration and the type of EBN samples studied.

MATERIALS AND METHODS

Materials

In this study, forty eight EBN samples were analysed. Twenty four of these were house nests harvested from different locations in West Malaysia, namely, Alor Setar, Bukit Mertajam, Kota Bharu, Segamat, Taiping and Teluk Intan. The twenty four cave nests were harvested from the caves located in East Malaysia (Bau and Sandakan) and Indonesia (Aceh and Medan). All EBN samples used in this study were raw genuine samples collected from different locations (see Figure 1) with the assistance of reliable suppliers and sponsors. All pre-processed samples were cleaned and air-dried under the same process. EBN samples were soaked in water and the feathers and impurities were removed using tweezers until the nests were devoid of visible feathers and impurities and followed by air-drying. Then, cleaned nests were dipped into liquid nitrogen for 10 seconds prior to grinding them into powder form. The samples were kept in air-tight bottles and stored at room temperature until further analysis.

Moisture Content

Moisture content of the samples was determined by volumetric Karl Fischer titration (784 KFP Titrino, Metrohm, Switzerland) following AOAC Official Method 2001.12.

Elemental Analysis

About 0.25 g of EBN powder was digested in a mixture of 3 mL H_2O + 2 mL HNO_3 + 1 mL H_2O_2 with a microwave digester (MARSXpress, CEM Corporation, Matthews, NC), following the method described in Saengkrajang et al. (2013). The digestion was carried out at 220ºC for 45 minutes until a clear transparent solution

was obtained. The digest was then made up to 50 mL with 2% HNO_3 solution and kept chilled in plastic bottles prior to mineral determination.

The concentrations of sodium (Na), potassium (K), calcium (Ca) and magnesium (Mg) were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES), Perkin Elmer optima 7000DV equipped with S10 autosampler and WinLab32™ for ICP V5.1 (Perkin Elmer, Waltham, MA). The calibration was performed with standard mixture from Perkin Elmer (Waltham MA) and all elements were determined at axial plasma view. The instrumental settings of the ICP-OES were as follows: the source equilibration delay was 15 seconds, plasma parameters were set at plasma 15 L/min, auxiliary 0.2 L/min, nebulizer 0.8 L/min and power 1300 W. Flow rate of sample was 1.5 mL/min with Argon as carrier gas. There was a washing step between the samples at the rate of 1.5 mL/min for 30 seconds. The wavelengths for each element were: Ca, 317.933 nm; Na, 589.592; Mg, 285.213 and K, 766.490.

Method Verification

The raw data were pre-processed and the concentration of each element was expressed in unit of mg/100 g dry matter basis to minimise data fluctuation. Calibration curves for Ca, Na, Mg and K were constructed using external standards method. Coefficient of determination, r^2 of calibration curves for the elements were all above 0.9900. Repeatability was determined by intra- and inter-day variation studies, while reproducibility was determined by two different analysts that conduct the same method. This method showed a very good precision in repeatability and reproducibility, with relative standard deviation (RSD) of elements determined ranged from 0.80 to 5.69%.

Statistical Analysis

Experimental data obtained were analysed using the statistical package SPSS version 22 for Windows (SPSS Inc., Chicago, IL). Independent samples t-test was conducted to determine significant difference between mean values. Pearson correlation analysis was used to study the direction (positive/negative) and strength (weak/moderate/strong) of the correlation between elements within each type of nest samples. Three pattern recognition techniques: hierarchical cluster analysis (HCA), principal component analysis (PCA) and linear discriminant analysis (LDA) were used to observe the possible pattern and trend in classification.

RESULTS AND DISCUSSION

Elemental Composition of the EBN Samples

Calcium (Ca), sodium (Na), magnesium (Mg) and potassium (K) composition of both house EBN and cave EBN from different locations and descriptive statistics of both types of EBN are tabulated in Tables 1 and 2, respectively. Based on the independent samples t-test result, it is evident that Ca content in cave EBN is significantly higher than house EBN but the Mg and

Na contents are significantly lower in cave EBN. Nonetheless, there is no significant difference observed in the K content in both types of EBN.

Since K is not significantly different for the two types of samples, mineral composition could better or more accurately be compared by its ratio after being normalised to K content. Generally, the average major minerals contents determined in this study were arranged in the decreasing order of Ca > Na > Mg > K, which is in accordance with the research findings of Norhayati et al. (2010). For cave samples,

the ratio of Ca:Na:Mg: K is 101:13:6:1, whereas for the house samples the ratio is 46:33:8:1. Obviously, calcium content in the cave EBN samples was slightly more than double of those found in the house EBN samples, and the reverse is true for Na content. The discrepancy in the element contents of both samples could largely be contributed by the inherent different environmental conditions prevailing in the cave and in the house farm (Sia & Tan, 2014).

Cave EBN is normally found as self-supporting nests that attached to vertical or

Table 1
Major minerals profile of house nests and cave nests.

Location	Type	Sample size	Ca	Mg	Na	K
Alor Setar	House Nest	5	706±32	122±11	632±78	18±2
Bukit Mertajam	House Nest	5	780±62	123±6	625±66	12±3
Kota Bharu	House Nest	5	665±51	127±8	633±100	14±1
Segamat	House Nest	3	777±98	138±11	548±150	20±1
Teluk Intan	House Nest	3	787±13	130±7	358±13	18±0.4
Taiping	House Nest	3	750±19	112±7	228±22	18±1
Medan	Cave Nest	4	1741±314	93±30	94±50	8±2
Aceh	Cave Nest	4	1389±334	102±19	112±69	13±4
Sandakan	Cave Nest	8	2263±207	130±23	294±166	33±10
Bau	Cave Nest	8	1203±42	90±3	274±24	6±1

Values are mean±standard deviation reported in mg/100g dry matter.

Table 2
Descriptive statistics for house and cave edible bird's nests.

	Minerals content (mg/100g dry matter)							
Element	House nests (n=24)				Cave nests (n=24)			
	Min.	Max.	Mean	STDEV	Min.	Max.	Mean	STDEV
Ca*	586	891	737	67	1141	2542	1677	504
Na*	203	795	536	167	38	630	224	131
Mg*	106	149	125	10	60	159	106	25
K	8	21	16	3	5	51	17	14

*Mean values are significantly different ($P<0.05$).

concave surface of a cave wall. Therefore, it is easy to rationalise high Ca content found in cave EBN. According to Northup and Lavoie (2010), mineral dissolution and precipitation processes in caves are microbially mediated reactions. Cave dissolution process involves iron-, sulfur- and manganese- oxidising bacteria, through which activities considerable acidity is being generated and subsequently used to dissolve cave wall that is rich in calcium carbonate. Meanwhile, the mineral precipitation process was reported to be either passive where microbial cells acts as nucleation sites or active, where bacterially produced enzymes control mineralisation. In passive mineralisation, dissolved metal (Ca^{2+}) was found to sorb onto amphoteric functional groups (such as carboxyl, phosphoryl and amino constituents) found on negatively charged cell walls, sheaths or capsules, following which carbonate (HCO_3^-) precipitates and in turn serves as nucleation site for calcium carbonate precipitation (Lowenstem & Weiner, 1989; Konhouser, 1997, 1998). It is believed that similar mechanism could have occurred by mineralisation on salivary strands (which is high in proteins) of a cave EBN.

On the other hand, Na content in the house EBN was found to be significantly higher than those cave EBN samples (Table 2). Interestingly, Na was also reported to be the predominant element in processed house-farmed EBN harvested from different locations in Thailand (Saengkrajang et al., 2013), pre-processed house-farmed EBN in Penang, Malaysia and pre-processed cave EBN in Sumatra Indonesia (Nurul Huda et al., 2008). Our raw data showed that Na content was extremely high in EBN harvested from Alor Setar, Bukit Mertajam and Kota Bharu house farms (Table 1 & Figure 1), which are located at the coastal locations facing the Malacca Straits. The Na content recorded was 2-3 folds higher when compared to the other samples harvested from other locations. Based on the report of Norhayati et al. (2013), this high Na content could be attributed to the accumulation of Na from marine aerosols through atmospheric deposition into the EBN. It is believed that sea salt concentration in the air could be high at these locations as a result of the persistent on-shore winds which generate sea water droplets and marine aerosols (sea sprays). The speculation was made based on the unique drinking behaviour of swiftlets, which capture the water droplets in the air. Therefore, the Na content in marine aerosol (swiftlet's saliva) is assumed to contribute to the nest Na content.

Besides the environmental factor, swiftlet diets could contribute partly to the difference in elemental profile of both types of samples. According to Lourie and Tompkins (2000), swiftlet's diets vary and are very much dependent on their foraging regions and food availability. Apart from this, White nest swiftlet's diet was discovered to be diverse and this species was predicted to survive and adapt well in urban areas (shop lot house farms). This could be a factor that yields the different minerals composition patterns in EBN harvested from different origins.
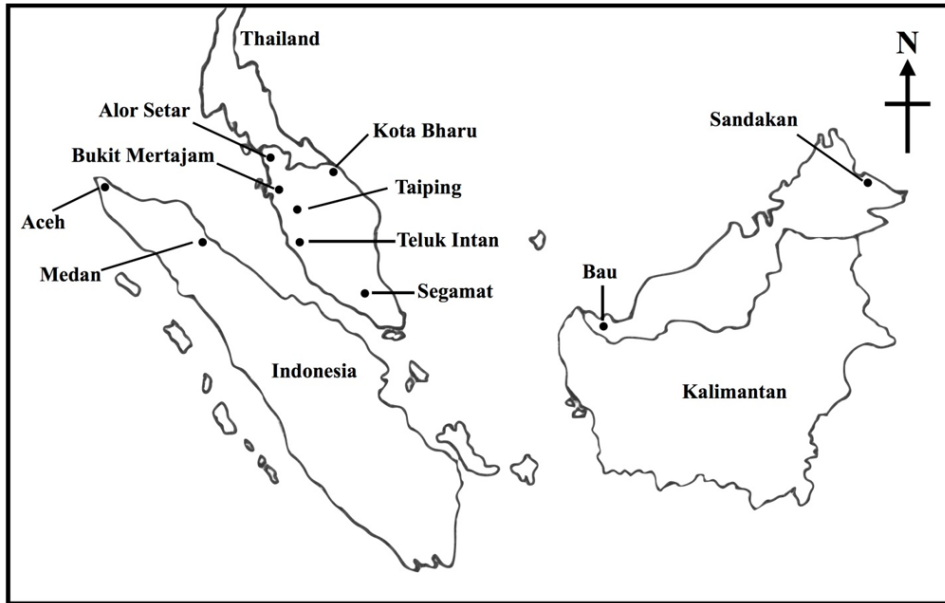
Figure 1. Edible bird's nest sampling points.

Pearson Correlation Analysis

The correlation matrix between mineral pairs of both types of EBN is presented in Table 3. Ca was found to demonstrate moderate positive correlation with Mg and it was significantly different at $r = 0.450$ ($P < 0.05$) for cave EBN samples. The Na content correlated significantly with K content at moderate values with $r = -0.477$ and 0.505 ($P < 0.05$) for house EBN and cave EBN, respectively. Interestingly, there were strong positive correlations between the mineral pairs in the cave EBN samples such as Ca and K ($r = 0.776$, $P < 0.01$), Na and Mg ($r = 0.609$, $P < 0.01$) and Mg and K ($r = 0.832$, $P < 0.01$). The significant relationship between the minerals leads us to further analyse the influence of macro- and micro-environmental factors on minerals composition of EBN.

Hierarchical Cluster Analysis (HCA)

Hierarchical cluster analysis (HCA) is an unsupervised classification method that discerns objects into groups based on the level of similarity between them based on the relative contribution of the variables. The clustering method used was the nearest neighbour (single linkage) method, measured based on squared Euclidean distance. A dendrogram was an easy visualisation aid produced with the samples of the same similarity level being grouped together. The use of HCA has successfully assigned the EBN samples into two main clusters, *i.e.* house EBN (n = 24) and cave EBN (n = 24), based on the dendrogram cut at a distance of 17.5 as presented in Figure 2. All the EBN samples were accurately classified into their own clusters which indicated that the elemental
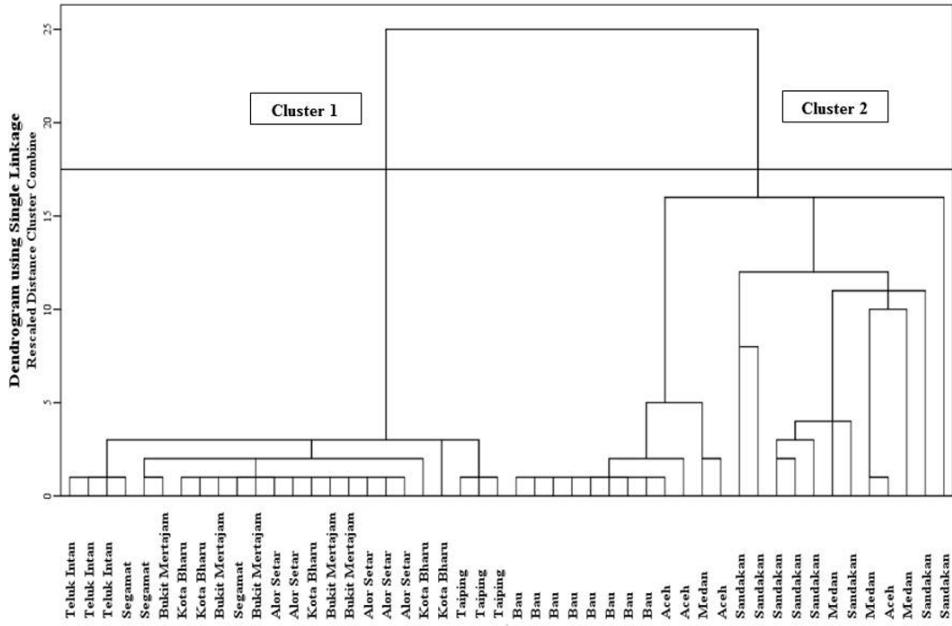
Figure 2. Dendogram of hierarchical cluster analysis. Cluster 1: house nests; cluster 2: cave nests

Table 3
Pearson correlation of minerals content in house and cave edible bird's nest.

House nests					Cave nests				
Element	Ca	Na	Mg	K	Element	Ca	Na	Mg	K
Ca	1				Ca	1			
Na	-0.069	1			Na	0.151	1		
Mg	0.338	0.275	1		Mg	**0.450***	**0.609****	1	
K	0.077	**-0.477***	0.208	1	K	**0.776****	**0.505***	**0.832****	1

 ** and * correspond to significance of correlation at the 0.01 level and 0.05 level (2-tailed), respectively.

composition could be appropriately used in classification of the type of EBN sample.

Principal Component Analysis (PCA)

Principal component analysis (PCA) is a chemometric tool used for dimension reduction of data set through which the most significant and important data would be extracted for further analysis (Abdi & William, 2010). Basically, PCA demonstrates primary evaluation and visualisation of between-class similarity based on the contributing variables variation direction in a multivariate space. PCA was carried out on a data matrix consists of EBN elemental profiles. The principal component (PC) scores and possible clustering results are illustrated in Figure 3A. Only two PC were extracted from the dataset to explain the total variability up to 90.64%. Two clusters
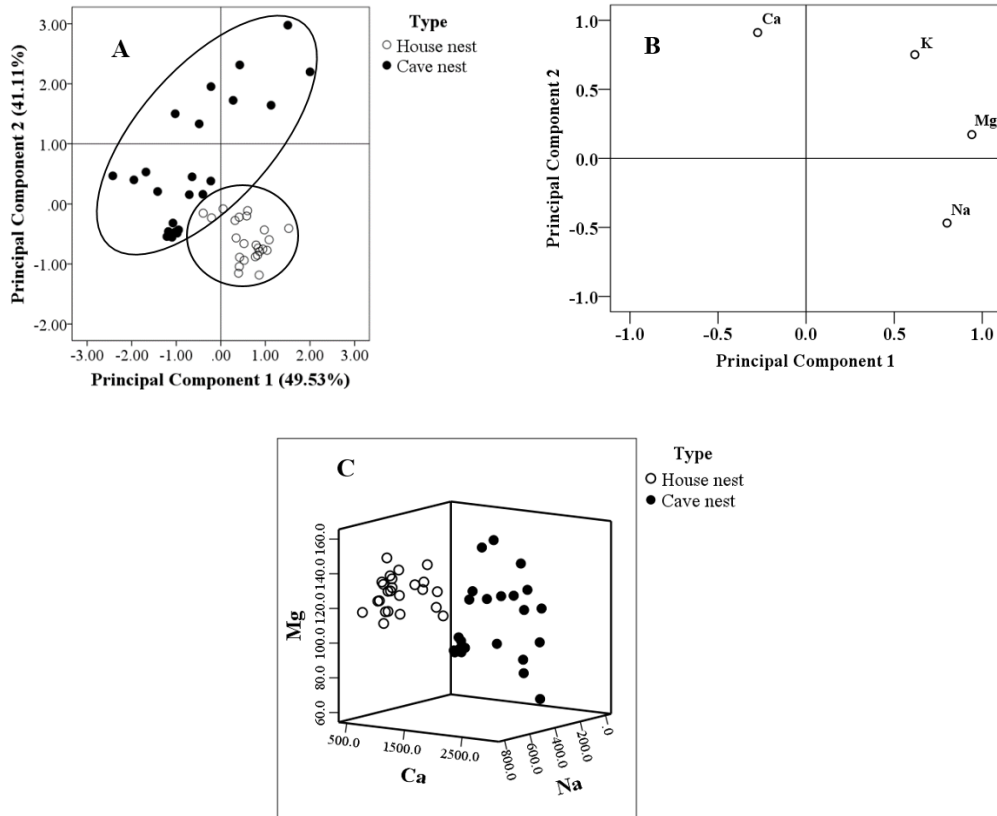
Figure 3. Principal component analysis was applied to study possible clustering between (A) house nest and cave nest, and their respective influential variables loaded as shown in (B). A simple 3-D plot of Ca vs. Na vs. Mg as illustrated in (C) gives a simple view of clustering potential

were identified and separated successfully at the diagonal by PC1 (explaining 49.53% of the variability) and PC2 (explaining 41.11%). Mg and Na, and Ca and K were the highly loading variables in PC1 and PC2, respectively, as shown in Figure 3B. In particular, the loading scores for Mg, Na, Ca and K were 0.941, 0.800, 0.911 and 0.751, respectively. A simple 3D-plot of Ca vs. Na vs. Mg concentrations was constructed to give a simple view of sample distribution or clustering potential as shown in Figure 3C, as these three variables contribute most of the variance. This 3D-plot is in good agreement with the PCA result (Figure 3A) that it provides a good discrimination pattern whereby house nest and cave nest are separated. As illustrated in Figure 3A, house EBN was observed to distribute more closely as compared to cave EBN. Geographical origin with different environmental conditions could be the key factor that contributes to the differences in EBN collected from different locations. Recently, authenticity assessment of commodities such as cabbages (Bong et

al., 2013), Croatian wines (Kruzlicova et al., 2013), Spanish cherries (Matos-Reyes et al., 2013) and Brazilian honey (Batista et al., 2012) through determination of mineral profiles analysed by chemometric analysis was found to be a valuable tool in classification according to geographical origins. Hence, PCA was further applied to investigate the possible groupings within class for both house and cave EBN.

As shown in Figure 4A, house EBN samples collected from the northern region (Alor Setar, Bukit Mertajam and Kota Bharu), whereas the remaining samples obtained from the central (Taiping and Teluk Intan) and the southern region (Segamat) in Peninsular Malaysia were separated into two clusters by PC1 which accounts for only 37.54% of the total variability. Na and K were the variables highly loaded in PC1, with the loading scores of -0.776 and 0.863, respectively, as illustrated in Figure 4B. Likewise, PC1 with the highly loading K, Mg and Ca variables, which explained 67.82% of variability (Figure 4C) categorised Sandakan cave EBN samples as one cluster and the other samples from Aceh, Bau and Medan as another cluster. As shown in Figure 4D, K, Mg and Ca were positively loaded in PC1 with the scores at 0.963, 0.897 and 0.731, respectively. The minerals profile of the cave EBN is associated to the cave wall the nest adheres to. The mineral profile of Sandakan cave EBN, which differs from the other locations may be attributed by the unique materials of the cave wall in Sandakan. This is evidenced by the geological survey that Sandakan

rocks, which consist predominantly of mudstone, sandstone and siltstone with minor coal seams and conglomerate (Lee, 1970), are different in composition from the caves in Bau which are composed of fossiliferous limestone (Wolfenden, 1965). The lithological variations between different locations are due to facies changes (Lee, 1970). The results are in good agreement with the findings discovered by Saengkrajang et al. (2013), Norhayati et al. (2010) and Nurul Huda et al. (2008) that nutritional composition of EBN could be distinguishable by breeding sites. However, it could be observed that even for the samples collected from the same location and within the same breeding season, the distributions were scattered. Hence, other contributing factors should be taken into considerations for future research studies. A better distinct separation between the samples harvested from different locations could be achieved by increasing the sample size.

Linear Discriminant Analysis (LDA)

Linear discriminant analysis (LDA) is a supervised pattern recognition approach which separates classes based on their dissimilarities by maximising the variance between classes and minimising the variance within classes (Roggo et al., 2003; Liu et al., 2012). A stepwise method was used to investigate if cave and house EBN could be differentiated by their elemental composition. Cross-validation procedure was carried out by employing the leave-one-out technique to evaluate the robustness
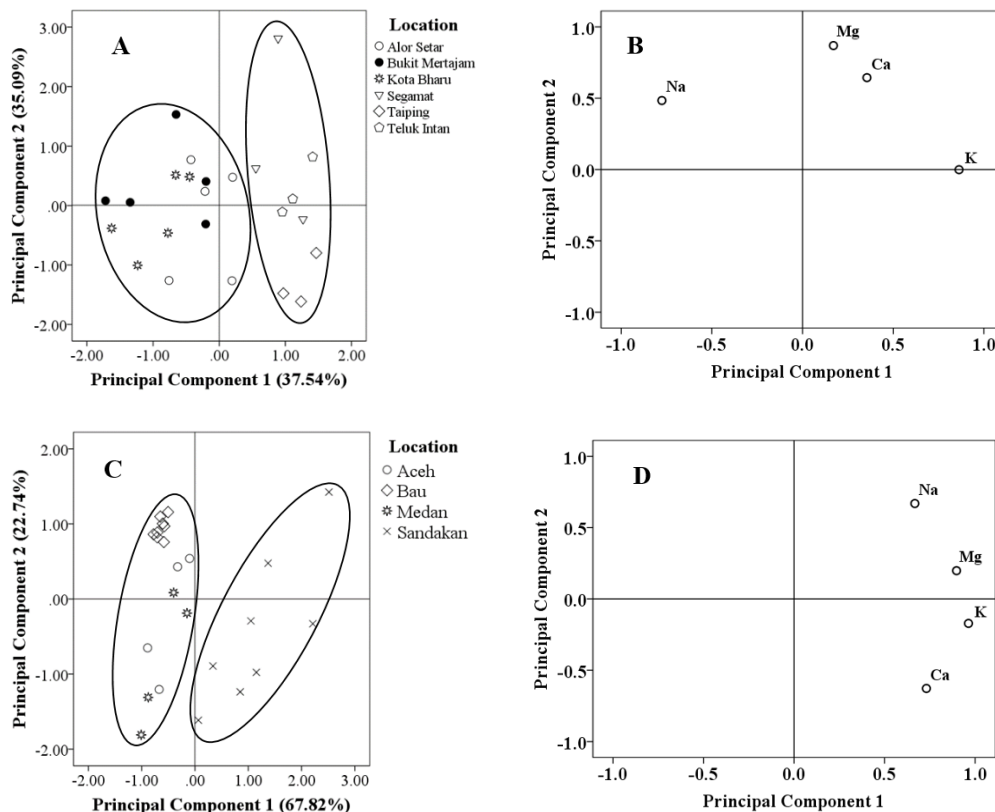
Figure 4. Principal component analysis was applied to study sample distribution within (A) house nest and (C) cave nest for geographical origins, and their respective influential variables loaded as shown in (B) and (D)

of the classification model. Each sample was classified based on the discriminant functions generated from the remaining samples and the accuracy of the classification was calculated as rate of cross-validation (Lachenbruch, 2006). LDA is used to assess the EBN samples with respect to the type based on the elemental composition. Four major elements (Ca, Na, Mg and K) were evaluated through LDA and only one linear discriminant function (DF) responsible in elucidating the differences between cave and house-farmed EBNs was derived. This DF explained 100% of the total variability

between two types of EBN and the relative contribution of each parameter identified is as depicted in Eq. (1).

$$Z = 0.616 \, Ca - 0.489 \, Na - 0.290 \, Mg - 0.012K \qquad [1]$$

Ca and Na exhibited strong contribution in discriminating cave EBN from house EBN, whereas Mg showed relatively lower contribution in explaining the variation between the cave EBN and house EBN. Scores of DF for EBN samples of different types correspond to the behaviour of the parameters in the DF as depicted in Figure 5.
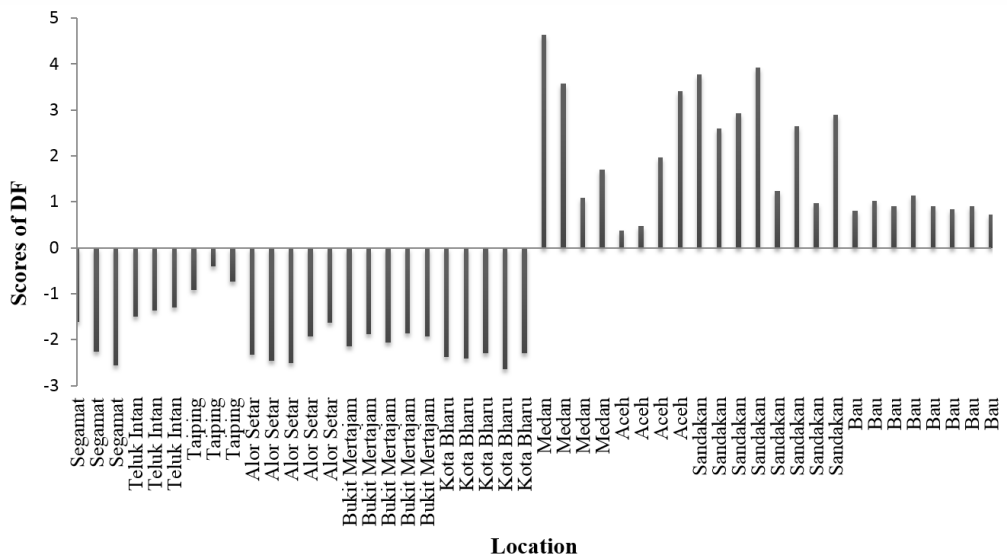
Figure 5. Scores for the discriminant function

Overall, all the house EBN samples showed negative contribution to the DF, whereas all the cave EBN samples demonstrated positive contribution. The sources of the samples were correctly identified in accordance to their own origins.

The membership of the EBN samples was predicted by employing a stepwise discriminant procedure, as shown in Table 4. A leave-one-out cross validation method was used to evaluate the robustness of this prediction model. Both overall classification and cross-validation classification were 100%, which implied that all samples, were correctly assigned to their own cluster. The results exhibited that this classification model was a very promising tool in discrimination of the EBN samples according to the types.

CONCLUSION

The use of elemental composition determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) combined with chemometric approach is verified to be a powerful tool in discriminating edible bird's nest based on types. Ca and Na were the elements which demonstrated strong contributions in the differentiation of two types of EBN samples. The robustness of the classification model has been validated and found to possess great predicting power at a classification rate and cross-validation rate of 100%.

ACKNOWLEDGEMENTS

Table 4

Classification of edible bird's nest samples and percentage of classification according to types through cross-validation method.

Type			Predicted Group Membership		Total
			House nest	Cave nest	
Original	Count	House nest	24	0	24
		Cave nest	0	24	24
	%		100	100	100[b]
Cross-validated[a]	Count	House nest	24	0	24
		Cave nest	0	24	24
	%		100	100	100[c]

[a]Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
[b]100% of original grouped cases correctly classified.
[c]100% of cross-validated grouped cases correctly classified.

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Path Analysis of Agronomic Traits of Thai Cassava for High Root Yield and Low Cyanogenic Glycoside

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ABSTRACT

Most high-yielding cassava cultivars have high cyanogenic glycoside (CNglcs) content in their roots and the CNglcs content of < 50 ppm in fresh root is considered safe for consumption. The root yield and CNglcs content which are agronomic traits involving several genes and environmental interactions can be evaluated only during the harvest time. In this study, 83 breeding lines and parents were evaluated for the variation and correlation between root yield and CNglcs content with 17 agronomic traits: root weight, leaf weight, stem weight, starch content, harvest index, root number, plant type, plant height, the first branch height, cyanide-equivalent contents in root and in leaf, chlorophyll content, carotenoid contents in leaf and in root, and cassava bacterial blight, fibrous and tuberous root-knot symptom scorings that may affect root yield and CNglcs content. The multiple regression and path analysis indicated that: a) harvest index, leaf weight and stem weight, and b) stem weight, starch content, CNglcs content in leaf, the first branch height and leaf weight could produce root weight with predicted $R^2 = 86.03$ and 47.05%, respectively. Also, a) chlorophyll content, CNglcs content in leaf, and root-knot symptom scoring, and b) carotenoid content in leaf and CNglcs content in leaf could be used in screening for low CNglcs content in root with predicted $R^2 = 52.20$ and 55.06%, respectively.

However, CNglcs content in leaf and root did not show any correlation with cassava bacterial blight and fibrous root-knot symptom scorings. Further evaluation and trial in other locations are required for the verification.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz, Euphorbiaceae) is the fourth most important staple crops after rice, wheat and maize. It is one of the cyanogenic plants such as bamboo (*Bambusa* spp., Gramineae) and sorghum (*Sorghum bicolor* (L.) Moench, Gramineae) and some other cultivated plants, with cyanogenic glycoside (CNglcs) present in its leaves and roots. It is cultivated for tuber production in Central America, the Caribbean, Central, East and West Africa, India, Sri Lanka, Southeast Asia and most tropical regions in the equatorial belt between 30°N and 30°S latitudes. It is also used to a less extent for leaf consumption in West Africa, Brazil, Indonesia, Malaysia, the Philippines and Thailand (Tindall, 1983). Thailand ranks next to Nigeria and Brazil on cassava annual production, but it was the world largest exporter of dried cassava at 1.4 million tons in 1973-1974 to 6.7 million tons in 1983-1985 before it dropped to 3.9 million tons in 1988 (Ratanawaraha et al., 2001). Cassava export from Thailand was accounted at 77% of the total world export in 2005 and in 2012 the area harvested was 1,362,080 ha with 29,848,000 metric tons production (FAO, 2015).

Cassava originated in the northeastern region of Brazil, western and southern Mexico, and part of Guatemala. It was known to be taken by the Portuguese in the latter half of the sixteenth century to West Africa and from there, it spread to East Africa; taken from Brazil to Réunion and Madagascar in 1736; recorded in Zanzibar in 1799; introduced from Mauritius to Sri Lanka in 1786 and reached Calcutta in 1794; and probably taken at an earlier date to the Philippines from Mexico (Purseglove, 1981; Tindall, 1983). It was also reported that cassava was transported directly from Brazil to Java, Singapore and Malaysia in 1850.

Cassava is not native to Thailand and it is not known exactly when it was introduced for cultivation. Sarakarn et al. (2001) reported that cassava was imported mainly as germplasms nearly 40 years ago, and since cassava production in Thailand is mostly for starch-based industrial purpose, the key objective of the cassava breeding programme has been to increase root yield and starch content. According to Ratanawaraha et al. (2001), some 20 cassava cultivars were introduced from Malaysia, Indonesia and Mauritius before 1960, and more clones were introduced from Indonesia in 1963, the Virgin Islands in 1965, and the first introduction from the International Centre for Tropical Agriculture (Centro Internacional de Agricultura Tropical - CIAT), Cali, Colombia was in 1975. They were observed and evaluated for root yield, starch, dry matter and HCN contents, harvest index and other agronomic traits.

It was known that CNglcs content of the cassava cultivars commercially cultivated in Thailand is relatively high due to the traits in the parent plants used in the breeding programme. However, most cassava lines core collection and germplasms

at the International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria, the International Centre for Tropical Agriculture (CIAT) in Cali, Colombia, and in Cameroon are known to have low CNglcs content (Bokanga, 1994). The known toxic principle found in all parts of the cassava plant is a CNglcs called linamarin. Bolhuis (1954) set the level of its toxicity at an intake of 50-60 mg daily for a European adult. Bhattacharya et al. (2009) reported that consumption of cassava with high CNglcs content, without proper processing, could cause acute or chronic intoxication due to the toxins and paralytic disease.

Mahunga (1994) reported that the CNglcs content in the root of cassava had a correlation with the root yield with a coefficient of 0.19 to 0.20. This correlation indicated low genetic linkage between the two traits leading to the effort in cassava breeding for high root yield with low CNglcs. However, Kizito et al. (2007) and Whankaew et al. (2011) reported two and five quantitative trait loci (QTLs), respectively associated with CNglcs content in cassava roots, indicated difficulty in selecting for roots with low CNglcs. Moreover, the environmental conditions can also affect the CNglcs content in roots. There were reports that nitrogen and fertiliser applications increased or reduced CNglcs content in cassava roots (Bokanga et al., 1994; Gleadow & Møller, 2014). Water deficit also has an effect on CNglcs content (Bokanga et al., 1994; Hular-Bograd et al., 2011; Vandegeer et al., 2013).

Therefore, an investigation into the agronomic traits associated with root yield and CNglcs would be of a benefit in selecting cassava with high root yield and low CNglcs content. However, there were studies showing difference on the correlation coefficients of these two traits. For instance, Moh (1976) found a positive correlation between CNglcs content in cassava leaf and root with a coefficient of 0.59, while Mahungu (1994) reviewed the relationships between CNglcs content of cassava and other agronomic traits. In addition, Cooke et al. (1978) and Mahungu et al. (1992) reported a positive correlation between CNglcs contents in cassava leaf and root with correlation coefficients ranging from 0.20 to 0.36.

The CNglcs content in the cassava plant may also be affected to some noticeable extent by the environmental variables such as an infestation by insect pests and infections by diseases such as bacterial leaf blight and root-knot nematodes. Bernays et al. (1977) demonstrated that yellow senescent leaves of cassava were readily eaten by the variegated grasshopper, *Zonocerus variegatus* (L.) (Orthoptera: Pyrgomorphidae) but green leaves were not, and this was apparently because the senescent leaves lacked CNglcs. Meanwhile, Bellotti and Arias (1993), Bellotti and Riis (1994) and Riis et al. (2003) also gave an account of cassava cyanogenic content and resistance to insect pests especially the burrowing bug, *Cyrtomenus bergi* Froschner (Hemiptera: Cydnidae), feeding on cassava roots.

Among the diseases, in Africa, Zinsou et al. (2005) reported that cassava bacterial blight caused by *Xanthomonas axonopodis* pv. *manihotis* and *X. campestris* pv. *manihotis* could reduce cassava yield up to 76%. In Thailand, Lertsuchatavanich et al. (2014) found a negative correlation between the HCN content in cassava leaves and the incidence of cassava bacterial blight caused by *X. axonopodis* pv. *Manihotis*, and the root-knot nematodes, *Meloidogyne* spp. (Tylenchida: Heteroderidae), which could cause up to 70% yield loss in cassava production (Lertsuchatavanich & Chinnasri, 2012). A preliminary screening of Kasetsart 50 with high CNglcs in the roots and resistant to the root-knot nematode is on-going (Udomsak Lertsuchatavanich, pers. comm.). Coyne et al. (2006) attempted to score the intensity of root-knot disease by using the root gall index at the harvesting period, which was found either positively or negatively correlated with the root yield depending on the cultivars. These reports suggested that high CNglcs content in the roots and leaves of cassava might have an implication in the pest and disease resistance mechanism.

The objective of this study was to find the variation of some agronomic traits for further stepwise multiple regression analysis for the correlations between the fresh root weight, CNglcs content in roots and other agronomic traits so as to choose the significant and highly correlated traits as the selection criteria for rapid screening of 83 cassava breeding lines. The 17 selected quantifiable and observable agronomic traits in this study were fresh root weight, fresh leaf weight, fresh stem weight, starch content in root, harvest index, root number, plant type, plant height, the first branch height, CNglcs contents in root and leaf, chlorophyll content in leaf, carotenoid contents in root and leaf, and cassava bacterial blight and fibrous and tuberous root-knot symptoms scorings. The data obtained were evaluated and analysed using the stepwise multiple regression analysis or the path analysis originally developed by Wright (1934). The ultimate objective was to verify the effects of the significantly correlated traits on the fresh root weight and CNglcs content in the root for verification and further investigation.

MATERIALS AND METHODS

Plant Materials and Field Experiment

Eighty-three cassava breeding lines were used for clonal selection trial carried out at Thai Tapioca Development Institute (TTDI) Experiment Station in Huai Bong, Dan Khun Thot, Nakhon Ratchasima (15º12.444′ N, 101º 42.911′ E). They were grown from stakes obtained from the cassava plants which were hybridised between the cultivars with high root yield and high CNglcs content in the root (Kasetsart 50, Huai Bong 60, Huai Bong 80 and Rayong 5), and the cultivars with low CNglcs content in the root but low root yield (Kolok, Rayong 2, and Ha Na Thi). They were planted along with the parent cultivars to serve as check every two rows. In each row, six plants were planted with the plant to plant distance of 1 m, while the row to row distance was 2 m.

The stakes were planted in May 2012 and the plants harvested in May 2013. Three plants in each row were selected and the agronomic traits measured were fresh root weight (RW), fresh leaf weight (LW), fresh stem weight (SW), starch content in root (SC), harvest index (HI), number of roots (RN), plant type (PT), plant height (PH), first branch height (FBH), and the cyanide-equivalent contents (HCN) in root and in leaf (R-HCN and L-HCN), chlorophyll content in leaf (CHLO), carotenoid contents in leaf (L-CA) and in root (R-CA) were measured in 28 selected lines having either white, cream, or yellow root flesh in appearance, whereas cassava bacterial blight (CBB) and cassava root-knot symptoms in fibrous and tuberous roots (FRK and TRK) with the scorings of 1 to 5, 1 to 4, and 1 to 5 respectively, were estimated in 60 breeding lines. The starch content in root (SC) was analyzed by Reimann Scale Balance (GENIX, Samutprakarn, Thailand) described in Prammanee et al. (2010). The harvest index (HI) was calculated from the fresh root weight (RW) divided by fresh stem weight (SW) of the whole plant. The plant types (PT) were scored from 1 to 5, ranging from highly branched plant type (1) to straight plant type (5), consecutively.

Cyanogenic Glycoside Analysis

The cyanide-equivalent contents in the root and in leaf were analysed following the modified methods described in Bradbury et al. (1999). Root parenchyma was collected using a 4 mm diameter cork borer to cut the largest section of root, using a portion with the final weight of 100 mg without cortex layer. The leaf tissue was cut from a fully expanded young leaf with a 1 cm^2 circular cork borer to obtain around 10 mg leaf. The fresh tissue was ground in a 15 ml tube containing 50 µl of phosphate buffer, pH = 8.0, and then sealed with the cap attached with picrate paper. The reaction was held at 30°C for 24 h before the picrate paper was soaked in 5 ml of water for 30 min for quantitating cyanide-equivalent content at 510 nm by spectrophotometer (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) using a standard curve of HCN generated from linamarin.

Chlorophyll and Carotenoid Analysis

The chlorophyll and carotenoid contents were analysed as follows. A leaf disc was cut from a fully expanded young leaf with a 1 cm^2 circular cork borer and put in a vial with 3 ml of *N, N*-dimethylformamide (DMF). The vial was held in the dark for 24 h before the solution absorbance was measured at 248, 647, and 664 nm, respectively, with a spectrophotometer (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). The chlorophyll *a* and *b* contents per leaf in mg per cm^2 were estimated using the formulae described in Equations [1] to [3] (Porra et al., 1989).

$$\text{Chlorophyll } a = 12.00 \, A_{664} - 3.11 \, A_{647} \quad [1]$$

$$\text{Chlorophyll } b = 20.78 \, A_{647} - 4.88 \, A_{664} \quad [2]$$

Total chlorophyll
    = Chlorophyll *a* + Chlorophyll *b*
                                    [3]

Meanwhile, the total carotenoid content per leaf in mg per cm^2 was estimated using the formula described in Equation [4] (Wellburn, 1994).

Carotenoid
    = (1000 A_{480} − 1.12 Chlorophyll *a*
    − 34.07 Chlorophyll *b*) /245   [4]

The total carotenoid content in roots was extracted by 80% (v/v) acetone and estimated in micromoles per g, as described in Equation [5] (Edwards et al., 1998).

Carotenoid
    = {(A_{480} + 0.114 A_{663} − 0.638 A_{645})
    × *V* × 1000} + 112.5 *W*   [5]

where, *V* is extraction volume, *W* is fresh root weight.

Cassava Bacterial Blight Scoring

The cassava bacterial blight disease symptoms caused by *X. axonopodis* pv. *manihotis* were estimated and scored from three plants per line and averaged from six leaves per plant, with two leaves selected from the top, middle and bottom portions of the stem. The severity was scaled into five levels (1 to 5), associated with 0, 5, 10, 15, or 20% leaf damage, consecutively as described in Teri (1978).

Cassava Root-knot Scorings

The root-knot symptoms caused by the nematodes *Meloidogynes* spp. were scored for fibrous root-knot (FRK) and tuberous root-knot (TRK) using three plants per line. The symptom scorings for the fibrous root-knot and tuberous rot-knot were somewhat different and the systems were adapted from Coyne et al. (2006), as follows.

Fibrous root-knot symptom scoring criteria:

Symptom Score	Severity
0	No root- knot symptom appeared
1	25% of fibrous roots have root knots
2	50% of fibrous roots have root knots
3	More than 50% of fibrous roots have small root knots
4	More than 50% of fibrous roots have large root knots

Tuberous root-knot symptom scoring criteria:

Symptom Score	Severity
0	No root knot appeared in fibrous roots and no effect on tuberous roots
1	Root knot appeared in fibrous roots but no effect on tuberous roots
2	Root knot appeared in fibrous roots and 25% of tuberous roots no longer developed
3	Root knot appeared in fibrous roots and 50% of tuberous roots no longer developed
4	Root knot appeared in fibrous roots and 75% of tuberous roots no longer developed
5	Root knot appeared in fibrous roots and 100% of tuberous roots no longer developed

Statistical and Path Analysis

Mean, standard deviation, coefficient of variation and range across all 83 breeding lines and parent lines were calculated

by using the values obtained from 17 agronomic traits of each line. They were then calculated for the phenotypic correlation coefficients. The effects of agronomic traits were formulated using the stepwise multiple regression analysis and the Pearson correlation coefficients (r) among the agronomic traits analysed. The multiple regression analyses were formulated using the stepwise method. The path analyses were calculated from correlation coefficients. The Statistic Tool for Agricultural Research STAR 2.0.1 software (IRRI, 2013) was used for all calculation and analysis.

RESULTS AND DISCUSSION

A. Variation Evaluation of Agronomic Traits

The mean ($\bar{x}$), standard deviation (SD), range and coefficient of variation (CV) of 17 agronomic traits, including the fresh root weight (RW), fresh leaf weight (LW), fresh stem weight (SW), starch content (SC), harvest index (HI), roots per plant (RN), plant type (PT), plant height (PH), the first branch height (FBH), cyanide-equivalent contents in root (R-HCN) and in leaf (L-HCN), chlorophyll content in leaf (CHLO), carotenoid contents in leaf (L-CA) and in root (R-HCN), as well as cassava bacterial blight (CBB) symptom scoring, and fibrous root-knot (FRK) and tuberous root-knot (TRK) symptoms scorings are summarised in Table 1.

Fresh Root Weight (RW), Fresh Leaf Weight (LW) and Fresh Stem Weight (SW)

The preliminary evaluation of agronomic traits revealed a high variation in fresh root weight (RW), fresh leaf weight (LW) and fresh stem weight (SW) with the CV values from 41.62 to 53.66%. The variation in dry matter content of cassava root was also reported in cassava in the sub-Saharan Africa by Kawaki et al. (2011). Among these traits, fresh leaf weight (LW) was found to have greater variation than fresh root weight (RW) and fresh stem weight (SW). It was also observed that many traits showed relatively high variation, which could be due to segregation of genes caused by the heterozygosity of cassava breeding parents used in this trial.

Harvest Index (HI) and Starch Content (SC)

However, the harvest index (HI) and starch content (SC) had lower percentages of CV, than the fresh root weight, being 22.07, 18.95 and 46.76%, respectively. The harvest index (HI) averaged 0.49±0.11, ranging from 0.22–0.75, indicating that the assimilate partitioning between the above-ground and underground parts of cassava lines used in this trial were mostly balanced. The starch content (SC) averaged 19.26±3.65%, ranging from 10.0–26.80% in this trial. The results obtained were somewhat lower than those obtained from 266 germplasm varieties evaluated by Yurasit (2007), with the range of 10.0–35.9%. The low coefficient variation of the starch content

(SC) of this trial might be limited by the genetic variation of selected parents compared to the germplasm evaluation.

Plant Type (PT), First Branch Height (FBH), Plant Height (PH) and Roots per Plant (RN)

The cassava lines in this clonal evaluation trial had different plant types (PT) from being straight to being branchy from 1 to 5. As a result, the variation in plant type (PT), averaging at 2.55±0.99, and the first branch height (FBH), averaging 81.99±29.56 cm and ranging from 0–240.00 cm, were relatively high compared with the plant height (PH) variation, averaging at 206.40±30.70 cm and ranging from 125.00–283.33 cm (Table 1). The number of roots per plant (RN) averaged 12.02±3.80 roots per plant, ranging from 4–38 roots per plant. The number of roots per plant (RN) which is a component of fresh root weight (RW) also had a high coefficient variation at 46.76%, averaging at 5.54±2.59 kg plant^{-1} and ranging from $1.00 – 15.60$ kg plant^{-1}. However, since the size of cassava root is not uniform, the number of roots per plant (RN) may not be a suitable parameter to be used for selecting for high root yield, not unless they could be clearly classified or categorised into a number of large and/or small roots.

Cyanide-Equivalent Contents

This study revealed that the cyanide-equivalent contents in root (R-HCN) and leaf (L-HCN) averaged at 110.30±62.33 ppm, ranging from $12.52 – 271.72$ ppm, and at 1,231.20±312.20 ppm, ranging from $512.90 – 2,248.10$, respectively (Table 1). The result indicated that the cyanide-equivalent content in the leaves was higher than that in the roots. The higher cyanide-equivalent content in fresh leaves compared to in fresh roots was obvious because CNglcs were shown to be synthesised in the leaves and translocate to the root tissues through the phloem (Selmar, 1994). It was also obvious that the root had a higher coefficient variation of cyanide-equivalent content than the leaf (i.e., 56.51 vs. 25.35%). The cyanide-equivalent contents in root obtained in this trial had a range from the innocuous group (<50 mg kg^{-1} root fresh weight) to the toxic group (>100 mg kg^{-1} root fresh weight) as reported in Bolhuis (1954, 1966) and reviewed in Lukuya et al. (2014). Nevertheless, the coefficient of variation of cyanide-equivalent content in the roots was much higher than that in the leaves (i.e., 56.51% to 25.35%). Therefore, the variation of the cyanide-equivalent content in the roots may not only be due to the cyanide-equivalent in the leaves, but also the CNglcs transported from the leaves to roots through the phloem, which may have complex enzymatic mechanisms involving diglucosidase (Selma, 1994) and hydroxynitrile lyase (Siritunga & Sayre, 2004). It is also well known that CNglcs accumulated in all cassava lines and there has never been any report of cassava with zero CNglcs content, except for the transgenic cassava lines, with *CYP79DI/ D2* knockout (Siritunga & Sayre, 2004).

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Table 1

Mean ± SD, range, and coefficient of variation of 17 agronomic traits in cassava breeding lines in clonal evaluation trial

Agronomic traits	Mean ± SD ($\bar{x}$)	Range	CV (%)
RW (Fresh root weight, kg plant[1])	5.54 ± 2.59	1.00 - 15.6	46.76
LW (Fresh leaf weight, kg plant[1])	2.70 ± 1.45	0.10 - 8.75	53.66
SW (Fresh stem weight, kg plant[1])	2.86 ± 1.19	0.40 - 7.85	41.62
SC (Starch content, %)	19.26 ± 3.65	10.00 - 26.80	18.95
HI (Harvest index, ratio)	0.49 ± 0.11	0.22 - 0.75	22.07
RN (Root number, number/plant)	12.02 ± 3.80	4 - 38	31.57
PT (Plant type, 1-5)	2.55 ± 0.99	1 - 5	38.79
PH (Plant height, cm)	206.40 ± 30.70	125.00 - 283.33	14.87
FBH (First branch height, cm)	81.99 ± 29.56	0 - 240	36.05
R-HCN (Cyanide-equivalent in root, ppm)	110.30 ± 62.33	12.52 - 271.72	56.51
L-HCN (Cyanide- equivalent in leaf, ppm)	1,231.20 ± 312.20	512.90 - 2,248.10	25.35
CHLO (Chlorophyll content in leaf, mg cm^{-2})	9.37 ± 1.93	6.19 - 13.54	20.58
L-CA (Carotenoid content in leaf, mg cm^{-2})	2.15 ± 0.33	1.54 - 2.97	15.56
R-CA (Carotenoid content in root, µg g^{-1})	3.94 ± 1.76	2.00 - 7.77	44.68
CBB (Cassava bacterial blight symptom, 1-5)	1.27 ± 0.52	1 - 5	40.62
FRK (Fibrous root-knot symptom, 1-4)	2.60 ± 0.89	1 - 4	30.80
TRK (Tuberous root-knot symptom, 1-5)	2.90 ± 0.95	1 - 5	36.64

The amounts of cyanide-equivalent content in the leaves and roots could vary with the environmental condition, especially under high nitrogen fertilisation (Bokanga et al., 1994; Gleadow & Møller, 2014), and with water deficits (Bokanga et al., 1994; Hular-Bograd et al., 2011; Vandegeer et al., 2013). In this experiment, the harvest time was during the early monsoon season of May 2013; therefore, the drought effect should not have any significant impact on the CNglcs transportation or storage in cassava breeding lines under this trial.

Chlorophyll and Carotenoid Contents

For the chlorophyll content in leaf (CHLO), the average content was 9.37±1.93 mg cm^{-2}, ranging from 6.19–13.54 mg cm^{-2} and the lower level of variation was 20.58%. However, the pattern of variation of the carotenoid content in leaf and in root (L-CA and R-CA) was similar to those of the cyanide-equivalent contents, i.e., higher variation in the roots than in the leaves. The average carotenoid contents in root (R-CA) and in leaf (L-CA) were 3.94±1.76 µg^{-1}, ranging from 2.00–7.77µg^{-1} and 2.15±0.33 mg cm^{-2}, ranging from 1.54 – 2.97 mg cm^{-2}, respectively. The carotenoid content, as appeared in the roots, varied from the

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white-coloured flesh, through the cream-colored flesh and to the yellow-coloured flesh.

Cassava Bacterial Blight Symptom Scoring

The incidence of cassava bacterial blight (CBB) disease caused by (*X. axonopodis* pv. *manihotis*) on cassava breeding lines in this trial, evaluated by using the symptom scoring of 1 to 5, was 1.27±0.52. This indicated a relatively low or rather negligible overall cassava bacterial blight incidence during the trial.

Root-Knot Symptom Scorings

Scorings of the root-knot nematodes (*Meloidogyne* spp.) infection causing fibrous root-knot (FRK) symptom and tuberous root-knot (TRK) symptom, from 1- 4 and 1- 5, averaged at 2.60±0.89 and 2.90±0.95, respectively. This indicated a moderate nematode incidence which caused both fibrous root-knot and tuberous root-knot symptoms.

B. Agronomic Traits and Path Analysis

Phenotypic Correlation Coefficients in Cassava Breeding Lines

The phenotypic correlation coefficients between 17 agronomic traits in cassava breeding lines in the clonal evaluation trial are summarised in Table 2. The stepwise multiple regression analysis on the effects of agronomic traits on fresh root weight (RW) and cyanide-equivalent content in the root

(R-HCN) from the clonal evaluation trial is shown in Table 3.

The path analysis of the agronomic traits evaluated, in terms of the standardised partial regression coefficients (*b*) and the correlation coefficients (*r*), which showed the effects of the harvest index (HI), leaf weight (LW) and stem weight (SW); as well as the stem weight (SW), starch content (SC), cyanide-equivalent content in leaf (L-HCN), the first branch height (FBH) and leaf weight (LW) on the root weight (RW), are illustrated and presented in Figure 1 and Figure 2, respectively.

The standardised partial regression coefficients (*b*) and the correlation coefficients (*r*), which showed the effects of the chlorophyll content (CHLO), cyanide-equivalent content in leaf (L-HCN) and tuberous root-knot symptom scoring (TRK), as well as the carotenoid content in leaf (L-CA) and the cyanide-equivalent content in leaf (L-HCN) on the cyanide content in root (R-HCN), are illustrated in Figure 3 and Figure 4, respectively.

Effects on Root Weight

It is evident in Table 2 that the fresh root weight (RW) showed a positive correlation with fresh stem weight (SW) (*r* = 0.51) and harvest index (HI) (*r* = 0.56) (Figure 1). This is probably because stems and roots are both the carbohydrate storage tissues. The stepwise analysis showed a standardised linear regression that the harvest index (HI), fresh leaf weight (LW) and stem weight (SW) had contributions to the fresh root

Table 2

Phenotypic correlation coefficients between 17 agronomic traits in cassava breeding lines in clonal evaluation trial

Traits[†]	RW	LW	SW	SC	HI	RN	PT	PH	FBH	R-HCN	L-HCN	CHLO	L-CA	R-CA	CBB	FRK	TRK
RW	1.00	0.26**	0.51**	0.38**	0.56**	0.38**	-0.26**	0.23*	0.25**	0.29**	0.36**	0.11	0.12	0	0.19	0.11	-0.09
LW		1.00	0.51**	-0.02	-0.49**	0.21*	-0.52**	0.40**	0.06	-0.15	-0.19	-0.04	-0.10	0.16	0.14	0.29*	0
SW			1.00	0.11	-0.24**	0.36**	-0.42**	0.53**	0.19*	0.07	0.22*	0.02	-0.01	0.01	0.22	0.25	-0.10
SC				1.00	0.35**	0.09	-0.38**	-0.14	0.03	0.20*	0.12	0.18	0.13	0.71	0.16	-0.05	-0.42**
HI					1.00	0.08	0.25**	-0.26**	0.22*	0.32**	0.33**	0.03	0.10	-0.02	-0.03	-0.15	-0.01
RN						1.00	-0.07	0.19*	0.23**	0.29**	0.24**	0.11	0.06	-0.40	-0.07	-0.06	-0.08
PT							1.00	0.01	0.18*	0.07	0.11	-0.07	-0.03	-0.63	-0.15	-0.08	0.21
PH								1.00	0.18*	0.04	0.12	0.18	0.15	-0.77*	0.11	0.30*	-0.09
FBH									1.00	0.17	0.06	0.15	0.14	0.28	0.09	0.15	0
R-HCN										1.00	0.48**	0.65**	0.67**	-0.43	0.17	0.04	-0.33*
L-HCN											1.00	0.19	0.25	-0.27	0.09	-0.02	-0.13
CHLO												1.00	0.95**	0.10	0.36	0.23	-0.45*
L-CA													1.00	0.45	0.26	0.21	-0.49*
R-CA														1.00	-0.39	0.45	0.07
CBB															1.00	0.08	-0.29*
FRK																1.00	0.04
TRK																	1.00

* significant at 95% confidence level, ** highly significant at 99% confidence level

†RW = Fresh root weight (kg plant⁻¹), LW = Fresh leaf weight (kg plant⁻¹), SW = Fresh stem weight (kg plant⁻¹), SC = Starch content in root (%), HI = Harvest Index (ratio), RN = Root number, PT = Plant type (1-5), PH = Plant height (cm), FBH = First branch height (cm), R-HCN = Cyanide-equivalent content in root (ppm), L-HCN = Cyanide-equivalent content in leaf (ppm), CHLO = Chlorophyll content in leaf (ppm), L-CA = Carotenoid content in leaf (mg cm⁻²), R-CA = Carotenoid content in root (µg g⁻¹), CBB = Cassava bacterial blight symptom (1-5), FRK = Fibrous root-knot symptom (1-4), TRK = Tuberous root-knot symptom (1-5)

Table 3

Stepwise multiple regression analysis of effects of agronomic traits on (A) Fresh root weight and (B) Cyanide-equivalent content in root from clonal evaluation trial

Traits†	R^2 (%)	P-value
(A) Fresh Root Weight (RW)		
	86.03	0
Regression model:		
RW = 0.89 HI + 0.44 LW + 0.49 SW		
HI (Harvest index)	49.46	0
LW (Fresh leaf weight)	11.68	0
SW (Fresh stem weight	24.89	0
Residual	13.97	
Regression model:	47.05	0
RW = 0.24 SW + 0.32 SC + 0.31 L-HCN + 0.23 FBH + 0.22 LW		
SW (Fresh stem weight)	12.29	0.012
SC (Starch content)	12.05	0
L-HCN (Cyanide-equivalent in leaf)	11.16	0
FBH (First branch height)	5.75	0.010
LW (Fresh leaf weight)	5.80	0.025
Residual	52.95	
(B) Cyanide-equivalent in Root (R-HCN)		
Regression model:	52.20	0
R-HCN = 0.53 CHLO + 0.37 L-HCN-0.04 TRK		
CHLO (Chlorophyll content in leaf)	32.84	0.011
L-HCN (Cyanide-equivalent content in leaf)	17.94	0.033
TRK (Tuberous root-knot symptom scoring)	1.42	0.031
Residual	47.80	
Regression model:	55.06	0
R-HCN = 0.59 L-CA + 0.33 L-HCN		
L-CA (Carotenoid content in leaf)	39.21	0.001
L-HCN (Cyanide-equivalent in leaf)	15.85	0.038
Residual	44.94	

†RW = Fresh root weight (kg plant^{-1}), LW = Fresh leaf weight (kg plant^{-1}), SW = Fresh stem weight (kg plant^{-1}), SC = Starch content in root (%), HI = Harvest index, FBH = First branch height (cm), R-HCN = Cyanide- equivalent content in root (ppm), L-HCN = Cyanide- equivalent content in leaf (ppm), CHLO = Chlorophyll content in leaf (mg cm^{-2}), L-CA = Carotenoid content in leaf (mg cm^{-2}), TRK = Tuberous root-knot symptom scoring

weight (RW), with $R^2 = 86.03\%$, as shown in Table 3. This model was, however, in contrast with the report by Boakyn et al. (2013) showing a non-significant correlation between the root weight (RW) and the so-called top weight consisting of leaf and stem weights. In this study, the harvest index (HI) had a direct effect on root weight (RW) with a standardised partial regression coefficient (b) = 0.89 (Figure 1), when calculated from the fresh root weight (RW), leaf weight (LW) and stem weight (SW). The path analysis in Figure 1 also showed various standardised partial regression coefficients with these parameters affecting the root weight (RW). Therefore, the stepwise multiple regressions were analysed without the harvest index (HI). It was also found that the leaf weight (LW), stem weight (SW), starch content (SC), the first branch height (FBH) and

cyanide-equivalent content in leaf (L-HCN) contributed significantly to the fresh root weight (RW), with $R^2 = 47.05\%$.

Ntawuruhunga and Dixon (2010) proposed a regression model to predict root yield using leaf area, petiole length, storage root number, root size, root girth, stem weight, and starch content. The agronomic traits used in the said model, which are common with our model, were the stem weight (SW), starch content (SC) and leaf weight (LW), assuming that the leaf area relates to the leaf weight. Our analysis showed that both stem weight (SW) and root weight (RW) were equally correlated with the leaf weight (LW) ($r = 0.26$) and the stem weight (SW) ($r = 0.51$ (Table 2 & Figure 2). Apparently, the stem weight (SW) was the only agronomic trait or parameter in the standardised regression
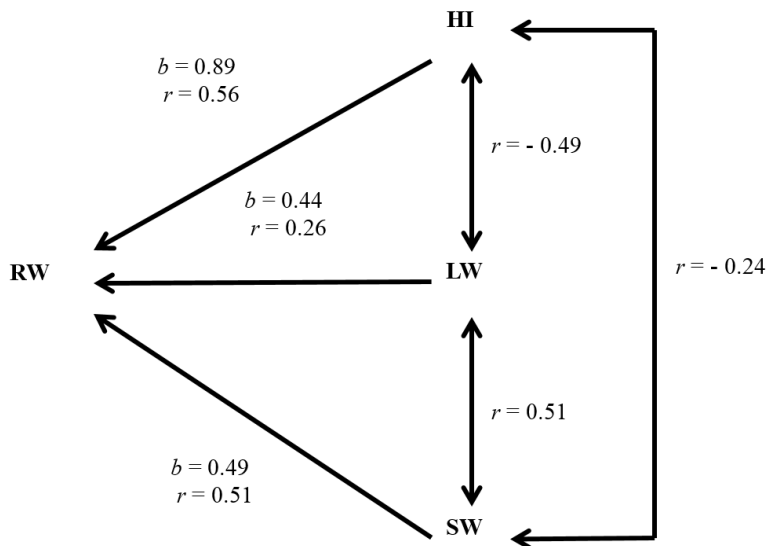

Figure 1. Path analysis effect of harvest index (HI) (ratio), fresh leaf weight (LW) (kg plant[-1]), and fresh stem weight (SW) (kg plant[-1]) on fresh root weight (RW) (kg plant[-1]). b = standardized partial regression coefficient, r = correlation coefficient
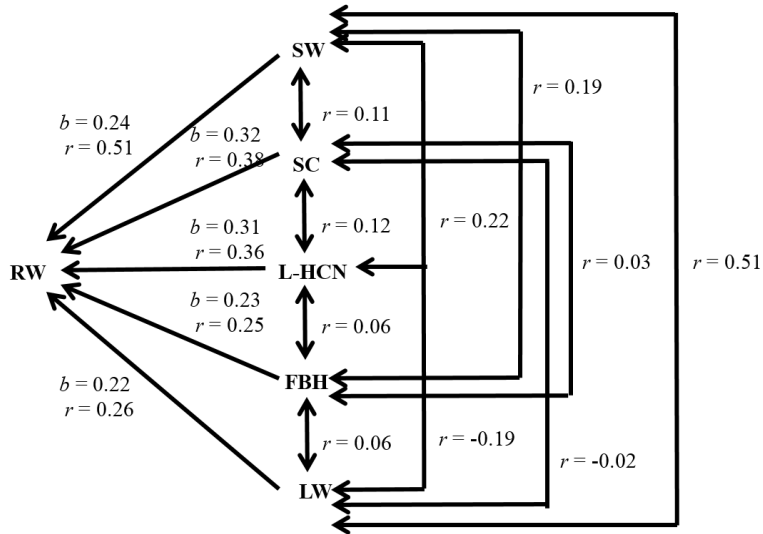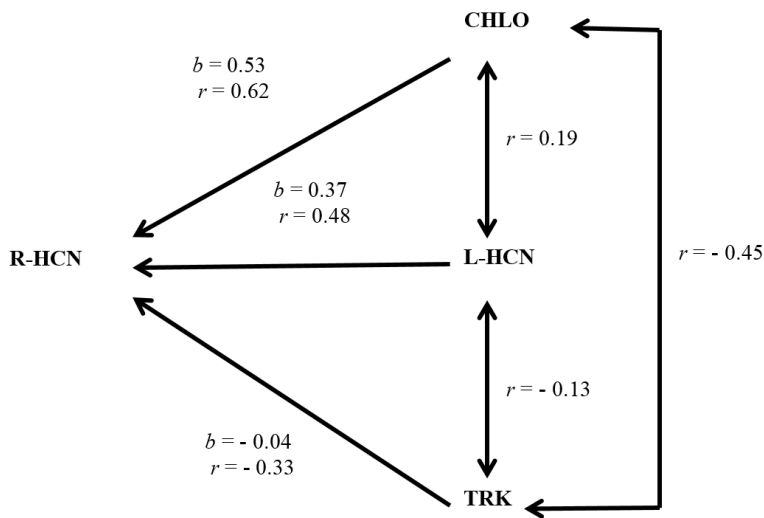
Figure 2. Path analysis effect of fresh stem weight (SW) (kg plant^{-1}), starch content in root (SC) (%), cyanide-equivalent content in leaf (L-HCN) (ppm), first branch height (FBH) (cm), and fresh leaf weight (LW) (kg plant^{-1}) on fresh root weight (RW) (kg plant^{-1}). b = standardized partial regression coefficient, r = correlation coefficient

Figure 3. Path analysis effect of chlorophyll content in leaf (CHLO) (mg cm^{-2}), cyanide-equivalent content in leaf (L-HCN) (ppm), and tuberous root-knot symptom scoring on cyanide-equivalent content in root (R-HCN) (ppm). b = standardized partial regression coefficient, r = correlation coefficient
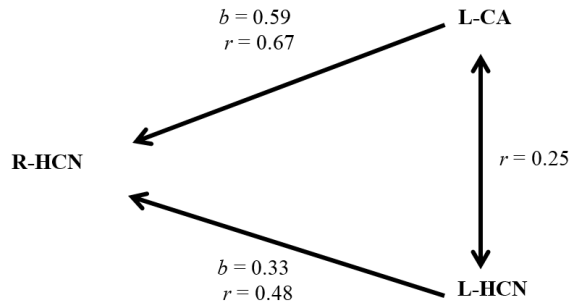
Figure 4. Path analysis effect of carotenoid content in leaf (L-CA) (mg cm^{-2}) and cyanide-equivalent content in leaf (L-HCN) (ppm) on cyanide equivalent content in root (R-HCN) (ppm). *b* = standardized partial regression coefficient, *r* = correlation coefficient

model which had a correlation coefficient (*r* = 0.51) higher than the standardised partial regression coefficient (*b* = 0.24) (Figure 2), while other four parameters [starch content (SC), cyanide-equivalent content in leaf (L-HCN), the first branch height (FBH), and leaf weight (LW)] showed no significant difference between the correlation coefficient and the standardised partial regression coefficient. Thus, the findings indicated that the stem weight (SW) had direct and indirect effects on the root weight (RW). The highest indirect effect of the stem weight (SW) on root weight (RW) was obviously through the leaf weight (LW) (Figure 2), while other traits tended to have a direct effect on the root weight (RW) rather than an indirect effect.

It was also observed that the cassava plants having branchy plant type and rather tall plant height tend to have a higher leaf weight (LW) and stem weight (SW) with significant correlations (*r* = - 0.52 and 0.53, respectively) (Table 2). However, the parameter which was included in the standardised regression analysis was the

first branch height (FH) instead of the plant height (PH) or the plant type (PT). The first branch height (FBH) had non-significant positive correlation with the stem weight (SW) and the leaf weight (LW) with *r* = 0.19 and 0.06, respectively (Table 2).

The starch content (SC) was also correlated with the root weight (RW), implying that starch is the most common carbohydrate stored in the roots. The correlation between the starch content (SC) and the harvest index (HI) also indicated the importance of carbohydrate partitioning to root. The root number (RN) also had a high positive correlation with root weight (*r* = 0.38), leaf weight (*r* = 0.21) and stem weight (SW) (*r* = 0.36). In this study, however, the root number (RN) was not included in the standardised partial regression model analysed by the stepwise method. A significantly positive correlation (*r* = 0.64) between the root number (RN) and root weight (RW) was also reported by Boakyn et al. (2013). Ntawuruhunga and Dixon (2010) also proposed a regression model with the root number (RN) and other root characters

in the model. In this study, the roots were not characterised by size or girth. Therefore, the root number used in Ntawuruhunga and Dixon (2010) might be accounted for the root storage capacity which could be reasonably included in the model.

Effects on Cyanide-Equivalent Content in Root

In this study, the cyanide-equivalent content in the root (R-HCN) had a positive correlation ($r = 0.29$) with the fresh root weight (RW). It was consistent with previous reports that the cyanide-equivalent content in cassava roots was correlated with the root yield, with coefficients ranging from 0.19 to 0.20 (Muhungu, 1994). Therefore, these two traits were likely unlinked. However, when the data were analysed separately within the parent group and breeding line group, the parent group had a lower positive correlation ($r = 0.22$) between these two traits than the breeding line group ($r = 0.35$), which is in contrast with the hypothesis that the parent lines have a higher correlation between the two traits.

It was also found in the trial that the root cyanide-equivalent content (R-HCN) was correlated with the cyanide-equivalent content in leaf (L-HCN), chlorophyll content in leaf (CHLO) and carotenoid content in leaf (L-CA) (Table 2). Interestingly, the cyanide-equivalent content in root (R-HCN) showed a higher correlation with the carotenoid content in leaf (L-CA) than the chlorophyll content in leaf (CHLO). However, it was found that the chlorophyll content in leaf (CHLO) and the carotenoid content in leaf

(L-CA) were highly correlated to each other, although the carotenoid content in leaf (L-CA) was calculated by subtracting the chlorophyll content in leaf (CHLO) in Equation [4].

Although it is known that the high nitrogen environment can increase nitrogen compound content, there are differences between the sink capacities of cyanogenic compounds in the vacuole and photosynthetic pigments in the chloroplast. As CNglcs are synthesised and could be stored in the leaf tissues in limited amounts, an excess may be translocated and stored in the root tissues. We found that in our trial, the cyanide-equivalent content in the leaves (L-HCN) had a lower variation than in the roots (R-HCN), even though the amount of CNglcs accumulated in the leaves was much higher than that in the roots. On the contrary, the chlorophyll and carotenoid contents in the leaves (CHLO and L-CA) seemed to increase under certain circumstances such as with high nitrogen fertilisation. As a result, the cyanide-equivalent content in the roots (R-HCN) could be altered with various nitrogen use efficiency of cassava breeding lines, and the chlorophyll and carotenoid contents in the leaves. However, there is a non-significant positive correlation between the carotenoid content in the root (R-CA) and leave (L-CA) in this trial. This result could be due to different expression levels of phytoene synthase isozymes, the key enzyme in carotenoid synthesis in the leaves and roots (Arango et al., 2010). It was also found in this trial that the chlorophyll content in leaf (CHLO) at 9.37 ± 1.93mg cm^{-2}, ranging

from 6.19-13.54mg cm^{-2} and carotenoid content in leaf (L-CA) at 2.15±0.33mg cm^{-2}, ranging from 1.54-2.97mg cm^{-2}, had lower coefficients of variation compared to the carotenoid content in root at 3.94±1.76 µg g^{-1}, ranging from 2.00 to 7.77 µg g^{-1}, which showed a noticeably higher variation of flesh colour, varying from white to cream and to yellow, which is similar to the report from the previous studies with the root carotenoid variation from 1.02 to 10.40 µg g^{-1} (Chavez et al., 2005). In this study, however, the chlorophyll and carotenoid measurements were carried out only in selected breeding lines.

The multiple regression or the path analysis showed the contribution of cyanide-equivalent content in leaf (L-HCN), chlorophyll content in leaf (CHLO), the tuberous root-knot symptom scoring (TRK) to the cyanide-equivalent content in the root (R-HCN) with R^2 = 52.20 (Table 3). Byju and Anand (2009) reported that under high nitrogen condition, the leaf tissue and chlorophyll content were abundant because nitrogen is an important macronutrient for plant growth that contributes to chlorophyll and CNglcs molecules. In our trial, however, the cyanide-equivalent content in root (R-HCN) showed a high positive correlation with the chlorophyll content in leaf (CHLO) and carotenoid content in leaf (L-CA) and was higher than the correlation with the cyanide-equivalent content in leaf (L-HCN).

The path analysis showed that both the cyanide-equivalent content in leaf (L-HCN) and chlorophyll content in leaf (CHLO) had a direct effect on the cyanide-equivalent content in root (R-HCN) with a low indirect effect through other traits, while the tuberous root-knot symptom scoring (TRK) had an indirect effect on the cyanide-equivalent content in leaf (L-HCN), which was mainly through the chlorophyll content in leaf (CHLO) (Figure 3). This implies that the effects of the root-knot disease scoring on cassava growth by reducing the nitrogen uptake capacity caused lower chlorophyll content in leaf (CHLO) and carotenoid content in leaf (L-CA), and consequently a lower cyanide-equivalent content in root (R-HCN).

The symptom scoring of cassava bacterial blight (CBB) and disease caused by the root-knot nematodes with fibrous root-knot (FRK) and tuberous root-knot (TRK) symptom scorings in this trial also showed higher coefficients of variation, which could be of a benefit in studying the correlations between the agronomic and disease-resistant traits. For disease symptom scoring analysis, cassava bacterial blight (CBB) showed no significant correlation with any agronomic traits in our findings.

Gleadow and Møller (2014) stated that cyanogenesis or the release of toxic HCN from endogenous CNglcs is an effective defense against generalist herbivores but less effective against fungal pathogens. Efforts are underway to genetically engineer CNglcs into some crops as a pest control measure, whereas in other crops, efforts are directed toward their removal to improve food safety. However, Lertsuchatavanich et al. (2014) reported a high positive correlation between the root CNglcs content

(R-HCN) and cassava bacterial blight (CBB) resistance. For the root-knot disease, the root-knot symptom scorings had no significant correlation with any agronomic traits but the score of inhibited growth of tuberous root-knot showed a negative correlation with the root starch content (SC) and cyanide-equivalent content in root (R-HCN) and the cyanide-equivalent in leaf (L-HCN) and carotenoid content in leaf (L-CA) (Table 2). Furthermore, in the locations where there is no incidence of root-knot disease, the tuberous root-knot (TRK) symptom scoring would not be included in the stepwise path analysis. The standardised regression analysis carried out also showed that the cyanide-equivalent content in leaf (L-HCN) and carotenoid content in leaf (L-CA) contributed significantly to the cyanide-equivalent content in root (R-HCN) with R^2 = 55.06 (Table 3).

As shown in the path analysis with the tuberous root-knot (TRK) symptom scoring (Figure 3), the path analysis without the tuberous root-knot (TRK) symptom scoring (Figure 4) also showed that both the cyanide-equivalent content in leaf (L-HCN) and carotenoid content in leaf (L-CA) had a direct effect on the cyanide-equivalent content in root (R-HCN), with a low indirect effect through one another agronomic trait.

CONCLUSION

Eighty-three cassava breeding lines were used for the clonal selection trial carried out from May 2012 to May 2013. As a consequence of the variation evaluation of the 17 agronomic traits chosen for further

determination, the multiple regression analysis employing the path analysis to select appropriate agronomic traits of Thai cassava lines yielded an evidence that the cassava lines, with high root yield and high CNglcs content in the roots (Kasetsart 50, Huai Bong 60, Huai Bong 80, and Rayong 5) and the cultivars with low CNglcs content in the roots but low root yield (Kolok, Rayong 2, and Ha Na Thi) used in this trial, had a significant correlation between the root yield and low CNglcs content in the roots.

This finding provided a future promising achievement in cassava breeding programme for high root yield but low CNglcs content in the roots. It could be speculated that some cassava lines with high root yield and low CNglcs in the roots would be eventually achieved. The CNglcs and carotenoid contents in the leaves may be indicative traits for screening for low CNglcs content in the roots, as evident from the correlations obtained in the trial. Moreover, it is also evident from the data analysed that the cassava plants, with high leaf weight (LW), high stem weight (SW), high starch content (SC), high first branch height (FBH) and high CNglcs content in leaf (L-HCN), contributed significantly to the high root weight (RW). However, the CNglcs content in root (R-HCN) and in leaf (L-HCN) did not show a correlation with cassava bacterial blight (CBB) and fibrous root-knot (FRK) symptom scorings. It was indicated that an artificial inoculation might be required for the disease symptom scoring in the future experiments. The relatively high scoring of the tuberous root-knot

(TRK) symptom might contribute to the low CNglcs content in leaf (L-HCN) as a consequence of some factors that decreased the plant growth by limiting the nutrient transport. Nevertheless, there is a need for additional data to be acquired and generated from further trials and evaluations in other locations with various environmental regimes for the reliable verification of the multiple regression models obtained from the path analysis carried out in our present investigation.

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Nutritional Compositions and Antioxidant Activities of Non-Polar and Polar Extracts of Germinated Brown Rice

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ABSTRACT

The objective of this study was to investigate the nutritional compositions and antioxidant activities (AA) of non-polar and polar extracts of germinated brown rice (GBR). Nutritional compositions such as moisture, ash, carbohydrate, fat, protein and fibre were determined. Energy and minerals content were determined by using bomb calorimetry and Atomic Absorption Spectroscopy (AAS), respectively. Total phenolic content (TPC) and total flavonoid content (TFC) of the extracts were determined by Folin-Ciocalteu method and aluminium chloride colorimetric method. The AA was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and [2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt (ABTS) radical scavenging capacity assay, ferric reducing antioxidant potential (FRAP) assay, and β-carotene bleaching assay. Total energy content of GBR sample was 390.95±11.31 kcal/100g and carbohydrate (54.30±1.04 g/100 g) was the most abundant nutrient. The predominant minerals in the GBR sample were sodium, potassium and magnesium. The polar extract showed significantly higher ($p < 0.05$) level in TPC, TFC and AA than non-polar extract except in β-carotene bleaching assay. Positive and strong correlations ($r > 0.90$, $p < 0.001$) existed between antioxidants (TPC and TFC) and AA. Therefore, polar extract was better than non-polar extract. The nutritional composition of GBR also provided an update for food composition database.

INTRODUCTION

Rice plays a prominent role in the food security of the world's population. It is a major staple food for more than half of the world population in general and some populous countries such as Asia and Africa in particular (Hu et al., 2012). Rice provided about third-quarter of the calorific intake for the population in Southeast Asia (Fitzgerald et al., 2009). It is also the main carbohydrate source for people in South-East Asia including Malaysia. In 2012, the worldwide rice production was about 926 million of metric tonnes, in which China was the highest rice production with 206 million of metric tonnes and Malaysia produced 2.75 million of metric tonnes of rice (FAO, 2014).

Recently, whole grain cereal has been associated with reduced risks of developing chronic diseases including coronary heart disease, diabetes mellitus and certain types of cancer (Hübner & Arendt, 2013). Germination of grain for a short period of time activates the hydrolytic enzymes and improves the contents of dietary fibre, minerals, vitamins, and phytochemicals including phenolic compounds and sterols (Chavan et al., 1989; Hübner & Arendt, 2013). Thus, it is believed that germinated grains rich in nutrients content and phytochemicals are better for the health-promoting benefits compared to non-germinated grains.

GBR has become a popular health food especially in Asian. GBR is produced by soaking brown rice grains in water to promote germination (Moongngarm & Saetung, 2010). It contains significantly higher amount of nutrients and is easier to be digested and absorbed, yet the texture is softer than brown rice (Chavan et al., 1989). Nutrients such as γ-amirobutyric acid (GABA), dietary fibre, inositols, ferulic acid, phytic acid, tocotrienols, some minerals, γ-oryzanol, and prolylendopeptidase inhibitor, are significantly increased in GBR (Latifah et al., 2010). In view of the nutritional value and the potential to become a novel functional food for human diet, there is a need to investigate the nutritional value of GBR for future industrial and domestic applications.

Thus, the objectives of this study were to determine the nutritional compositions of GBR and to evaluate the TPC, TFC and AA of non-polar and polar extracts of GBR in local brown rice varieties.

MATERIALS AND METHODS

Chemicals and Reagents

Methanol, n-hexane and dimethyl sulfoxide (DMSO), sodium acetate trihydrate, ferric trichloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous sulphate ($FeSO_4 \cdot 7H_2O$) and hydrochloride acid (HCl) were purchased from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3), sodium nitrite, sodium hydroxide (NaOH), potassium persulphate, aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), glacial acetic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium peroxodisulfate ($K_2S_2O_8$), ABTS [2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt, 2,4,6-tripyridyl-1,3,5-s-triazine (TPTZ), gallic acid, quercetin, 3,5-Di-tert-4-butylhydroxytoluene (BHT),

ascorbic acid and Folin-Ciocalteau reagent were purchased from Sigma Chemical Co (St. Louis, MO, USA). All chemicals and reagents used in the study were of analytical grade.

Sample Preparation

Brown rice (*Oryza sativa* L. mixed varieties MR219 and MR220), local rice varieties that are massive produced and sold in the market, was obtained from Padiberas Nasional Berhad (Bernas), Malaysia. The rice was germinated according to the pre-optimised conditions established in the Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia (UPM), Selangor, Malaysia as described previously (Sani et al., 2012). Briefly, brown rice was washed twice with clean tap water before incubation at 37°C for 24 hours until germinated. Then, the GBR was dried in an oven at 50°C. It was ground to powder using a stainless steel blender (Waring Commercial, Torrington, CT, USA) before further analysis.

Nutritional Composition Analysis

Moisture content was determined through direct drying method as described by Tee et al. (1996) using an air oven (Memmert Universal, Schwabach) set at 105ºC until constant weight of the sample was obtained. Meanwhile, lipid content was determined by using hexane extraction, facilitated by the Soxtec Avanti 2050 apparatus (Foss Tecator AB, Höganäs, Sweden), as described by AOAC (2006) (Official Method, 2003.05). Protein content was determined according to Kjeldahl method (AOAC, 2000) facilitated by the Kjeltec 2200 Auto Distillation Unit (Foss Tecator AB, Höganäs, Sweden), and the conversion factor used was 5.95. The total available carbohydrate content was determined using Clegg-anthrone method (Peris-Tortajada, 2004). Dietary fibre was determined using enzymatic-gravimetric method, as described by AOAC (1995) (Official Method 991.42 and 993.19), facilitated by the Fibertec system (Fibertec System E 1023 filtration module, Tecator, Höganäs, Sweden). Dry ashing method was used to determine the ash content by incinerating the sample in a furnace (Carbolite, Parsons Lane, Hope, UK) set at 550°C until whitish/greyish ash was obtained (Tee et al., 1996). The remaining inorganic material was cooled and weighed. The results were expressed as g/100 g of sample. Energy content was determined by bomb calorimetry (IKA C5003, IKA Werke, Germany) according to the manufacturer's protocol and expressed as kcal/100 g of sample. The resulting ash was further prepared for determination of mineral contents as described by Tee et al. (1996). The content of sodium, potassium, magnesium, calcium, iron and zinc were then measured using the Model nov AA 400 atomic absorption spectrometer (Analytik Jena AG, Jena, Germany). Calibration curves for each of the minerals were determined using the standard solutions before the readings of sample were obtained. The results for mineral contents were expressed as mg/100 g of sample.

Extraction of Non-Polar and Polar Components

Non-polar and polar components of GBR were extracted as described by Jang and Xu (2009) with slight modifications. Briefly, 10 g weighed sample was mixed with 30 ml of hexane in the ratio of 1:3 (w/v) and was vortex for 30 sec before placed in a 60ºC water bath for 20 min. Then, the mixture was centrifuged at 3500 rpm for 15 min before the supernatant was collected and filtered through filter paper (Whatman No.1). The extraction was repeated for another two times under the same condition. The three supernatants were combined into non-polar fraction and placed in a rotary evaporator (Büchi Rotavapor R-200, Büchi Labortechnik AG, Switzerland) below 40°C. Subsequently, the defatted GBR was spread on an aluminium foil and placed under a laboratory hood to dry before mixed with 30 ml methanol (1:3, w/v) to perform polar components extraction. The procedure for polar extraction was the same as the non-polar components extraction, except methanol was used as the extraction solvent. The extracts were reconstituted with DMSO before stored at -20°C until further analysis.

Measurements of Antioxidant Groups

TPC of GBR was determined based on Folin-Ciocalteu reagent (diluted 10-fold) method (Velioglu et al., 1998). After incubation at room temperature for 90 min with added of Na_2CO_3 solution (60 g/l), the reaction was measured at 725 nm versus blank using a spectrophotometer (UV-1800, Shimadzu

Co., Japan). A standard calibration curve was plotted using gallic acid (0.005–0.25 mg/ml) for quantification purpose and the results were expressed as mg gallic acid equivalent (GAE)/100 g of sample.

TFC of GBR was determined by aluminium chloride colorimetric assay reported by Liu et al. (2008) with modification. Briefly, 0.6 ml of appropriately diluted extract was mixed with 0.06 ml of 5% sodium nitrite and incubated for 5 min. Then, 0.06 ml of 10% aluminium chloride was added to the mixture. After 6 min of incubation, 0.6 ml of 1 M sodium hydroxide was added to the mixture. The end volume of the reaction mixture was made up to 1.5 ml with DMSO. Absorbance of the reaction was read at 510 nm against a blank. Quercetin (6.25-100 µg/ml) was used to construct a calibration curve for quantification and the results were expressed as mg quercetin equivalent (QE)/100 g of sample.

Measurements of Antioxidant Activities

DPPH free radical scavenging assay was performed based on Brand-Williams et al. (1995). Briefly, 3.9 ml of 60 µM DPPH radical solution prepared in methanol and 0.1 ml of various concentrations of extract were mixed well. The absorbance of the mixture was measured at 515 nm by using the spectrophotometer after it was incubated at the room temperature for 30 min under dark condition. Reagent solution without test extract was used as the control. Ascorbic acid at different concentrations (7.8125-125 µg/ml) was used as the standard

of comparison. The DPPH free radical scavenging activity was calculated as follows:

Scavenging activity (%)

$$= \frac{A_{515}Control - A_{515}Extract}{A_{515}Control} \times 100 \quad [1]$$

where, A_{515}Extract and A_{515}Control are the absorbance of reagent with extract and reagent without extract, respectively.

FRAP assay was adapted from the method described by Benzie and Strain (1996). The FRAP reagent contained 2.5 ml of 10 mM TPTZ solution in 40 mM HCl plus 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 ml of 300 mM acetate buffer at pH 3.6 in a ratio of 1:1:10 (v/v/v). The FRAP reagent was prepared fresh daily and warmed to 37°C in a water bath prior to use. FRAP reagent (3 ml) was mixed thoroughly with 0.1 ml of DMSO. Immediately, the absorbance was measured at 593 nm (0 min) using the spectrophotometer (A_0). Then, 3 ml FRAP reagent was mixed thoroughly with 0.1 ml of extract and incubated for 30 min at 37º C. Absorbance of the mixture was measured at 593 nm reference to blank (A_{30}). The absorbance of extract was the differences in absorbance (A_{30}) and absorbance (A_0). Ferrous sulphate (0.1-1.0 mM) was used to prepare the calibration curve for quantification. The FRAP value was expressed as mM of Fe (II) equivalent/100 g of sample.

ABTS radical scavenging assay was determined as described by Re et al. (1999). ABTS radical cation (ABTS•+) was obtained by reacting 7 mM ABTS in distilled water with 2.45 mM potassium peroxodisulfate and allowing the mixture to stand in the dark at room temperature for 12–16 hr before use. The ABTS•+ solution was diluted with ethanol to obtain an absorbance of 0.70±0.02 at 734 nm. Aliquots of various concentrations of extracts (20 µl) were allowed to react with 1 ml of the diluted ABTS solution and the absorbance was measured after 10 min incubation using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with BHT (100 µg/ml). The percentage inhibition was calculated as follows:

ABTS radical scavenging activity (%)

$$= \frac{A_{734}Control - A_{734}Extract}{A_{734}Control} \times 100 \quad [2]$$

where, A_{734}Extract and A_{734}Control are the absorbance of reagent with extract and reagent without extract, respectively.

β-Carotene bleaching method was performed as Jayaprakasha et al. (2007). β-Carotene solution was prepared by dissolving β-carotene (0.4 mg) in 0.4 ml of chloroform, 40 mg of linoleic acid and 400 mg of Tween 20 in round bottom flask. Chloroform was completely evaporated at 40°C using a vacuum evaporator. Then, 100 ml of distilled water was added with vigorous shaking. Aliquots (2 ml) of this emulsion were transferred into different test tubes containing 0.1 ml of extracts. The tubes were incubated at 50°C in a water bath and the absorbance was measured at 470 nm at zero time (t=0). After that, the

absorbance readings were recorded at 15 min intervals for 120 min at 470 nm by using the spectrophotometer. The same procedures were done on the control and blank. BHT (100 µg/ml) and ascorbic acid (100 µg/ml) were used for comparison purposes. The antioxidant activity (AA) was calculated as the percent of inhibition relative to the control using the following equation from Al-Saikhan et al. (1995), as follows:

$$AA\,(\%) = \frac{DR_{Control} - DR_{Extract}}{DR_{Control}} \; x \; 100 \tag{3}$$

where, $DR_{control}$ and $DR_{Extract}$ represent the degradation bleaching rates of β-carotene without and with the extract at 0 and 120 min, respectively.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) of three replicates. Significant differences at p< 0.05 between the means were determined using independent T-test in IBM SPSS software version 21.0. Pearson correlation test was performed to determine the correlations between the antioxidant groups and AA of the GBR extracts.

RESULTS AND DISCUSSION

Nutritional compositions analysis

Germination of grains has not only changed the texture, flavour and taste of the grains, but also their content of nutrients and bioactive compounds (Kaukovirta-Norja et al., 2008). The nutritional compositions of GBR sample are listed in Table 1. The weights of GBR sample before and after moisture test were measured. The GBR sample consisted of 14.04% water content and 85.96% dry matter. The macronutrient compositions of GBR sample were total available carbohydrate, 54.30±1.04 g; lipid, 2.11±0.09 g; and protein, 11.03±0.10 g. The carbohydrate content of GBR sample was relatively lower than polished rice (79.00 g/100 g) and unpolished husked rice (75.98 g/100 g), as described in Nutrient composition of Malaysian foods (Tee et al., 1997). The decrease of total carbohydrate content in the germinated grains could be attributed by the utilisation of simple sugar as an energy source to start germination (El-Adawy, 2002). Meantime, previous studies reported that germinated red aromatic brown rice from Thailand (Wichamanee & Teerarat, 2012), GBR cultivar Koshihikari from Japan (Ohtsubo et al., 2005) and GBR cultivar RD-6 form Northeast of Thailand (Moongngarm & Saetung, 2010) contained higher carbohydrate contents than GBR sample in this study, with 70.69 g/100 g, 71.30 g/100 g and 77.70 g/100 g, respectively. A study done by Palmiano and Juliano (1972) revealed that the starch content of rice grain decreased progressively during germination. Thus, the germination time of the rice grain might be one of the possible factors that contributed to the differences in carbohydrate contents. In contrast, the content of protein in the GBR sample was higher than non-germinated rice with only 7.09 g/100 g in polished rice and 8.02 g/100 g in unpolished husked rice (Tee et al., 1997). At the same time, the

GBR sample also showed higher protein content than germinated rice from some previous studies [e.g., 5.49 g/100 g (Megat Rusydi et al., 2011), 8.2 g/100 g (Ohtsubo et al., 2005), 8.34 g/100 g (Wichamanee & Teerarat, 2012), 8.47 g/100 g (Mohd. Esa et al., 2011) and 8.98 g/100 g (Moongngarm & Saetung, 2010)]. Bau et al. (1997) suggested that the synthesis of enzyme proteins or a compositional change following the degradation of other constituents during germination could possibly explain the increase of protein content after germination. Meanwhile, the lipid content in the GBR sample was close to the study reported by Wichamanee and Teerarat (2012), who found value of fat of germinated red aromatic brown rice was 2.08 g/100g. Wichamanee and Teerarat (2012) and Megat Rusydi et al. (2011) suggested that the lipid content decreased after germination. This could be explained by the fact that lipid is used to produce energy during sprouting process (Wichamanee & Teerarat, 2012). However, the lipid contents of polished rice (0.49 g/100 g) and unpolished husked rice (1.78 g/100 g) were lower compared with the result in this study (Tee et al., 1997). Nevertheless, further validation studies are still needed. Hence, carbohydrate is the most abundant macronutrient in GBR, which majorly contributes to the GBR sample calories content with 390.95±11.31 kcal/100 g.

The total dietary fibre of GBR in this study was 9.18±0.91 g/100 g, where the content of insoluble dietary fibre (8.63±0.84 g/100 g) was significantly higher ($p < 0.05$) than soluble dietary fibre (0.55±0.12 g/100 g).

Table 1
Nutritional composition of GBR based on 100 g of raw sample

Components	Contents
Moisture (g/100 g)	14.04±0.10
Lipid (g/100 g)	2.11±0.09
Protein (g/100 g)	11.03±0.10
Carbohydrate (g/100 g)	54.30±1.04
Total dietary fibre (g/100 g)	9.18±0.91
Insoluble dietary fibre	8.63±0.84
Soluble dietary fibre	0.55±0.12
Ash (g/100 g)	1.19±0.01
Energy calories (kcal/100 g)	390.95±11.31
Minerals (mg/100 g)	
Sodium	34.41±0.85
Potassium	166.2± 5.77
Magnesium	86.43±7.15
Calcium	15.67±0.92
Iron	1.923±0.07
Zinc	1.848±0.01

The total dietary fibre of GBR was higher than non-germinated rice with only 0.38 g/100 g in polished rice and 0.48 g/100 g in unpolished husked rice (Tee et al., 1997). It can be explained by the formation of primary cell walls (pectin substances) in the middle lamella (Lee et al., 2007). These results were similar to other studies done on GBR (Megat Rusydi et al., 2011; Mohd. Esa et al., 2011), but higher than germinated rice in Thailand and Japan (Ohtsubo et al., 2005; Moongngarm & Saetung, 2010; Wichamanee & Teerarat, 2012). This also indicated that GBR in this study is a good fibre source among the rice

as intake of dietary fibre reduces the risk of developing several non-communicable diseases (Anderson et al., 2009). The ash content in this study was 1.19±0.01 g/100 g, which was higher than polished rice (0.49 g/100 g) (Tee et al., 1997) and unpolished husked rice (1.02 g/100 g) (Tee et al., 1997) but lower than those reported by Moongngarm and Saetung (2010) and Wichamanee and Teerarat (2012) on Thailand cultivars. Potassium was the highest mineral (166.2±5.77 mg/100 g) in GBR, followed by magnesium (86.43±7.15 mg/100 g), sodium (34.41±0.85 mg/100 g), calcium (15.67±0.92 mg/100 g), iron (1.923±0.07 mg/100 g) and zinc (1.848±0.01 mg/100 g). These results were similar those reported by Mohd. Esa et al. (2011).

Antioxidant Groups of Non-Polar and Polar Extracts

The phenolic compounds including flavonoids have been typically characterised as the compounds that exerted antioxidant activities which are important in the maintenance of health and protection from several diseases like coronary heart disease and cancer (Kähkönen et al., 1999). The results of antioxidant groups of non-polar and polar extracts of GBR sample are presented in Table 2. TPC was expressed in terms of gallic acid equivalent from a standard curve (y = 6.4748x+0.0255, r² = 0.9996). The polar extract of GBR sample contained significantly higher (p< 0.05) TPC (224.65±7.39 mg GAE/100 g of sample) than non-polar extract (41.60±2.15 mg GAE/100 g of sample). These results

were implied that primarily the phenolics content in GBR were present in polar extract more than in non-polar extract. Phenolic acids such as ferulic, vanillic, caffeic, p-hydroxybenzoic, protocatechuic, p-coumaric and syringic acids are the most abundant polar compounds comprise in cereals (Kähkönen et al., 1999; Ma et al., 2013). The amount of TPC reported by Moongngarm and Saetung (2010) of methanolic extract on GBR cultivar RD-6 was only 84.3±6.35 mg GAE/100 g of the sample, which was lower than the TPC in this study. Alternatively, TFC was expressed in term of quercetin equivalent from a standard curve (y = 1.2073x-0.0009, r² = 0.9995). The TFC of GBR was similar to TPC, where the polar extract contained significantly higher (p< 0.05) TFC (125.31±11.91 mg QE/100 g of sample) than non-polar extract (11.06±1.67 mg QE/100 g of sample). TFC was represented about half of the TPC compounds in the polar extract and about one-quarter in the non-polar extract of GBR. Flavones such as apigenin, luteolin and tricetin are the predominant flavonoids in rice (Kim et al., 2008).

Antioxidant Activities of Non-Polar and Polar Extracts

Antioxidant compounds exert their activities through different ways. These include reducing agents, free radical scavengers, complexers of pro-oxidant metals and quenchers of singlet oxygen (Gordon, 1990). The existence of different antioxidant components in plant sources has become a relatively challenging task to measure their

AA. In this study, several methods have been employed to determine the AA of the non-polar and polar extracts of GBR sample.

In DPPH free radical scavenging assay, the absorbance of the DPPH radical decreased due to the presence of hydrogen donor from the antioxidant, resulting in the decolouration from purple to yellow. It is a rapid and highly sensitivity way in measuring the antioxidant efficiency of the sample (Blois, 1958). The DPPH free radical scavenging activity of the non-polar and polar extracts of GBR sample (Figure 1) was in dose-dependent manner, where the highest scavenging activity was at 5 mg/ml in non-polar extract (5.16±0.77%) and polar extract (29.94±1.17%), respectively. However, the ascorbic acid as a positive reference showed a better scavenging activity with 65.15±2.76% at concentration 125 µg/ml. In contrast, both non-polar and polar GBR extracts showed better AA in ABTS radical scavenging activity (Figure 2) compared to those obtained in DPPH reaction. The polar extract of GBR sample at 4 mg/ml (42.84±3.33%) and 5 mg/ml (47.01±1.55%) was shown to have better ability to reduce ABTS•+ than positive reference, BHT at

100 µg/ml (35.82±1.43%). In addition, there was a significant difference (p< 0.05) of scavenging activities between polar and non-polar extracts in both DPPH assay and ABTS assay. The scavenging activity of the non-polar extract was only shown at the concentration of 3 mg/ml and above in DPPH assay, indicating that the polar extract of GBR showed better AA than lipophilic extract.

The trend for the FRAP assay of GBR extracts (Table 2) did not vary markedly from their DPPH and ABTS results. The FRAP value was expressed as mM Fe (II) E/100 g of the sample with reference to ferrous sulphate (y = 0.7075x+0.0118, r² = 0.9992). The FRAP value of the non-polar extract (650.42±5.28 mM Fe (II) E/100 g of sample) was significantly lower (p< 0.05) than the polar extract (2063.13±37.40 mM Fe (II) E/100 g of sample). On the contrary, the β-carotene bleaching activity of the non-polar extract (37.84±4.32%) was not significantly different from polar extract (35.28±6.44%) in Table 2. The decrease in the absorbance of β-carotene in the presence of extracts with the oxidation of β-carotene and linoleic acid is shown in Figure 3.

Table 2

Comparison of the antioxidant groups and antioxidant activities of the non-polar and polar extracts of GBR

Antioxidant	Non-polar extract	Polar extract
Antioxidant groups		
TPC (mg GAE/100 g of sample)	41.60±2.15[a]	224.65±7.39[b]
TFC (mg QE/100 g of sample)	11.06±1.67[a]	125.31±11.91[b]
Antioxidant activities		
FRAP value (mM Fe(II)/ 100 g of sample)	650.42±5.28[a]	2063.13±37.40[b]
β-Carotene bleaching (%)	37.84±4.32[a]	35.28±6.44[a]

[a-b]Values within a row followed by different letters are significantly different at p<0.05. FRAP, ferric reducing antioxidant potential; TFC, total flavonoid content; TPC, total phenolic content.

Interestingly, the β-carotene bleaching activity of non-polar extract initially showed inferior activity than the polar extract but later it showed slightly better activity at 105 min and 120 min. This result suggested that although non-polar extract showed less effectiveness in antioxidant efficiency, it might have a more prolonged inhibition effect on the radical cation decolourisation than polar extract.
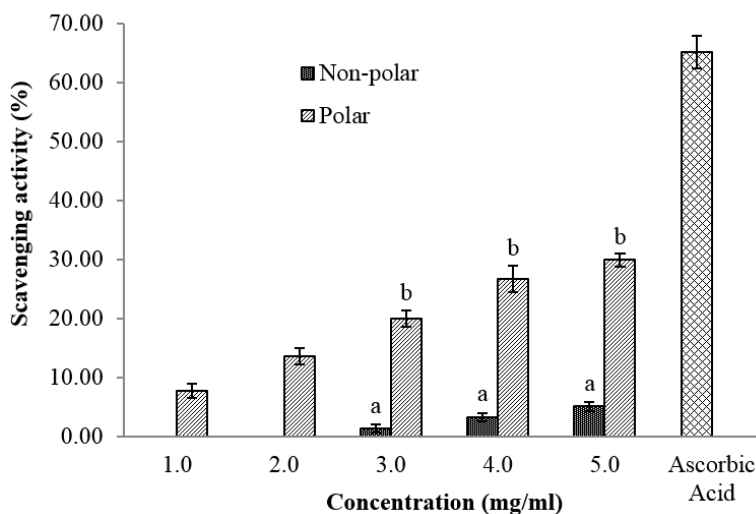

Figure 1. DPPH free radical scavenging activity of the non-polar and polar extracts of GBR at various concentrations and ascorbic acid (125 µg/ml). [a-b]Values with different letters are significantly different at $p < 0.05$ among the same concentration.
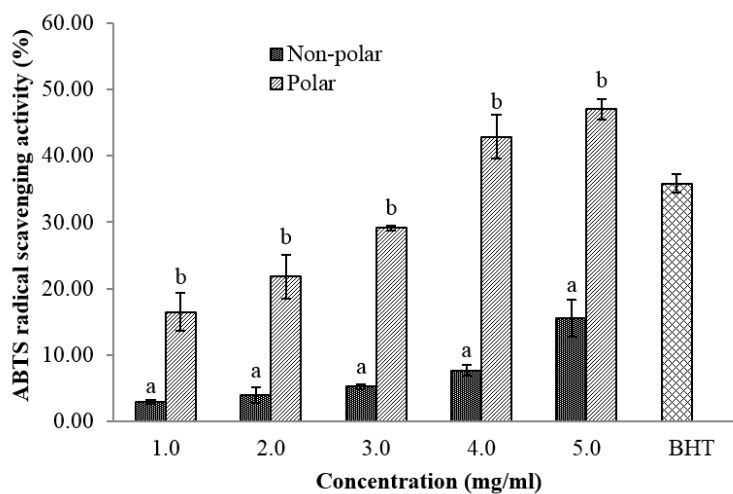
Figure 2. ABTS radical scavenging activity of the non-polar and polar extracts of GBR at various concentrations and BHT (100 µg/ml). [a-b]Values with different letters are significantly different at $p < 0.05$ among the same concentration.
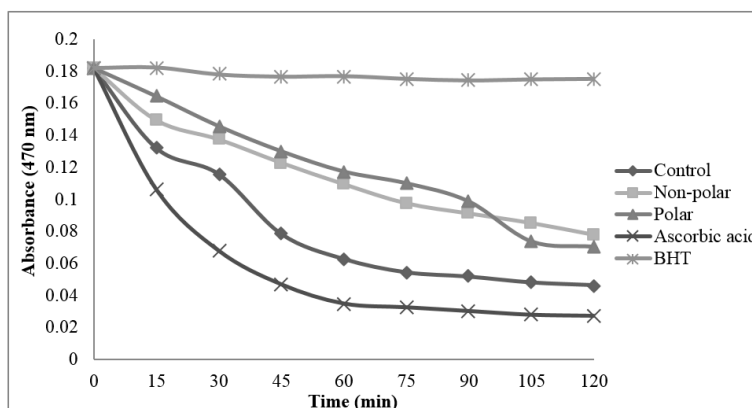
Figure 3. Absorbance value of the non-polar and polar extracts of GBR at 4mg/ml using β-carotene bleaching method. Higher absorbance at 470 nm indicates higher antioxidant activity.

In general, the results of this study concur with a previous report in 100 different kinds of food including fruits, vegetables, nuts, dried fruits, spices, cereals, infant and other foods, in which the AA of the non-polar extract of samples was lower than the polar extract (Wu et al., 2004). Jang and Xu (2009) also reported that the polar extracts of purple rice bran showed significantly higher phenolic content and free radical scavenging activity than the non-polar extract. Both the non-polar and polar extracts of GBR sample showed AA in this study; however, the polar extract was generally better. The antioxidant properties of rice sample is the result of combined activity of a wide range of compounds including vitamin E (α-tocopherol, α-tocotrienol, γ-tocopherol and γ-tocotrienol) (Xu et al., 2001), γ-oryzanol components (Xu et al., 2001), polyphenols (Hudson et al., 2000) and possibly other components.

Correlation between Antioxidant Groups and Antioxidant Activities

The relationships between antioxidant groups (TPC and TFC) and AA (DPPH free radical scavenging activity, FRAP value, ABTS radical scavenging activity and β-carotene bleaching activity) of the GBR sample are shown in Table 3. Very strong and positive correlations were observed between the antioxidant groups and AA (r>0.90 and p<0.001) according to Guildford's (1973) Rule of Thumb. This might relate to the phenolic and flavonoid compounds present in the non-polar and polar extracts. The presence of the hydroxyl group of the phenolic compounds and the second hydroxyl group in the *ortho* and *para* position forming resonance-stabilised phenoxyl radicals (Chen & Ho, 1997) clarified the antioxidative activity of the extracts. Moreover, flavonoids are one of the most diverse and widespread group of natural compounds in phenolics (Agrawal,

Table 3
Correlation coefficient (r) between antioxidant groups and antioxidant activities of GBR extracts and between assays

Antioxidant assay	TFC	DPPH	FRAP	ABTS	β-Carotene bleaching
TPC	0.997*	0.987*	0.997*	0.990*	-0.304
TFC	1	0.975*	0.989*	0.981*	-0.357
DPPH	0.975*	1	0.996	0.996*	-0.203
FRAP	0.989*	0.996*	1	0.996*	-0.251
ABTS	0.981*	0.996*	0.996*	1	-0.186
β-Carotene bleaching	-0.357	-0.203	-0.251	-0.186	1

*Correlation was significant at the 0.01 level (2-tailed). ABTS, ABTS radical scavenging capacity; DPPH, DPPH radical scavenging capacity; FRAP, ferric reducing antioxidant potential; TFC, total flavonoid content; TPC, total phenolic content.

1989). This also explained the reason of a strong and positive relationship existed between TPC and TFC (r=0.997, p<0.001). However, no significant relationship (p>0.05) was found between antioxidant groups with β-carotene bleaching.

Furthermore, very strong and significant correlations were also found between the various methods used to determine the AA of the GBR extracts (r>0.90 and p<0.001). However, just like the correlation between antioxidant groups with β-carotene bleaching, no significant correlation was found between β-carotene bleaching with other methods (p>0.05). Unlike the others, the β-carotene bleaching assay is based on hydrogen atom transfer, which might explain the divergence between the results obtained with the β-carotene bleaching assay and those obtained with other assays that are based on single-electron transfer mechanism (Huang et al., 2005).

CONCLUSION

The nutritional composition of the GBR sample obtained in this study is useful to provide update for food composition database. GBR could be used as a new functional food as it is low in carbohydrate but high in protein and fibre contents. The level of TPC and TFC in the GBR polar extract and their AA was significantly higher than that in the non-polar extract. Further studies could focus on their AA using *in vivo* model.

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Effect of Elemental Sulphur Timing and Application Rates on Soil P Release and Concentration in Maize

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ABSTRACT

High pH soil accounts for more that 30 percent of world's soils and poses problems to plant nutrient availability. As a cheap and readily available source of soil acidulates, elemental sulphur may be a useful material for alleviating some alkaline soil problems. To elucidate the role of elemental sulphur as a soil amendment for plant production in a high pH soil, maize plants were grown under greenhouse conditions for 45 days after 0, 20 and 40 days of soil incubation at different rates of elemental sulphur (0, 0.5, 1 and 2 g S kg^{-1} of soil). Soils were sampled two times (before and after planting) and subjected to soil pH and available P determination. The results showed with each unit increase in S rate, soil pH decreases by 1.52 units. In addition, while sulphur application increased available P before planting, it failed to increase P supply to maize at harvest. Supporting the role of elemental S on soil P availability, with increasing S application rate the P concentration in maize root, stem and leaves was successively decreased. This relationship can be explained by the dilution of P in increasing leaf biomass and the similar concomitant increase of both zinc and manganese nutrient concentrations with increasing sulphur application rate. Overall, soil acidification by elemental sulphur application resulted in P reduction in soil labile pools and intensified P deficiency in maize..

Keywords: Soil acidification, Phosphorous, Mn and Zn

INTRODUCTION

It is well known that the availability of essential nutrients affects yield and yield components of crops (Ye et al., 2011). The availability of nutrients in soils, as the major source for plant nutrients, depends

on soil characteristics especially soil pH (Chien et al., 2011; Lindsay, 1979; Shenker et al., 2005; Wang et al., 2006). Fertilisation and addition of acidifying amendments are common practices in high pH soils to enhance nutrient availability and improve plant performance. Elemental sulphur, as a soil amendment, is of special interest to increase soil nutrient solubility since it possesses slow release acidifying characteristic and is readily available (Chien et al., 2011). The acidifying function of S originates from its microbial oxidation to sulphuric acid over time (Vidyalakshmi et al., 2009). There are contrasting reports on the effects of elemental S on soil pH and nutrient availability (Klikocka, 2011; Safaa et al., 2013; Skwierawska et al., 2012). For instance, the effectiveness of elemental sulphur application on nutrient solubility was not observed in some soils (Sameni et al., 2004; Shenker & Chen, 2005; De la Fuente et al., 2008; Skwierawska et al., 2012). However, the positive effect of elemental sulphur on soil nutrient solubility as a result of soil pH reduction has been well documented (Cui et al., 2004; Ye et al., 2010). As reported by Lambers et al. (2008), high concentrations of hydrogen ions (low pH) cause modest increases in nutrient input by increasing weathering rate. Protons first displace cations from the exchange complex on clay minerals and soil organic matter. In addition, the availability of ions is strongly affected by pH because this affects their oxidation state and solubility (Lambers et al., 2008).

As different soil types may show diverse responses to soil acidification as an effective strategy for soil nutrient solubility enhancement (Wang et al., 2006), it is necessary to find the optimum sulphur rate to obtain optimum pH for each specific soil in which nutrient solubility increased and concurrently extreme soil acidification and its consequences such as nutrient toxicity for plants were avoided. While the effectiveness of elemental sulphur on Bintang Series soil pH reduction was documented (Karimizarchi et al., 2014), the minimal research data on the impacts of elemental S addition on soil phosphorous release and plant uptake for this soil have been released. Therefore, the present study was carried out to elucidate the effects of elemental sulphur rates and timing, as well as soil acidity on phosphorous solubility in Bintang Series soil and phosphorus uptake by maize. In addition, the phosphorous interactions with Mn and Zn in maize root, stem and leaves are also discussed.

MATERIAL AND METHODS

Site Description and Soil Characterisation

The soil sample for this study was taken from Bukit Bintang, Perlis (located in Malaysia with the geographical coordinates of 6°31′01.61″N, 100° 10′ 12.43″ E). The area, Bukit Bintang, is developed from limestone parent materials and is under natural vegetation (forest). 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 in a reciprocal shaker. Total carbon,

nitrogen and sulphur were determined by CHNS LECO analyser. Meanwhile, soil mechanical analysis was done using the pipette method (Gee et al., 1986) and texture class was determined using the United States Department of Agriculture (USDA) soil textural triangle. Titrimetric method was used for determination of total calcium carbonate (Bashour et al., 2007).

Growth Conditions and Plant Materials

A pot experiment was conducted to elucidate the effects of elemental sulphur application time and rate on maize growth and soil phosphorous release. A completely randomised block design with factorial treatment combination was used with the following factors: (i) Elemental sulphur application at 4 rates including 0, 0.5, 1 and 2 g S per kg of soil; and (ii) elemental sulphur application times including 0, 20 and 40 days before planting of maize. Each pot contained 10 kg soil and received three plants which were thinned to one within one week. The plants were grown for 45 days in the greenhouse. By weighing each pot, the plants were irrigated daily to maintain 90% of soil field capacity moisture content. All the plants were supplied with fertilisers based on the recommendations by Malaysian Agricultural and Development Research Institute; 120 kg N ha^{-1} in the form of urea, 80 kg P_2O_5 in the form of triple superphosphate and 100 kg K_2O in the form of muriate of potash. There were four replications for each of treatments that were randomised in four rows.

Plant Available Soil Nutrient Extraction and Determination

To evaluate the effect of elemental S and soil pH on nutrient solubility, the soluble fraction of soil nutrients was extracted. The mobile fraction of soil nutrients can be extracted by water, neutral or buffered salts (Hlavay et al., 2004; Jones, 2001; Ye et al., 2011). As buffered extractants may hinder the effect of S on soil nutrient solubility, neutral and un-buffered solution, $CaCl_2$ for micronutrients and water for macronutrients were used as five g air dried soil was shaked for 2 hours with 25 ml of $0.01 M CaCl_2$ solution. To obtain a clear solution, it was centrifuged for 15 minutes at 3000 rpm and then filtered. For macronutrients, 10 g air dried soil was shaken for 1 hour with 50 g distilled water. It was centrifuged for 15 minutes at 3000 rpm and filtered. The extracted nutrients were determined by ICP-OES (Perkin Elmer, Optima 8300).

Plant Biomass Nutrient Extraction and Determination

Maize leave, shoot and root tissues were washed separately in deionized water then dried at 65°C and weighed. After grounding, the weighed plant tissues were ashed in a muffle furnace at 480 °C for about 10 h. After cooling, it was dissolved in 10 ml of diluted acid mixture (Jones, 2001). Then, the mixture was filtered into a 50ml volumetric flask through Whatman No. 40 filter paper. Element concentrations including Mn and Zn were determined by ICP-OES (Perkin Elmer, Optima 8300). Phosphorous content of the plant was measured by a Technicon Auto-Analyser.

Statistical Analysis

The relationship between plant and soil properties was subjected to different regression models at a probability level of 0.05 with the help of Sigmaplot software. Using SAS 9.1, Anova analysis and DMRT test at $\alpha = 0.05$ were employed to determine the significant differences between the treatments.

RESULTS AND DISCUSSION

Physico-Chemichal Properties of Bintang Series Soil

With the tentative USDA classification of Ultisol, the physicochemical characteristics of Bintang series soil are presented in Table 1. Being Silt loam in texture, the soil was found to be slightly alkaline in nature with the pH value of 7.5, which is affected by limestone parent materials from nearby hills. Base saturation is high (56 percent), however, the calcium carbonate content of the soil was not detected. Low calcium carbonate content that can be attributed to the high precipitation of the area implies that the soil buffering capacity is low and does not need high amount of acidic soil amendment to reduce soil pH. Supporting our initial assumptions on high pH soils, soil was poor in total carbon, nitrogen and sulphur, with 1.75, 0.12 and 0.004 percent, respectively. This could lead to their shortage for plants.

Table 1
Selected physicochemical properties of Bintang Series soil
(means ± SD, n=3)

Soil property	Unit	Value or Concentration
pH(H_2O)	-	7.51±0.1
$CaCO_3$	%	Tr
Total C	%	1.75±0.05
Total N	%	0.12±0.01
Total S	%	0.004±0.01
C/N	-	16.58± 1.2
C/S	-	437.50± 1.26
CEC	$cmol_+ kg^{-1}$ soil	11.50±0.35
BS	%	56.0 ± 2.0
Texture	-	Silt loam
FC	%	20.00±0.84

Tr: Traces; BS; Base Saturation, FC; Field Capacity, Ex.; Exchangeable

Effects of Elemental S on Soil pH

As it was hypothesised, soil pH was greatly affected by sulphur application rates and timing (Table 2). For instance, incubation of soil for 40 days with sulphur application rates of 0.5, 1 and 2 g kg^{-1} soil before planting decreased the pH from the background of 7.51 to 6.66, 5.45 and 4.8, respectively. In addition, soil pH was significantly affected by growth stages (Table 2). Averaged across timing, the values of soil pH for sulphur application rates of 0, 0.5, 1 and 2 g S kg^{-1} soil were 7.45, 6.89, 6.31 and 5.86 at planting and 6.93, 6.29, 5.26 and 3.94 at harvest, respectively. The dependence of soil pH to incubation time and growth stage showed that oxidation of elemental sulphur was time consuming and that incubation time of 20 days was not enough for complete oxidation of applied S in this study. As it

can be seen from the Table 2 that there is no significant difference in soil pH between incubation times for all sulphur application rates at harvest. This result indicates that elemental sulphur had been totally oxidised to sulphate at harvest under conditions of this experiment.

Interestingly, soil pH for treatments not receiving elemental sulphur was significantly different during the growing season. Averaged across timing, the figure was 6.93 at harvest and 7.45 before planting. This can be attributed to low buffering capacity of Bintang series soil, irrigation and fertiliser management and the interactions between soil and plant during the growing season. The soil pH dependence to timing and growth stages for un-treated soil can be attributed to low buffering capacity of Bintang series soil, irrigation and fertiliser management and the interactions between soil and plant during the growing season. This issue was elucidated by Bolan et al. (2003), who reported a decrease in soil pH in soils with low buffering capacity due to generation of H^+ through C, N and S.

In order to drive a method for predicting the likely outcome of S addition in Bintang Series soil, the relationship between sulphur rate and soil pH was modelled (see Figure 1). Regarding the soil pH at harvest, the relationship between soil pH and sulphur application rate was linear, pH = 6.94 – 1.52 S and $R^2 = 0.98^{**}$. In other words, with each unit increase in S rate, soil pH decreased by around 1.52 units. Averaged across timing, soil pH was 7.03, 6.29, 5.26 and 3.94 for sulphur application rates of 0, 0.5, 1 and 2 g S kg^{-1} soil, respectively. In line with our results, Owen et al. (1999) reported the linear decrease in soil pH, from 7 to 4.8, by application of elemental sulphur up to 4 tons per ha in a laboratory study. In addition, the relationship between S rate and soil pH for S application range of 0 to 12 tons per hectare was fitted best by exponential model. It should be noted that the relationship between S rate and soil pH change is of special interest and needs to be studied for each specific soil.

Table 2

Soil pH changes in response to elemental sulphur timing (0, 20 and 40 days application before planting) and application rates (g S kg^{-1} soil) at planting and at harvest.

Sulphur rate	Soil pH							
	At planting				At harvest			
	0	20	40	Mean	0	20	40	Mean
0	7.51Aa	7.44Aab	7.42Ab	7.45Aa	6.99Aa	6.92Aa	6.88Aa	6.93Ab
0.5	7.26Ba	6.75Bb	6.66Bb	6.89Ba	6.30Ba	6.23Ba	6.34Ba	6.29Bb
1	7.22Ca	6.27Cb	5.45Cc	6.31Ca	5.35Ca	5.27Ca	5.17Ca	5.26Cb
2	7.34Ca	5.44Db	4.80Db	5.86Da	3.90Db	3.86Db	4.06Da	3.94Db

Means within column followed by the same capital letter and means within rows followed by the same small letter are not significant at the 0.05 level, according to DMRT test at 5% level.

Effects of Elemental S and Soil Acidity on Soil Phosphorous Release

Extractable P was greatly affected by sulphur application rate and maize growth stage (Table 3). Averaged across S timing, application of elemental sulphur increased labile P concentration in soil solution at planting from the background of 0.13 to 0.27 and 0.47 mg kg^{-1} for third and fourth sulphur application rates, respectively. In other words, application of 1 and 2 g S kg^{-1} of soil increased labile P concentration by 145 and 318 percent compared

to the untreated soil at planting. As stated by Ye et al. (2011), the release of P associated with Ca, Al and Fe due to pH reduction could be the primary mechanism by which elemental S application increased P availability at planting. The replacement of PO_4 with SO_4 from exchangeable surfaces is another mechanism responsible for the increased P concentration. However, the increased P availability at planting decreased toward the end of the growing season.

Indicating the transitory effects of elemental sulphur on phosphorous availability, P concentration for untreated soil decreased by 85 percent from planting to harvest and that of treated soils decreased to undetectable amounts at harvest. In line with our finding, the limited long-term effects of sulphur on P availability have been reported by Modaihsh et al. (1989) and Ye et al. (2011). Modaihsh et al. (1989) reported that the incubation of soil with elemental S up to 18 weeks significantly decreased $NaHCO_3$ extractable P. Ye et al. (2011) explained their observation by leaching and runoff, and these two mechanisms can be ignored in the present study as bottom-closed pots were used. This observation can be explained by the conversion of labile P to non-labile forms such as Ca, Al, Mn and Fe bound forms over time and plant uptake (Devau et al., 2009). As concentrations of these elements had increased from planting to harvest (data was not shown), the re-precipitation or adsorption of P by Ca, Mg,
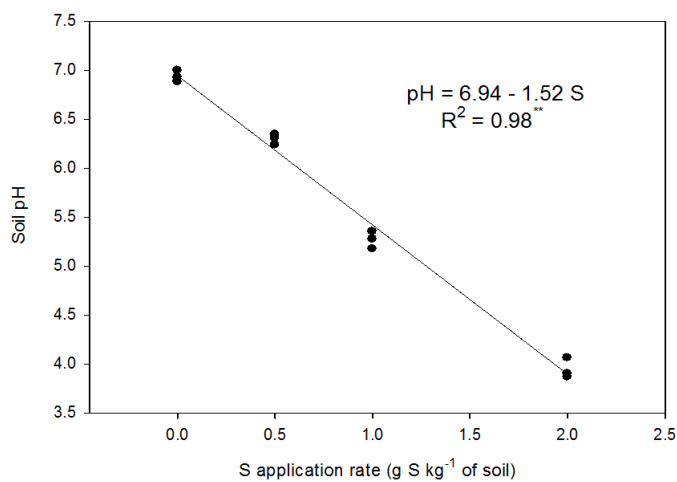
Figure 1. Soil pH changes in response to elemental sulphur application rate.

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Mn, Fe and Al could be considered as the possible reason for lower P at harvest in our conditions. The importance of Ca activity on the solubility of P has been stated by Foth and Ellis (1988). They showed that with increasing Ca activity, the solubility of P from rock phosphate had decreased. It is also known that Ca activity may not be closely related to pH. For this reason, the consequences of using rock phosphate even in very acid soils are sometimes unpredictable. Adhami et al. (2007) also reported the association of P with Mn. Using x-ray adsorption near edge structure (XANES) spectroscopy, the existence of Ca phosphates in acid soils and Al phosphates in calcareous soils has been reported by Harrell (2005). This conclusion is more supported by the negative and significant correlation between extractable P and all nutrients, except that of K, Mn and Mg (data were not shown). It indicates the precipitation of P by nutrients such as Ca and Al. The decreased concentration of P with increased S rate at harvest can be attributed to its adsorption by soil minerals as previously described (Lumsdon, 2012). The decrease in available

P and exchangeable Ca by soil acidification was reported by Owen et al. (1999). Bolan et al. (2003) also reported the precipitation of P with increases in Fe and Al concentrations due to soil pH reduction. Using geochemical modelling for a better understanding of soil process, Devau et al. (2009) showed that iron-oxides and gibbsite were the predominant P-adsorbing soil constituents at acidic and alkaline conditions, whereas P was mainly adsorbed by clay minerals at intermediate pH values.

In addition, there was no specific relationship between soil pH and P concentration under conditions of our experiment. The complexity of the solubility of P was previously documented by Jones et al. (2005) who had demonstrated that the availability of P is highly dependent upon soil pH and that the maximum availability could be obtained at pH 6.5. In neutral to high pH soils, the available P concentration is largely controlled by the solubility of P minerals that are dominated by calcium phosphates (Ca-P). However, at pH levels below 6, it is controlled by Al and Fe phosphates (Al-P and Fe-P). The poor

Table 3
Soil P changes in response to elemental sulphur timing (0, 20 and 40 days application before planting) and application rates (g S kg^{-1} soil at planting and harvest.

Sulphur rate	Soil P (mg kg^{-1} soil)							
	At planting				At harvest			
	0	20	40	Mean	0	20	40	Mean
0	0.16Ca	0.08Ca	0.15Ba	0.13Ca	Tr	0.04a	0.03Aa	0.02Ab
0.5	0.09Ca	0.11Ca	0.12Ba	0.11Ca	Tr	Tr	0.02A	Tr
1	0.32Ba	0.23Ba	0.27Aa	0.27B	Tr	Tr	Tr	Tr
2	0.54Aa	0.55Aa	0.34Ab	0.48A	Tr	Tr	Tr	Tr

Means within column followed by the same capital letter and means within rows followed by the same small letter are not significant at the 0.05 level, according to DMRT test at 5% level. Tr = traces.

negatively significance correlation between P and Al, Cu, Fe, Zn and Ca concentrations (i.e., less than 0.3), under conditions of our study, demonstrates that the solubility of P was complex and controlled by several factors. This is in line with the findings of De la Fuente et al. (2008), who reported the temporary effect for solid olive mill waste on soil nutrient solubility.

Effects of Elemental Sulphur on Nutrient Concentration in Maize

There is a successive decreasing trend between leaves, stem and root P concentration and S rate (Figure 2). It means that with increasing S application rate, the P deprivation has been intensified. As it can be seen, leaf, stem and root P concentrations varied from the maximum of 0.13, 0.12 and 0.073 percent in untreated soil to the minimum of 0.074, 0.086 and 0.06 percent for the soil treated with 2 g S kg^{-1}. Although there is a decreasing trend between S rate and P concentration in maize, the P concentration in stem and roots had the tendency to increase at highest S rate. This can be because of the profound decrease in dry matter production that can result in the increase of P concentration. Our finding is in contrast with the positive effect of elemental S on P concentrations in maize reported by Kayser (2000).

The decreasing trend in P concentration due to sulphur addition can be related to the interactions of P with other nutrients. For instance, the negative effects of Mn and Zn application on P uptake in common bean had previously been reported in glasshouse experiments (Fageria, 2002). Our results indicate that the increase in Zn concentration in maize tends to result in lower leaf, stem and root P concentration (Figure 3). As clearly shown in the graph, there is a negative, strong and linear relationship between P and Zn concentration in all parts of maize. Therefore, it can be concluded that Zn has an antagonistic effect on phosphorous uptake in maize. This conclusion is more supported by the Zn concentration in maize. The concentration of Zn at the sulphur application rates of 0.5, 1 and 2 g S kg^{-1} soil was 103.63, 121.13 and 166.73 respectively and all are more than the adequate range for maize (20-100 mg kg^{-1}), as recommended by Barker and Pilbeam, (2007). The interactions of P and Zn are diverse and have been previously reported by Fageria (2002). For instance, P-induced Zn deficiency because of the high application rates of P fertiliser to soils low in available Zn has been well documented (Marschner et al., 2012). The researchers proposed that Zinc deficiency increases the permeability of the plasma membrane of root cells to P, as well as to Cl and B, and may even lead to B toxicity. In addition, the negative effect of high Zn concentration on P uptake was also reported by Fageria (2002). He showed that with the application of Zn, P uptake in common bean was decreased (Fageria, 2002).

The high Zn concentration in maize under the conditions of our experiment can be attributed to the release of Zn due to the application of elemental sulphur in Bintang Series soil (Table 4). As clearly presented in Table 4, addition of elemental S at a rate of

0.5, 1 and 2g kg^{-1} increased Zn availability more than 7, 49 and 164 times, respectively.

Indicating the negative interaction between Mn and P, there is a downward trend between P and Mn contents of maize leaves, stem and root (Figure 4). As can be seen, with increasing Mn concentration in stem and leaves (up to 300 and 500 mg kg^{-1} dry weight, respectively), the P concentration decreased. However, with further increase in Mn concentrations (i.e., up to 500 and 800 mg kg^{-1} dry weight in stem and leaves, respectively), P concentration tended to increased. This slight increase in P concentration, in spite of the increased

Table 4
The availability of soil Zn and Mn (mg kg^{-1}) in response to elemental sulphur application rate.

Sulphur rate (g kg^{-1} soil)	Nutrient concentration (mg kg^{-1})	
	Mn	Zn
0	1.61 D	0.030 C
0.5	7.26 C	0.20 C
1	26.67 B	1.47 B
2	73.41 A	4.94 A

†Means within column followed by the same letter are not significant at the 0.05 level, according to Tukey test. Values denoted the means across incubation time.
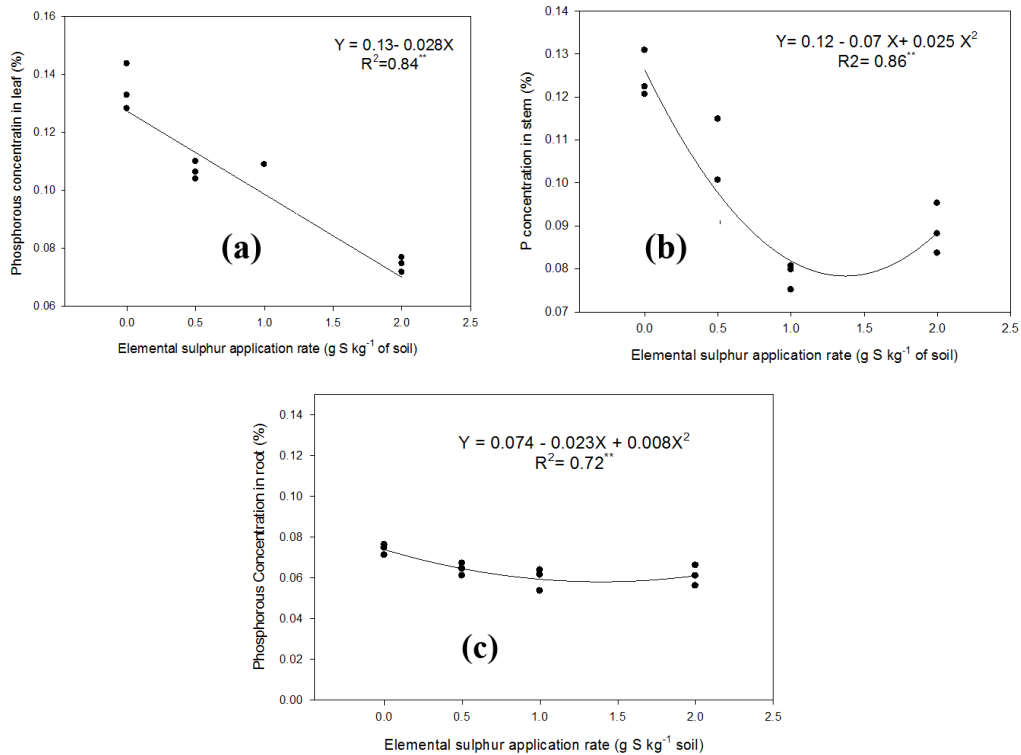
Figure 2. Effect of elemental sulphur on phosphorous concentration in maize leaves (a), stem (b) and root (c).

Mn concentration and its toxicity effects, can be explained by the biomass reduction at highest sulphur application rate synchronised with the highest Mn concentration. The antagonistic effects of Mn and P observed under conditions of our experiment are more supported by the Mn concentration in maize. The concentration of Mn at the sulphur application rates of 0.5, 1 and 2 g S kg^{-1} soil was 81.69, 199.68 and 691.72 respectively, and all are more than the adequate range for maize (50-160 mg kg^{-1}) recommended by Barker and Pilbeam, (2007). The antagonistic effects of Mn and P observed under conditions of our experiment are in line with the previous findings.

For instance, the negative effect of Mn and Al toxicity on P uptake was reported by Bolan et al. (2003). Known as lime-induced P-sparing effect, they reported that soil alkalinisation can decrease Mn and Al toxicity and increase P uptake. It should be noted that the increased Mn concentration in maize leaves, stem and root can be explained by the significant increase in soil Mn concentration due to addition of elemental sulphur (Table 4). As can be seen, with application of elemental sulphur at the rates of 0.5, 1 and 2g kg^{-1}, Mn availability increased more than 4, 16 and 45 times, respectively.

Effect of Elemental Sulphur on Maize growth

Maize leaf, stem and root dry matter production was significantly affected by sulphur application rate (Figure 5). In terms
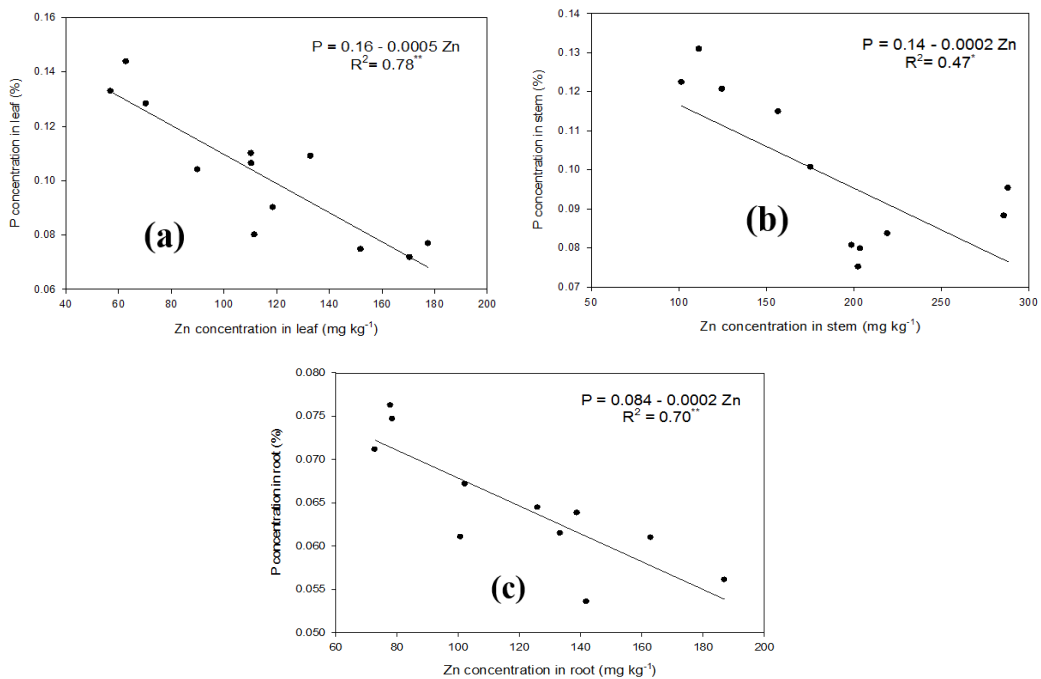
Figure 3. Interaction between P and Zn concentration in maize leaves (a), stem (b) and root (c).

of maize leaves, with the increasing S rate from 0 to 0.5 and 1 g kg⁻¹ soil, the leaves biomass production increased by 29.11 and 25.66 percent, respectively. As the leave biomass at S rate of 1 g kg⁻¹ soil was equal to 97.32 percent of that at S rate of 0.5 g kg⁻¹ soil, it seems that the maximum leave production can be achieved at S application range of 0.5 to 1 g kg⁻¹ soil. The similar trend in stem and root production as a function of elemental sulphur application rate was found and illustrated in Figure 5. Being 59 and 44 percent, the increases in stem dry matter production due to application of 0.5 and 1 g S kg⁻¹ soil are greater than leaves. Interestingly, the increases in the root production were also found to be greater than stem production, with 81 and

69 percent for S rates of 0.5 and 1 g kg⁻¹ soil compared to the un-treated soil.

The negative effect of sulphur application becomes severe at S rate of 2 g kg⁻¹ soil, where the maximum sulphur had been applied. This is mainly due to the fact that there is P deficiency in all the treatments. Thus, it should be noted that the value of P is smaller than 0.3 percent, indicating a P deprivation (Barker et al., 2007).

Based on the results of soil analysis (Karimizarchi et al., 2014), the deficiency of P in plants grown in Bintang Series soil was previously predicted. Therefore, the soil was provided with phosphorous fertiliser. However, it seems that more P fertiliser is needed. As stated by Hinsinger et al. (2008),
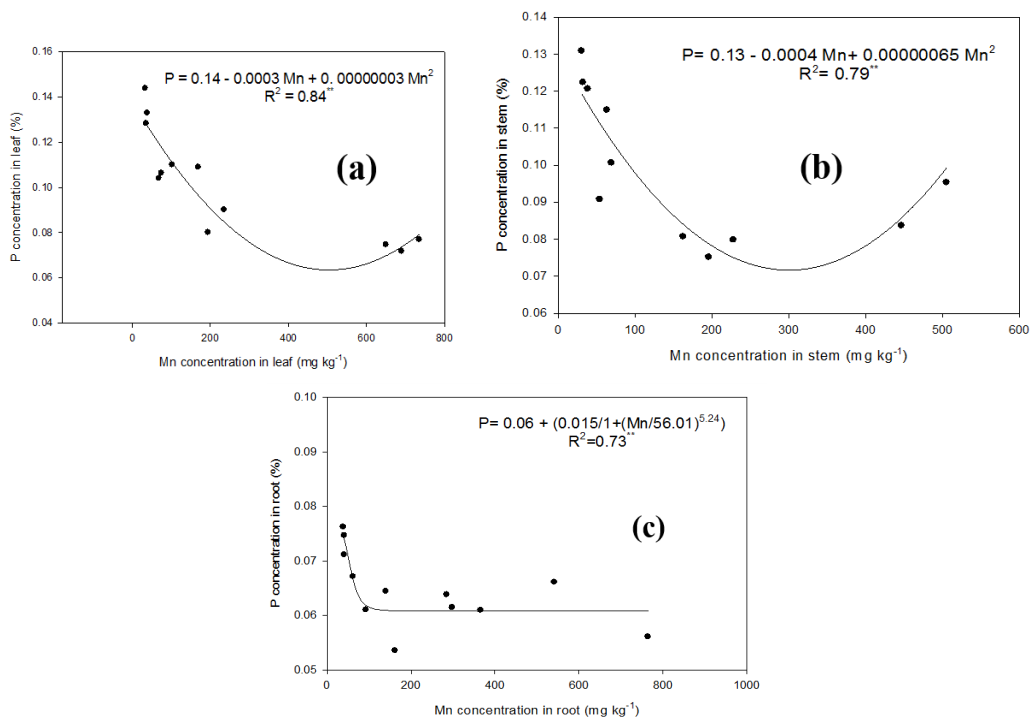
Figure 4. The interaction between Mn and P concentration in maize leaves (a), stem (b) and root (c).
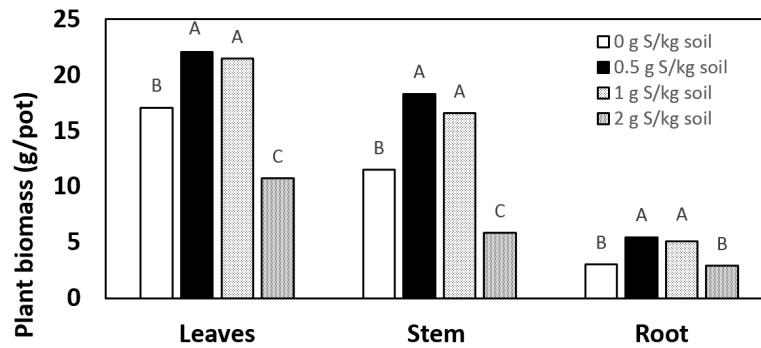
Figure 5. Effect of elemental sulphur application rates (0, 0.5, 1 and 2 g S kg^{-1} soil) on leaf, stem and root dry weight in maize. Means with the same plant part (leaves, stem or root) with a common letter are not significantly different at the 5% level based on DMRT.

the bioavailability of P and K, known as poorly mobile nutrients, depends on the nutrient availability in the soil (including both concentration as well as buffer power). As soil acidification intensified P deficiency in plants and decreased available P in soil, employing foliar application of P can be considered as another option to rapidly alleviate P deficiency. At the same time, enrichment of soil P pools is recommended. This issue can be considered as a future direction.

CONCLUSION

As application of elemental sulphur up to 1 g S kg^{-1} of soil improved maize performance and alleviated S deficiency; thus, it can be used as a soil amendment for crop production. However, as acidification of Bintang Series soil by elemental sulphur decreased available P and reduced P concentration in maize, it can be concluded that the application of elemental sulphur should be accompanied by external sources of P fertilisers for maximising maize production.

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Natural Product Compounds from *Calophyllum depressinervosum*

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ABSTRACT

Our detailed study on the phytochemistry of the stem bark of *Calophyllum depressinervosum* resulted in the isolation of four xanthones and one coumarin. The xanthones are trapezifolixanthone (1), macluraxanthone (2), ananixanthone (3), caloxanthone C (4) and the coumarin calonolide E2 (5). The structures of these compounds were elucidated using spectroscopic analysis such as 1D and 2D-NMR, GCMS, IR and UV .

Keywords: Natural product compounds, *Calophyllum depressinervosum*

INTRODUCTION

The genus *Calophyllum* comprises 180-200 tree species, which are distributed in the tropical rain forest with some occurring in Malaysia (Cechinel et al, 2009). *Calophyllum depressinervosum* species is one species from this genus which grows abundantly in Malaysia. This species is also known as *Bintagor lekok* by local Malaysians (Whitmore

et al., 1973). Previous phytochemical studies have shown that the *Calophyllum* genus is a valuable source of secondary metabolites such as xanthones, coumarins chromenes and flavonoids (Ee et al., 2006). These secondary metabolites have also been shown to give good bioactivities against the HIV virus and they possess good anti-proliferative activities against cancer cell lines (Mah et al., 2015). Many *Calophyllum* species are economically important for the timber industry especially for housing, shipbuilding, furniture, etc. (Sarangwood et al., 2009). This paper reports detailed structural elucidation of trapezifolixanthone (1) and spectroscopic data for macluraxanthone (2), anixanthone (3), caloxanthone C (4) and calanolide E2 (5).

EXPERIMENTAL

Plant Material

The stem bark of *Calophyllum depressinervosum* was collected from the Sri Aman district in Sarawak, Malaysia, and identified by Associate Professor Dr. Rusea Go from the Biology Department, Universiti Putra Malaysia. A voucher specimen was deposited in the herbarium of Biology Department, Faculty of Science, Universiti Putra Malaysia.

General

Infrared spectra were measured using universal attenuated total reflection (UATR) technique on Perkin-Elmer 100 Series FT-IR spectrometer. EIMS were recorded on a Shimadzu GCMS-QP 5050A spectrometer (column, SGE BPX5 30meter x 0.25 mm I.D x 0.25µm film thickness, temperature, 200ºC). The NMR spectra were obtained using a JEOL 500MHz FTNMR spectrometer using $CDCl_3$ as a solvent and tetramethylsilane (TMS) as internal standard. The UV spectra were recorded in EtOH on a shimadzu UV-160A, UV-Visible Recording Spectrophotometer. The melting points were obtained on a Leica Galen III instrument.

Extraction and Isolation

The dry and powdered stem bark of *Calophyllum depressinervosum* (2.1kg) was extracted three times by soaking in hexane at room temperature for 72 hours. The same procedure was repeated for another solvent, i.e. dichloromethane. Each extract was dried under reduced pressure using a rotary evaporator to obtain the hexane (27.7g) and dichloromethane (26.8g) extracts. These extracts were chromatographed in a silica gel glass column under vacuum using a stepwise gradient system (hexane/dichloromethane, dichloromethane/ethyl acetate and ethyl acetate/methanol). Further purifications of the hexane extract using another silica gel column (gravity) gave trapezifolixanthone (1) (98.9mg), macluraxanthone (2) (157.6mg), ananixanthone (3) (90.7mg) and calanolide E2 (5) (19.4mg). Meanwhile, further purifications on the dichloromethane extract using gravity silica gel column chromatography gave caloxanthone C (4) (16mg).

Trapezifolixanthone (1). Yellow needle crystal; m.p 161-163ºC (literature 140-142ºC, Daud et al., 2014). UV λmax : 3196, 1629, 1575, 1127 (EtOH) nm : 287, 260, 241, 210 EI-MS m/z: 378, 363, 154. ^{1}H NMR (500MHz, $CDCl_3$ and ^{13}C NMR (125MHz, $CDCl_3$) (Table 1).

Macluraxanthone (2). Yellow needle crystal; m.p 170-174ºC (literature 170-172ºC, Iinuma et al., 1994). UV $λ_{max}$: 3417, 2971, 1582, 1193 (EtOH) nm : 346, 295, 251, 204 EI-MS m/z: 394, 379, 339, 162. ^{1}H NMR (500MHz, $CDCl_3$): δ13.89 (s, 1-OH), δ7.57 (d , 1H, *J*=8.05Hz, H-8), δ6.97 (d, 1H, *J*=8.05Hz, H-7), δ6.67 (d, 1H, *J*=9.10Hz, H-10), δ6.50 (dd, 1H, *J*=10.8&17.2Hz, H-2'), δ5.69 (d, 1H, *J*=9.10Hz, H-11), δ5.02 (d, 1H, *J*=17.2Hz, H-3b'), δ4.86 (d, 1H, *J*=10.8Hz, H-3a'), δ1.71 (s, 6H, H-4'&H-5'), δ1.46 (s, 6H, H-13&H-14). ^{13}C NMR (125 MHz, $CDCl_3$) δ180.9 (C-9),

δ158.6 (C-1), δ156.1 (C-4a), δ155.2 (C-3), δ151.7 (C-2'), δ151.1 (C-5), δ145.9 (C-5a), δ132.9 (C-6), δ127.5 (C-11), δ116.2 (C-8), δ115.5 (C-10), δ113.5 (C-8a), δ113.4 (C-4), δ112.9 (C-7), δ106.6 (C-3'), δ104.8 (C-2), δ102.8 (C-9a), δ78.2 (C -12), δ41.0 (C-1'), δ29.2 (C-4'&C-5'), δ27.2 (C-13&14).

Anixanthone (3). Yellow needle crystal; m.p 168-170ºC (literature 170-171ºC, Joaquim et al., 1998). UV λ$_{max}$: 3217, 2918, 1573 (EtOH) nm : 302, 283, 261 EI-MS m/z: 378, 363, 335, 154. ^{1}H NMR (500MHz, CDCl$_3$): δ13.22 (s, 1-OH), δ7.78 (d, 1H, *J*=8.02 Hz, H-8),7.30(d,1H, *J*=8.02 Hz, H-6), δ7.23 (t, 1H, *J*=8.02, H-7), δ6.79 (d, 1H, *J*=10.3Hz, H-10), δ5.65 (d,1H, *J*=10.3Hz, H-11), δ5.25 (t, 1H, *J*=6.87Hz, H-2'), δ3.36 (d, 2H, *J*=6.87Hz, H-1'), δ1.81(s, 3H, H-4'), δ1.68 (s, 3H, H-5'), δ1.49 (s, 6H, H-13&H-14). ^{13}C NMR (125 MHz, CDCl$_3$) δ180.8 (C-9), δ160.6 (C-1), δ158.6 (C-3), δ149.3 (C-4a), δ144.3 (C-8a), δ144.1 (C-10a), δ131.7 (C-3'), δ127.5 (C-11), δ124.0 (C-7), δ 121.9 (C-2'), δ121.2 (C-5), δ120.1 (C-6), δ117.2 (C-8), δ115.0 (C-10), δ112.3 (C-2), δ103.2 (C-9a), δ100.7 (C-4), δ78.1 (C-12), δ28.2 (C-14&C-13), δ25.8 (C-5'), δ21.3 (C-1'), δ18.0 (C-4').

Caloxanthone C (4). Yellow needle crystal; m.p 211-213ºC (literature 217ºC, Iinuma et al.,1994). UV λ$_{max}$: 3436 ,2935, 1600, 1594, (EtOH) nm : 387, 294, 284, 233 EI-MS m/z: 378, 363, 335, 154. ^{1}H NMR (500MHz, CDCl$_3$): δ13.42 (s, 1-OH), δ7.72(d, 1H, *J*=9.15 Hz, H-8), δ7.23 (t, 1H, *J*=9.15Hz, H-7), δ7.20 (d, 1H, *J*=9.15Hz, H-6), δ6.78 (d, 1H, *J*=10.3Hz, H-10), δ6.77 (dd, 1H, *J*=10.3&17.2Hz, H-2'), δ5.63

(d, 1H, *J*=10.3Hz, H-11), δ5.24 (d, 1H, *J*=17.2Hz, H-3b'), δ5.07 (d, 1H, *J*=10.3Hz, H-3a'), δ1.64 (s, 6H, H-4'&H-5'), δ1.51 (s, 6H, H-13&H-14). ^{13}C NMR (125 MHz, CDCl$_3$) δ181.4 (C-9), δ159.4 (C-3), δ156.7 (C-1), δ155.8 (C-2'), δ154.0 (C-4a), δ145.4(C-5), δ144.2 (C-5a), δ127.4 (C-11), δ124.2 (C-7), δ120.5 (C-8a), δ119.7 (C-6), δ116.1 (C-8), δ116.0 (C-10), δ113.2 (C-4), δ105.6 (C-2), δ104.1 (C-3'), δ103.6 (C-9a), δ78.4 (C-12), δ41.4 (C-1'), δ28.3 (C-4'&C-5'), δ28.0 (C-13&C-14).

Calanolide E2 (5). Yellowish oil. UV λ$_{max}$: 2952, 1624, 1128 (EtOH) nm : 311, 297, 281, 240 EI-MS m/z: 388, 329, 271, 215, 107. ^{1}H NMR (500MHz, CDCl$_3$): δ12.37 (s, 7-OH), δ6.59 (d, 1H, *J*=10.31Hz, H-9), δ5.45 (d, 1H, *J*=10.31Hz, H-10), δ4.50 (m, 1H, H-3'), δ3.68 (m, 1H, H-4), δ2.84 (dd, 1H, *J*=9.16&14.89Hz, H-3a), δ2.67 (dd, 1H, *J*=9.16&14.89Hz, H-3b), δ2.53 (m, 1H, H-2'), δ1.81 (m, 1H, H-14a), δ1.46 (m, 1H, H-14b), δ1.43 (s, 3H, H-12), δ1.35 (s, 3H, H-13), δ1.34 (d, 3H, *J*=5.73Hz, H-4'), δ1.15 (m, 2H, H-15), δ1.12 (d, 3H, *J*=6.87Hz, H-5'), δ0.85 (t, 3H, *J*=6.87Hz, H-16). ^{13}C NMR (125 MHz, CDCl$_3$) δ201.1 (C-1'), δ179.3 (C-2), δ160.1 (C-8a), δ159.9 (C-5), δ157.3 (C-7), δ125.7 (C-10), δ115 (C-9), δ108 (C-4a), δ102.6 (C-6), δ101.0 (C-8), δ78.2 (C-11), δ76.1 (C-3'), δ44.2 (C-2'), δ38.6 (C-3), δ35.5 (C-14), δ30.5 (C-4), δ28.5 (C-13), δ28.1 (C-12), δ20.8 (C-15), δ16.3 (C-4'), δ14.0 (C-16), δ9.3 (C-5').

RESULTS AND DISCUSSION

Trapezifolixanthone (1) (98.9 mg) was isolated as yellow needle crystals with

a melting point of 145-148ºC (literature 140-142ºC, Daud et al., 2014). The EIMS spectrum showed a molecular ion peak at 378, which is consistent with the molecular formula $C_{23}H_{22}O_5$. The ion fragment peak at m/z 363 was due to the loss of a methyl group. The fragment ion peak at m/z 154 was due to the loss of a pyrano ring and the prenyl group that are attached to the xanthone skeleton.

The FTIR spectrum for compound 1 gives typical IR absorptions for xanthones at 3196 cm^{-1}, 1629 cm^{-1}, 1575 cm^{-1}, and 1127 cm^{-1}. The strong absorptions at 3196 cm^{-1} and 1629 cm^{-1} were representative for hydroxyl and conjugated carbonyl stretching. Meanwhile, the absorption at 1575 cm^{-1} was due to the stretching of an aromatic group.

The ^{1}H NMR spectrum for compound 1 exhibited the presence of one chelated hydroxyl group at δ13.05 (OH-1). The presence of a 3-methylbut-2-enyl substituent in compound 1 was indicated by the ^{1}H NMR signals at δ5.23 (t, 1H, *J*=5.73Hz, H-2'), δ3.49 (d, 2H, *J*=5.73Hz, H-1'), δ1.85 (s, 3H, H-5') and δ1.71 (s, 3H, H-4'). In the COSY experiment, the nature of the allylic coupling systems within the prenyl moiety was clearly demonstrated. It showed COSY couplings between the olefinic proton C-2' and the benzylic proton of C-1'. The signal at δ3.49 (H-1') in the proton NMR showed a long range coupling with the carbon signal at δ107.1 (C-4) , δ153.8 (C-4a) and δ158.3(C-3) in the HMBC spectrum hence confirming the prenyl unit to be positioned at C-4.

The ^{1}H NMR spectrum for compound 1 also revealed the presence of a pyrano ring attached to the xanthone skeleton. The ring ^{1}H NMR spectrum shows a pair of ortho-coupled proton with a coupling constant value of 10.3Hz at δ6.74 and δ5.61 for H-11and H-10. The HMBC spectrum shows a cross peak for the proton signal at δ1.47 (H-13 and H-14) with the carbon signal at δ78.4 (C-12). This indicates the two methyls to be directly bonded to the carbon at δ78.4 (C-12). The ^2J correlation of the proton at δ5.61 (H-10) with the carbon signal at δ104.8 (C-2) suggests the pyrano ring to be attached to the carbons at δ104.8 (C-2) and δ158.3 (C-3).

The ^{13}C NMR and DEPT spectra for (1) exhibited 23 carbon signals, which consist of four methyls, one methylene, six methines and eleven quaternary carbons including one carbonyl signal at δ181.1 (C-9). The carbons at δ181.1 (C-9), δ158.3 (C-3), δ156.1 (C-1), δ153.8 (C-4a), δ144.5 (C-5a) and δ144.3 (C-5) were shifted downfield due to the electronegative element oxygen, making them more deshielded. The carbon at δ181.1 (C-9) peak is generally weak due to slow relaxation of the quaternary carbon and is highly deshielded.

The COSY spectrum for (1) shows protons coupled to each other, indicating the positions of adjacent protons. One doublet and triplet signals with coupling constant 8.02Hz were observed at δ7.73 (H-8) and δ7.23 (H-7) indicating the ortho-coupled proton in a benzene ring. The meta-coupled protons in the left benzene ring at δ7.73 (H-8) and δ7.28 (H-6) signal were assigned to
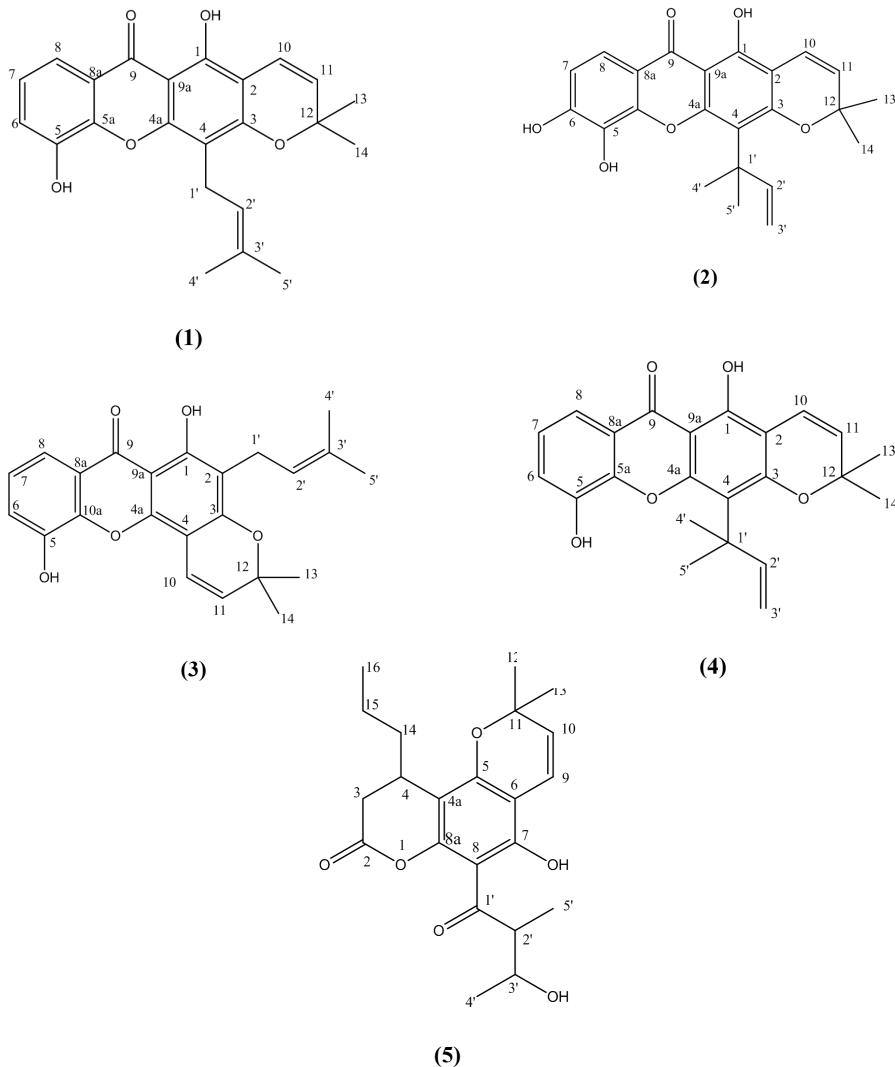
Figure 1. Structures of xanthones and coumarin

their respective carbons at signals δ116.9 (C-8) and δ119.9 (C-6) via the HMQC spectrum.

Based on the information given by the 1D and 2D NMR data, compound 1 was identified as trapezifolixanthone previously isolated from *Calophyllum hosei* (Daud et al., 2014).

Table 1
^1H NMR (500 MHz, CDCl$_3$) and ^{13}C NMR (125 MHz, CDCl$_3$)

Position	δ_H	δ_C	HMBC, δ
1	13.05 (s, OH)	156.1	156.1 (C-1), 104.8 (C-2)
2		104.8	
3		158.3	
4		107.1	
4a		153.8	
5	5.74 (s, OH)	144.3	144.3 (C-5), 119.9 (-6)
5a		144.5	
6	7.28 (d, 1H, J = 8.02Hz)	119.9	144.3 (C-5), 116.9 (C-8)
7	7.23 (t, 1H, J = 8.02Hz)	124.0	144.3 (C-5), 120.9 (C-8a)
8	7.73 (d, 1H, J = 8.02Hz)	116.9	144.5 (C-5a), 119.9 (C-6)
8a		120.9	
9		181.1	
9a		103.1	
10	5.61 (d, 1H, J = 10.3Hz)	127.6	104.8 (C-2), 78.4 (C-12)
11	6.74 (d, 1H, J = 10.3Hz)	115.8	158.3 (C-3), 78.4 (C-12)
12		78.4	
13	1.47 (s, 6H)	28.4	127.6 (C-10), 78.4 (C-12)
14			28.4 (C-13&C-14)
1'	3.49 (d, 2H, J = 5.73Hz)	21.8	158.3 (C-3), 153.8 (C-4a), 131.7 (C-3'), 122.7 (C-2'), 107.1 (C-4)
2'	5.23 (t, 1H, J = 5.73Hz)	122.7	25.7 (C-4'), 18.0 (C-5')
3'		131.7	
4'	1.71 (s, 3H)	25.7	131.7(C-3'),122.7(C-2'), 18.0(C-5')
5'	1.85 (s, 3H)	18.0	131.7(C-3'),122.7(C-2'), 25.7(C-4')

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Seroprevalence and Detection of Contagious Bovine Pleuropneumonia (CBPP) in Northeast States of Peninsular Malaysia

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ABSTRACT

Contagious Bovine Pleuropneumonia (CBPP) is a highly infectious disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* Small Colony (*Mmm*SC). It is a disease recognised by OIE that needs to be controlled or eradicated through surveillance system. This study establishes the sero-prevalence of CBPP, as well as attempts to isolate and identify the agent of CBPP from cattle in Kelantan and Terengganu, the northeastern states of Peninsular Malaysia, where cattle movement was high. A total of 3,242 sera from 428 farms were processed between 2011 and 2014 using the competitive ELISA (c-ELISA). The animal-level prevalence ranged between 5% (46/917) in Terengganu and 9% (220/2325) in Kelantan, whereas the herd prevalence ranged between 12% (22/210) in Terengganu and 24% (53/218) in Kelantan. The overall animal-level prevalence was 8% (266/3242), while the herd prevalence was 17% (75/428). Two hundred and four nasal swabs from 18 positive herds in Kelantan and 163 lung and mediastinal lymph node samples were processed for isolation of *Mmm*Sc before confirmation by PCR and immunoperoxidase. Forty-one samples showed turbidity in PPLO broth and 15 lung and mediastinal lymph node samples had 'fried-egg colony' growth on the PPLO agar, which were suggestive of *Mycoplasma* infection. However, all were negative for *Mmm*SC by PCR and immunoperoxidase. Thus, the northeastern part of Peninsular Malaysia remains positive serology for CBPP without isolation.

INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is a highly infectious acute, sub acute and chronic disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* Small Colony (*Mmm*SC) (Okaiyeto et al*.,* 2011; Schubert et al*.,* 2011; OIE 2014a, 2014b). CBPP is one of the diseases recognised by OIE that needs to be controlled or eradicated through a national surveillance protocol (OIE, 2014a). Disease transmission requires close contact up to 200 metres, particularly during animal movements (Okaiyeto et al*.,* 2011; Schubert et al*.,* 2011; OIE 2014a, b). CBPP is currently important in many parts of Africa, but the status in Asia is uncleared (OIE, 2014a).

The standard test for screening of CBPP is the Campbell and Turner complement fixation test (CFT) or competitive enzyme-linked immunosorbent assay (c-ELISA) (OIE, 2014b). The findings can be used to make reliable estimates on the distribution of the CBPP, which enables the implementation of a national CBPP control programme. Isolation usually uses culture media of heart-infusion broth or Bacto pleuropneumonia-like organisms (PPLO), while identification can be done by biochemical and immunological tests, and the polymerase chain reaction (PCR) (Bashiruddin et al., 1994; FAO, 2002; Swai et al*.,* 2013; OIE, 2014b). This study uses the serological test to estimate the prevalence of CBPP in the northeastern part of Peninsular Malaysia before positive animals were sampled for isolation and identification of *Mmm*SC.

MATERIALS AND METHODS

Sampling

A total of 3,242 serum samples from 428 cattle farms, both private and government farms, were collected from Kelantan and Terengganu located at the northeastern part of Peninsular Malaysia for the CBPP National Surveillance Programme. Kelantan and Terengganu recorded the most number of cattle and most number of animal movements in Peninsular Malaysia, while none of the farms had programme against bovine pleuropneumonia. A total of 2,325 serum samples were taken from 218 herds in Kelantan and 917 samples were extracted from 210 herds in Terengganu. The samples were subjected to CBPP competitive enzyme-linked immunosorbent assay (c-ELISA) kit to detect the specific antibodies to *Mycoplasma mycoides* subspecies *mycoides* Small Colony (MmmSC) according to the manufacturer's (IDEXX, Switzerland) guidelines.

Following cELISA serological testing, 204 deep nasal swabs from 18 cELISA-positive herds were collected. Furthermore, a total of 163 cELISA-positive cattle were slaughtered for consumption before lesions in the lung, while mediastinal lymph node samples were noted and collected for laboratory investigations. The nasal swabs were collected using the modified Dacron swab before they were swirled into the PPLO broth with supplement and

antibiotics (OIE, 2014a). All the samples were kept refrigerated at 4°C for 24 h or at –20°C for longer storage. The suspected positive cultures showing classical 'fried-egg' appearance were used to identify *Mycoplasma mycoides* cluster using the polymerase chain reaction (PCR). The same lung and lymph node samples were fixed in 10% formalin for immunoperoxidase (IP) studies.

MmmSC *Isolation*

To isolate *Mmm*SC from the organ samples, approximately 1 g of the tissue was minced in broth medium using a sterile scissor before the sample was diluted ten-folds. The sample was then inoculated into 3 ml of broth. The nasal swab was inoculated directly into the broth. Three serial dilutions were made before all the inoculated broths were incubated aerobically at 37°C. During incubation, the broths were checked daily for growth, indicated by a colour change from pink to yellow and appeared cloudy with silky, fragile filaments.

The broth culture was harvested at 3 to 7 days post-inoculation with a sterile syringe and filtered through a 25-mm filter containing 0.45-µm membranes to reduce contaminating bacteria. Approximately 0.2 ml of the filtrate was spread onto agar plates, while another 100 µl was re-introduced into the PPLO broth to passage further. The plates were incubated at 37°C in 5% CO_2 incubator. The plates were examined daily with inverted microscope (30X) for the presence of *Mycoplasma* colonies between 3 and 14 days of incubation. The plates were

discarded if there was no growth. On agar medium, the *Mmm*SC colonies appeared small (1 mm in diameter) and had the classical appearance of 'fried eggs' with a dense centre (OIE, 2014b).

Detection of MmmSC *by polymerase chain reaction*

The DNA was extracted by using InstaGene Matrix (Biorad, USA) according to the manufacturer's instructions. Quick Taq® HS DyeMix (Toyobo Life Science, Japan) was used as master mix reagent. The *M. mycoides* species-specific primers MM450 (Sequence: 5'-GTA-TTT-TCC-TTT-CTA-ATT-TG-3') and MM451 (Sequence: 5'-AAA-TCA-AAT-TAA-TAA-GTT-TG-3') were used (OIE, 2014b).

The optimal amplification cycle for both primer sets was performed in a thermal cycler consisted of pre-denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension for 5 min at 72°C and hold at 4°C indefinitely. The 1.5% agarose (Pronadisa, Spain) was prepared and electrophoresis was carried out at 100 volts for 30 min. The amplified product of 574 bp was observed in positive samples by ultraviolet transillumination.

Detection of MmmSC *by immunoperoxidase staining*

The paraffin-embedded tissue samples were sectioned at 3 – 4 µm thick, collected on coated silanized slides, placed in an oven at 60°C for 15 min, and de-parafinised using

xylene and rehydrated by 100% alcohol. A commercially available kit (Nichirei, Japan) was used for the immunoperoxidase staining according to the manufacturer's recommendation. Slides were quenched by soaking in 3% H_2O_2 solution and washed with 1x Phosphate Buffer Solution (PBS) twice. The rehydrated slides were immersed in a citrate buffer solution (Dako, USA) and digested using the autoclave method at 121°C for 10 min. The slides immersed in PBS were then applied 5% goat serum before the primary antibody was applied for 30 min. After washed with Phosphate Buffer Solution-Tween 20 (PBST), the tissues were incubated with a commercial secondary antibody N-Histofine® Simple Stain MAX PO (MULTI) (Universal Immuno-peroxidase Polymer, Anti-Mouse and Anti-Rabbit by Nichirei, Japan) for 30 min at room temperature. Then, 3-amino-9-ethylcarbazole chromogen (AEC) (Nichirei, Japan) was applied for 5 min at room temperature and counter stained with Mayer's Hematoxylin staining solution for 1 min. The immunoperoxidase staining was considered positive if there was a red colored deposit, particularly in macrophage-like cells in the lung and lymph nodes.

RESULTS

Sero-Prevalence

Tables 1 and 2 show the results of c-ELISA on sera collected from Kelantan and Terengganu between 2011 and 2014. Terengganu showed significantly ($p<0.05$) lower herd and animal prevalence. The herd prevalence was 10% in Terengganu and 24% in Kelantan (Table 1), while the animal prevalence was 5% in Terengganu and 9% in Kelantan (Table 2). The overall animal prevalence of CBPP in Kelantan and Terengganu between 2011 and 2014 was 8% (266 of 3242), while the overall herd prevalence was 17% (75 of 428).

Table 1

The prevalence of CBPP among herds in Kelantan and Terengganu between 2011 and 2014

State	No. of herd	No. of positive herd	Prevalence %
Kelantan	218	53	24.31[a]
Terengganu	210	22	10.48[b]
Total	428	75	17.52

[a,b] Different superscripts indicate significant difference ($p<0.05$)

Table 2

The prevalence of CBPP in animals from Kelantan and Terengganu between 2011 and 2014

State	No. of animals	No. of positive samples	Prevalence %
Kelantan	2325	220	9.46[a]
Terengganu	917	46	5.02[b]
Total	3242	266	8.20

[a,b] Different superscript indicates significant difference ($p<0.05$)

In Kelantan, there was an increasing trend in the animal sero-prevalence during the first 3 years (2011 to 2013) of surveillance from 0% in 2011 to 14.95% in 2013. The prevalence, however, decreased to 6.47% in 2014 (Table 3). Terengganu showed slight fluctuating pattern with a drop in prevalence in 2012 and 2014 (Table 4).

Isolation and Identification of MmmSC

Table 5 shows the results of attempted isolation of *Mmm*SC in broth, as indicated by the turbidity in the broths. A total of 41 samples showed broth turbidity; 10 (4.9%) of the 204 samples of nasal swab, 19 (11.6%) of the 163 samples of lungs and 12 (7.4%) of the 163 samples of lymph nodes. The overall percentage of samples showing turbidity was 7.7%.

The 41 turbit broths were subsequently re-inoculated onto agar and these resulted in 15 samples showing growth of small colonies of 1mm in diameter with classical 'fried-egg' appearance of *Mycoplasma.* They were 9 (5.5%) of the lungs and 6 (3.7%) of the lymph node samples. Similarly, the 41 suspected positive samples of turbit broth, which were subjected to PCR, revealed no amplification (Figure 1).

Out of the 163 lung and lymph node samples obtained from the sero-positive animals, two (1.2%) lungs had lesions

Table 3
Yearly prevalence of CBPP in Kelantan

Year	No. of samples	No. of positive samples	Prevalence %
2011	132	0	0
2012	460	12	2.61
2013	1110	166	14.95
2014	623	42	6.47

Table 4
Yearly prevalence of CBPP in Terengganu

Year	No. of samples	No. of positive samples	Prevalence %
2011	113	5	4.42
2012	477	2	0.42
2013	292	36	12.33
2014	35	3	8.57

Table 5
Results of attempted isolation of MmmSC in broth as shown by increased turbidity of the PPLO broth

Type of samples	No. of sample	No. of samples showing turbidity
Nasal swabs	204	10 [4.9%]
Lung tissue	163	19 [11.6%]
Lymph node tissue	163	12 [7.4%]
Total	530	41 [7.7%]

of *Mmm*SC infections, which included widening of the interlobular septa and hepatisation of the lung parenchyma with a mixture of oedematous fluid and fibrin (Figure 2). However, immunoperoxidase did not reveal positive staining.

DISCUSSION

This study revealed that sero-prevalence of CBPP in Malaysia increased each year and this was likely due to animal movements, which happened regularly in the states of Kelantan and Terengganu (Ramanoon et al*.,* 2013). In the infected herds, some sero-positive animals had lung lesions, while others did not show lesions and were identified as *Mmm*SC carriers. The ability to detect antibodies is optimal during the first month but showed decreasing ability in the following 6 months (Schubert et al., 2011; OIE, 2014a, b). Thus, c-ELISA is suitable in an epidemiological study as it is possible to detect new infected herd and estimate the distribution of CBPP for
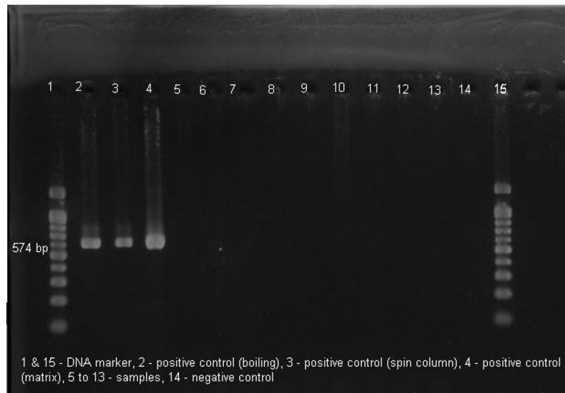
Figure 1. Results of PCR on the suspected samples where none of the samples (Lanes 5-14) were positive while the controls (Lanes 2-4) revealed the 574 bp bands
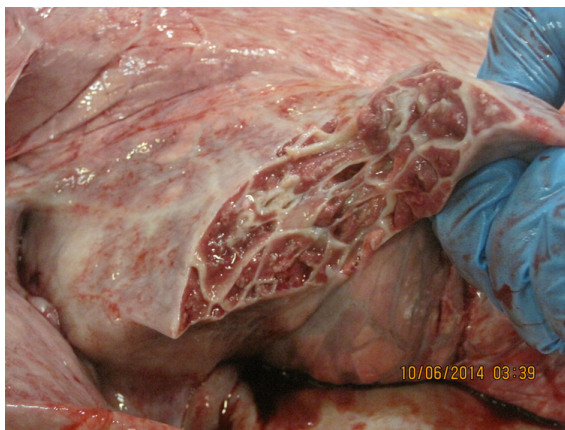
Figure 2. Gross lesions of the affected lung of cattle of suspected contagious bovine pleuropneumonia showing visibly distended interlobular septa

implementation of control programme. Other than the serological surveillance, abattoir surveillance is also suitable to estimate the prevalence of CBPP (Msami, 2009). Nevertheless, a definitive diagnosis is made by isolation and identification of the organism from tissues of sick, freshly dead or euthanised animals (McAuliffe et al., 2003).

The conventional methods of diagnosis are based on culture and serological tests (McAuliffe et al., 2003). This is because there is no single conventional or serological test that can detect all infected animals. On the other hand, PCR can overcome some problems of the conventional tests, particularly issues pertaining to time-consuming, cross-reaction and insensitivity (Ameera et al., 2010). Therefore, suspected positive *Mmm*SC in this study was subjected to PCR since it could detect the *Mmm*SC even though the antibiotic treatment had been done (Taylor et al., 1992; Bashiruddin et al., 1994; Hotzel et al., 1996; Dominique et al., 2004; Woubit et al., 2004; OIE, 2014b). In this study, both PCR and immunoperoxidase staining could not detect *Mmm*SC from any of the suspected positive samples. This might be due to the chronic stage of the disease during sampling (Sadique et al., 2012) and error in the preservation and transportation of samples that reduced the chances of isolating *Mmm*SC (Dominique et al., 2004; Mondal et al., 2004). Furthermore, Chazel et al. (2010) revealed that if there was several *Mycoplasma* species present in one sample, the isolation step tends to prefer the most rapidly growing species

such as *M. bovirhinis* and *M. arginini* and suppress the slower growing that are mostly pathogenic species including *Mycoplasma mycoides* cluster. However, Provost et al. (1987) concluded that the failure might due to the fact that the disease is not present in the country and the pathological findings might have been confused with other causes of pleuropneumonia such as *Pasteurella* species (Stark et al., 1995).

CONCLUSION

In conclusion, this study indicated that low cattle populations in the Northeastern States of Peninsular Malaysia showed sero-prevalence to CBPP. However, none revealed the presence of *Mmm*SC, which was likely due to the absence of this organism in this country.

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—SEPTEMBER 2015

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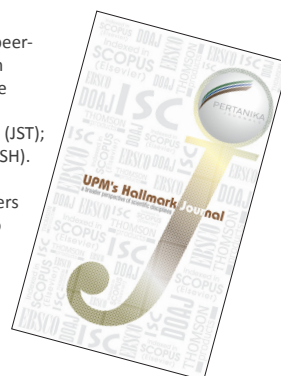
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