Journal of Tropical Agricultural Science

About the Journal

Pertanika is an international peer-reviewed journal devoted to the publication of original papers, and it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields. Pertanika Journal of Tropical Agricultural Science which began publication in 1978 is a leading agricultural journal in Malaysia. After 29 years as a multidisciplinary journal, the revamped Pertanika Journal of Tropical Agricultural Science (JTAS) is now focusing on tropical agricultural research. Other Pertanika series include Pertanika Journal of Science and Technology (JST) and Pertanika Journal of Social Sciences and Humanities (JSSH).

JTAS is published in **English** and it is open to authors around the world regardless of the nationality. It is currently published four times a year, i.e. in **February**, **May**, **August** and **November**.

Goal of *Pertanika*

Our goal is to bring the highest quality research to the widest possible audience.

Quality

We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 12 weeks. The elapsed time from submission to publication for the articles averages 5-6 months.

Indexing of *Pertanika*

Pertanika is now over 33 years old; this accumulated knowledge has resulted in Pertanika JTAS being indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge [BIOSIS & CAB Abstracts], EBSCO, DOAJ, AGRICOLA, ISC, MyAIS and Rubriq.

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.

We also have views on the future of our journals. The emergence of the online medium as the predominant vehicle for the 'consumption' and distribution of much academic research will be the ultimate instrument in the dissemination of research news to our scientists and readers.

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*.

Editorial Statement

Pertanika is the official journal of Universiti Putra Malaysia. The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

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.

Pertanika Journal of Tropical Agricultural Science
Vol. 38 (1) Feb. 2015

Contents

Selected Articles from International Conference on Crop Improvement (ICCI) 2013

Guest Editor: Siti Nor Akmar Abdullah

Foreword

Welcome to the **First Issue 2015** 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 **12 articles**, of which **one** is a short communication and **8** are regular research papers and **3 articles** are from International Conference on Crop Improvement (ICCI) 2013. The authors of these articles are from **Malaysia, Nigeria, Bangladesh** and **Iran**.

In the short communication, researchers from the Department of Chemistry, Faculty of Science, Universiti Putra Malaysia report on a new natural product compound benjaminin from *Calophyllum benjaminum* (*Sahimi, M. S. M., Ee, G. C. L., Mahaiyiddin, A. G., Daud, S., Teh, S. S., See, I.* and *Sukari, M. A*). The first research paper, a collaborative work between Universiti Sains Islam Malaysia, Universiti Kebangsaan Malaysia and International Islamic University Malaysia, documents species-richness and distribution of sea cucumbers (Phylum Echinodermata: Class Holothuroidea) in Malaysia (*Kamarul Rahim Kamarudin, Gires Usup, Ridzwan Hashim* and *Maryam Mohamed Rehan*). The second research paper reports on the inherent variability in growth and development of two ecotypes of the sesame plant (*Sesamum* sp.) in Malaysia (*Tan, S. P., Rosli, B. M., Adam, B. P.* and *M. M. Morshed*). The next research paper from the Federal University of Agriculture, Nigeria, reports on the growth trajectory of the Nigerian indigenous and exotic strains of chicken embryos during incubation under Nigerian conditions (*Oke, O. E., Obanla, L. O., Onagbesan, O. M.* and *Daramola, J. O.*)

In the next research paper, a group of researchers reports on the response to foot and mouth disease (FMD) vaccination among local Malaysian cattle of various vaccination backgrounds from endemic and non-endemic FMD areas (*A. Abbo Hamad, L. Hassan, M. Z. Azmie, P. Loganathan, T. Jaafar, S. S. Arshad, J. Hashim, H. Amir, O. Norlida, M. A. Syarifah Asiah* and *M. M. Salih*).

The next research paper, from Nigeria, reports on the performance, haematological parameters and faecal egg count of semi-intensively managed west African dwarf sheep to varying levels of cassava leaves and peel supplementation *(Fasae, O. A., Amos, A. O., Owodunni, A.* and *Yusuf, A. O.*). The seventh research paper, from Bangladesh, reports on the development of fertiliser recommendation for aquatic taro (*Colocasia esculenta*) in grey terrace soil (*S. Noor, M. R. Talukder, M. K. R. Bhuiyan, M. M. Islam, M. A. Haque* and *S. Akhter*). The next research paper, which is from Universiti Putra Malaysia and it

reports on lithium levels in coastal areas of Peninsular Malaysia, is an assessment based on mangrove snail *Nerita lineata* and surface sediments (*Wan Hee Cheng, Chee Kong Yap, Mohamad Pauzi Zakaria, Ahmad Zaharin Aris* and *Tan Soon Guan*) and the last research paper reports on the potent antioxidative components from Langsat (*Lansium domesticum*) peel (*Kee, M. E., Khoo, H. E., Sia, C. M.* and *Yim, H. S.*).

I conclude this issue with three articles arising from the International Conference on Crop Improvement (ICCI) 2013: screening for optimum concentrations of boron, copper and manganese for the growth of three-month-old oil palm seedlings in a solution culture (*Tengoua, F. F., Hanafi, M. M., Idris, A. S.* and *Syed-Omar, S. R.*); genetic divergence and evaluation of yield potential of *Jatropha curcas* accessions collected from Peninsular Malaysia (*Arolu, I. W., Rafii, M. Y., Hanafi, M. M., Mahmud, T. M. M.* and *Askani, S.*); and the last article is a report on the effects of over-expressing ethylene responsive transcription factor on expression of selected fruit ripening-related genes in oil palm (*Elaeis guineensis* Jacq.) mesocarp (*Nur Annies Abd Hadi, Siti Nor Akmar Abdullah, Azzreena Mohamad Azzeme, Ahmed Al-Shanfari* and *Halimi Mohd Saud*).

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.

Chief Executive Editor
Nayan Deep S. KANWAL, FRSA, ABIM, AMIS, Ph.D.
nayan@upm.my

Editorial

Challenges and Opportunities of Agro-Bio Sciences in the Nano Era

Vijay K. Arora

UTM Distinguished Visiting Professor and IEEE-EDS Distinguished Lecturer, Wilkes University, U. S. A.

The scope of *Pertanika Journal of Tropical Agricultural Science (JTAS)* embraces a wide area of the agricultural and biological sciences. While the fundamental sciences in plant and human biology have not changed much, there is a shift in probing the living organism both in plants as well as in humans and animals through the power of microscopes, or nanoscopes, using current technology. The nanometer is one-billionth of a metre and it allows us to image organisms with greater resolution. As surface-to-volume ratio increases on the nanoscale, the dangling bonds at the surface become more reactive, creating a number of opportunities for sensors as well as actuators. Nanotechnology has been heralded as a new industrial revolution combining all basic sciences (physics, chemistry, biology, mathematics, computer and material) in engineering new systems and devices. The development of new nanotech-based tools and equipment may help to increase efficiency and overcome challenges faced by the agricultural industry and by health agencies in controlling diseases in search

for a healthy habitat for humanity through vigilant resource management.

At the time of independence from the British Raj, the Indian subcontinent was largely an agrarian society, experimenting with a mixed economy of capitalism and socialism. Although independence from the British Raj returned power to the people for a large part of Asia, it was a time of turmoil and social unrest. Formation of the Islamic State of Pakistan created mass migration of the people of same race; China was gripped by the Cultural Revolution launched by Mao Zedong; North Vietnam, led by the revolutionary Ho Chi Minh, fought for control of South Vietnam in a civil war; and Indonesia under Sukarno was in a state of confrontation against Malaysia and Singapore. The far-reaching impact of the Cold War had crept into Asia. The energy that should have gone into nation building went into claiming a fair share of hard-won freedom. In the new millennium of the 21st century, a new renaissance has engulfed Asia. Asia is regaining the stature it once enjoyed as the birthplace of the world's

major civilisations and religions. Part of the reason is the mobility of the work force that is global in the sense that products designed in one country are produced in another country for consumption in other countries of the globe to reap the benefits of political stability and knowledge no matter where they are found. Governments of the world are being asked about the value they add to the national economy. In their turn, governments question academia for the outcomes of their investment in higher education. How much smarter are students on graduation after spending three to six years in a university? There are also challenges facing the present generation: global terror, nuclear proliferation, hacking of the Internet, religious and cultural conflicts and climate change, just to name a few. These challenges are ecological with underlying age-old similarities: dealing with how we will share the planet's resources, of how we as humans relate to one another, of how we choose to live our lives.

Modern agriculture makes extensive use of chemicals. The process can be transformed through nanotechnology in making production more effective and less harmful to the environment. Precision-farming methodologies through the implementation of nanotechnology in the form of small sensors and monitoring devices will enable us to make accurate decisions related to plant growth and soil suitability. Consumer-friendly and eco-friendly nano delivery systems for nutrients and pesticides have started to find their place in the market. On the other hand, we are

also finding healthy mind-body connections that are not fully documented because of religious dogma attached to them. Spirituality is universal or ever present, which makes us question the meaning of our existence. Religions are man-made. The only way to overcome that conflict is to examine ourselves, ask ourselves whether we are drawing nearer to the ideals we profess or departing from them. Aristotle's words still resonate in enlightened minds: "We are what we repeatedly do. Excellence, therefore, is not an act, but a habit." A research mind will habituate open communications by freely expressing the ideas for advancement of humanity through science-spiritualty connection. In fact, there is no conflict between the two as both science and spirituality are in search of truth. As Newton's first law of inertia tells us, we are by our nature peace-loving minds. Newton's second law pushes us towards change in the light of our necessities that is driven by forces external to our inner self. The third law of action and reaction disturbs our peace as we spend considerable energy in reacting. That is where famine and hunger arises as valuable resources are diverted to fund wars. The outcome of our actions is now embedded in defining the educational outcomes of the Washington Accord (washingtonaccord.org), which has 17 participating countries, as we embrace 2015. The concept of 1Malaysia is being floated in the true spirit of the Arabic word *wahdat al-wujud*: unity of all beings to unite minds or thinking forces that are centred on outcome-based education (OBE).

Pertanika JTAS with its scope of uniting agro-bio sciences in the welfare of humankind and managing resources through capturing the power of thinking minds is a valuable resource to put your intellectualism on paper so others can benefit. As we usher in the new year of 2015, I envision *Pertanika* becoming a powerhouse of transformative science and technology for a better world habitat. *Pertanika* will be a place for creative interplay of curiosity, reason and necessity. With the engagement of its readers, *Pertanika* will be a wellspring of ideas and innovation, overflowing with benefits for planet Earth's habitat. Our wants are insatiable but our resources are limited. It is through scientific discourse that we may be able to manage well our resources by deriving synergy, instituting a process of quality deployment and ongoing improvements for limited agro, bio and intellectual resources through careful strategic planning and understanding the diversity of Nature. *Pertanika* is the ideal forum to unite these disparate thinking processes of the mind for the betterment of humanity. See the world around you, feel its pulse, reach out and touch lives -- make a difference. Catch the wave of opportunity. Catch it with your mind. And catch it with your heart and soul in creating mind-body-soul integration for a healthy world habitat.
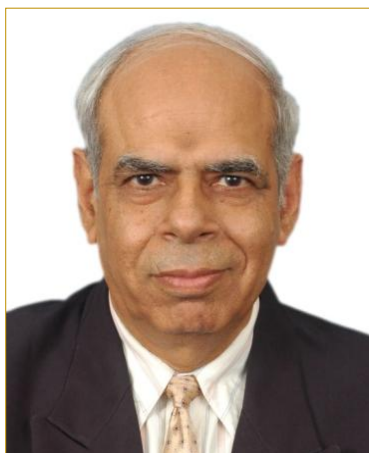
Vijay K. Arora:
Distinguished Professor & noted international educator and IEEE-EDS Distinguished Lecturer

Professor Arora, noted international educator and IEEE-EDS Distinguished Lecturer, resurrects nanoengineering integration in his most recent book entitled *Nanoelectronics: Quantum Engineering of Low-Dimensional Nanoensembles*, soon to be released by CRC Press: Taylor and Francis Group. Professor Arora obtained his Ph.D from the University of Colorado. He has held distinguished appointments at the University of Tokyo, National University of Singapore, Nanyang Technological University, University of Western Australia, and Universiti Teknologi Malaysia (UTM), in addition to several short-term visiting assignments around the world. Presently, he is Distinguished Visiting Professor at UTM on leave from Wilkes University, U. S. A., where he holds tenure as Professor teaching electrical engineering, physics and engineering management. Professor Arora was accorded Leading Educators of the World 2005, Leading Scientists of the World 2005 and Man of Achievement 2005 by International Biographical Centre of Cambridge, England. He is listed in a number of Who's Who biographies. He has been invited to give keynote lectures and presentations internationally. His publications include more than 100 papers in reputed journals and many uncounted publications in conference proceedings and numerous invited/keynote lectures. Professor Arora serves on the editorial board of a number of journals. He was chair of NanoSingapore2006, NanotechMalaysia2010 and EscienceNano2012 conferences. He can be reached at vijay.arora@wilkes.edu.

Short Communication

A New Natural Product Compound Benjaminin from *Calophyllum benjaminum*

Sahimi, M. S. M., Ee, G. C. L.*, Mahaiyiddin, A. G., Daud, S., Teh, S. S., See, I. and Sukari, M. A.

Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Our detailed study on the chemical constituents of the stem bark of *Calophyllum benjaminum* and *Calophyllum javanicum* has resulted in one new coumarin benjaminin (1), five xanthones fuscaxanthone C (2), β-mangostin (3), thwaitesixanthone (4), dombakinaxanthone (5) and caloxanthone A (6), together with four common triterpenes friedelin (7), β-sitosterol (8), lupeol (9) and stigmasterol (10). The structures of these compounds were elucidated using NMR, FTIR and GCMS.

Keywords: Benjaminin, *Calophyllum benjaminum*, *Calophyllum javanicum*, Clusiaceae, coumarin, xanthone

INTRODUCTION

Calophyllum genus is one of the many genera of the *Guttiferae* family. This particular genus comprises more than 180 species that are widely distributed in Southeast Asia. *Calophyllum benjaminum* and *Calophyllum javanicum* are among the many *Calophyllum* species that grow in Malaysia. Some *Calophyllum* species are used in folk medicine to treat rheumatism, wound and inflammation. Previous studies have indicated this genus as rich in secondary metabolites such as xanthones (Morel *et al*. 2000), coumarins (Ee *et al*. 2011), and chromanone acids (Cottiglia *et al*. 2004). Some of the compounds isolated from *Calophyllum* species exhibited significant biological effects such as cytotoxic activities (Guilet *et al*. 2001), anti-human immunodeficiency virus (HIV), antimicrobial (Yimdjo *et al*. 2004) and anti-

tumour promoter activities (Itoigawa *et al.* 2001). As part of our ongoing research on *Calophyllum* species from Malaysia, we reported here the first phytochemical study on *Calophyllum benjaminum* and *Calophyllum javanicum*.

MATERIALS AND METHODS

Extraction and Isolation

The ground stem bark of both plants was extracted with a series of solvents in increasing polarity, starting with hexane, followed by chloroform, ethyl acetate and methanol respectively for three days for each solvent. The extracts were evaporated to dryness using a rotary vacuum evaporator and the dry extract purified and isolated using column chromatography (Merck silica gel). Eluting solvents, which were of increasing polarity, ranged from 100% hexane to mixtures of hexane and chloroform, mixtures of chloroform and ethyl acetate, ethyl acetate with methanol and 100% methanol. Repeated purifications of the chloroform extract of *Calophyllum benjaminum* gave benjaminin (1) (11mg) together with fuscaxanthone C (2) (9mg). Meanwhile, β – mangostin (3) (5mg) and calaxanthone A (6) (10mg) were obtained from the methanol extract. The chloroform extract of *Calophyllum javanicum* gave dombakinaxantone (5) (7mg) and thwaitesixanthone (4) (8mg). Meanwhile, the ^{1}H and ^{13}C NMR spectral data for compounds 2 - 6 (see Fig.2) are in agreement with the data given in the literature (Ito *et al.*, 2003; Mahabusarakam *et al.*, 1987; Iinuma *et al.*, 1994; Ranjith *et*

al., 1997; Dahanayake *et al.*, 1974). *Benjaminin* (1): Yellowish Oil; FTIR: 3296, 2930, 1706, 1621 cm^{-1}

^{1}H-NMR(CDCl$_3$, 400MHz): *d* 12.28 (7-OH, *s*), 5.19 (1H, *t*, *J* = 6.29 Hz, H-2″), 4.13 (1H, *m*, H-3‴), 3.72 (3H, *s*, 5-OCH$_3$), 3.51 (1H, *m*, H-4), 3.25 (2H, *m*, H-1″), 2.74 (2H, *d*, *J* = 6.85 Hz, H-3), 2.53 (1H, *m*, H-2‴), 1.74 (3H, *s*, H-5″), 1.70 (2H, *m*, H-1′), 1.67 (3H, *s*, H-4″), 1.49 (3H, *d*, *J* = 6.87, H-CH$_3$-3‴), 1.24 (2H, *m*, H-3′), 1.22 (2H, *m*, H-4′), 1.19 (3H, *d*, *J* = 6.87 Hz, H-CH$_3$-2‴), 1.11 (2H, *m*, H-2′), 0.82 (3H, *t*, *J* = 6.87, H-5′).

^{13}C-NMR (CDCl$_3$, 100MHz): *d* 200.42 (C-1″), 179.27 (C-2), 164.96 (C-5), 160.17 (C-7), 158.60 (C-8a), 131.80 (C-3″), 122.90 (C-2″), 115.26 (C-6), 115.19 (C-4a), 78.76 (C-3‴), 61.92 (OCH$_3$), 46.21 (C-2‴), 39.20 (C-3), 33.87 (C-1′), 32.67 (C-4), 31.95 (C-3′), 27.78 (C-2′), 25.80 (C-4″), 22.68 (C-1″), 22.64 (C-4′), 19.69 (C-CH$_3$-3‴), 17.99 (C-5″), 14.15 (C-5′), 10.34 (C-CH$_3$-4‴)

RESULTS AND DISCUSSION

Benjaminin (1) was obtained from the chloroform extract of *Calophyllum benjaminum* as a yellowish oil with molecular formula $C_{25}H_{36}O_6$. The EIMS spectrum gave a molecular ion peak at m/z 432. The FTIR spectrum of benjaminin displayed absorbtion peaks at 3296, 2925, 1705, 1626 cm^{-1}, while the UV spectrum gave a maximum absorbtion at 400nm.

The ^{1}H NMR signals were assigned through their COSY and HMBC correlations. The ^{1}H-NMR spectrum gave one singlet signal at δ 12.28 implying a hydroxyl peak. One olefinic proton signal at δ 5.19 (*t*, 1H,

$J = 6.30$ Hz), three methine signals at δ 2.53 (*m*, 1H), 3.51 (*m*, 1H), and 4.13 (*m*, 1H) and six methylene signals at δ 1.70 (*m*, 2H), 1.11 (*m*, 2H), 1.24 (*m*, 2H), 1.22 (*m*, 2H), 3.25 (*m*, 2H) and 2.74 (*d*, 2H, $J = 6.87$ Hz) were also observed. The olefinic signal was assigned to the olefinic (H-2″) proton of the prenyl side chain at C-6 while the methylene signals belong to the four methylene protons of the n-pentyl side chain at C-4 (H-1′, H-2′, H-3′ and H-4′) and the prenyl group (H-1″) and H-3, respectively. Five methyl signals at 0.82 (*t*, 3H, $J = 6.87$ Hz, 5′-CH$_3$), 1.74 (*s*, 3H, 5″-CH$_3$), 1.67 (*s*, 3H, 4″-CH$_3$), 1.19 (*d*, 3H, $J = 6.87$ Hz, 2‴-CH$_3$) and 1.49 (*d*, 3H, $J = 6.87$ Hz, 3‴-CH$_3$) were duly assigned to the five methyl groups of the side chains at C-4, C-6 and C-8. One methoxyl group at δ 3.72 (*s*, 3H) was seen to be attached to C-5 through their ^3J correlation in the HMBC spectrum. The ^{13}C-NMR spectrum gave 25 signals and from the analysis of the DEPT experiment, it was concluded

that the molecule had five methine carbons, six methylenes, and six methyls plus nine quaternary carbons including the two carbonyl carbons at δ 179.27 and 200.42.

The complete elucidation of the structure was achieved with the aid of the HMBC analysis (Fig.1) after assigning the protons to their direct bonding carbons by the HMQC spectrum. The attachment of the n-pentyl group to C-4 was justified by the ^2J correlation between H-4 and C-1′. H-1″of the prenyl group was seen to have a cross peak with C-6 (^2J) and a ^3J correlation with C-7 and C-5. Hence, the attachment of this prenyl side chain to C-6 is obvious. C-7 was concluded to be bonded to an OH group from its ^3J correlations with C-8 and C-6 and its ^2J correlation with C-7. The methoxyl protons correlated to C-5 via a ^3J correlation hence proving its attachment. The doublet signal at δ 2.74, which integrated for

(1)
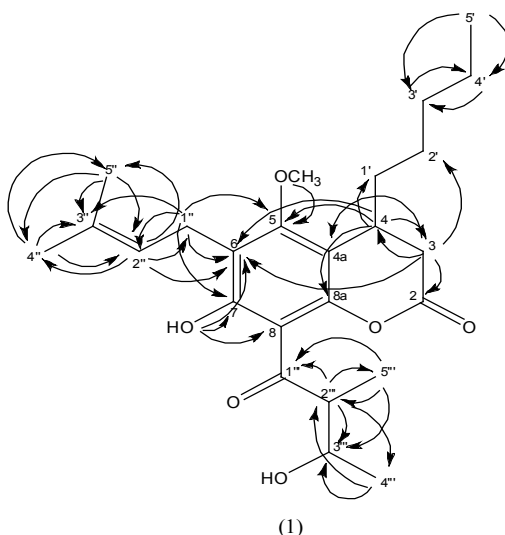
Fig.1: HMBC correlations in benjaminin (1)

Sahimi, M. S. M., Ee, G. C. L., Mahaiyiddin, A. G., Daud, S., Teh, S. S., See, I. and Sukari, M. A.
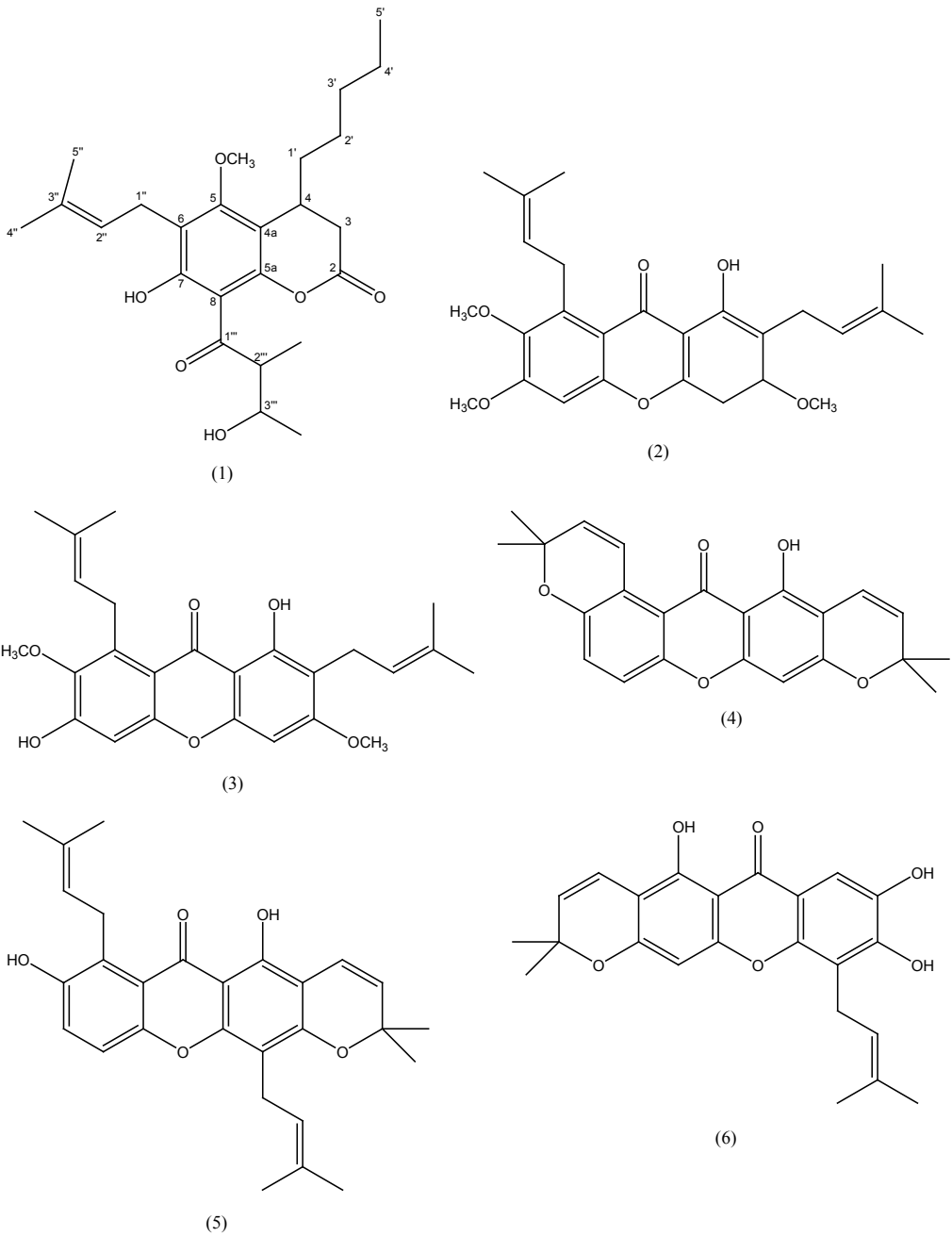
Fig.2: Structures of compounds 1-6

4

Pertanika J. Trop. Agric. Sci. 38 (1) 1 - 6 (2015)

2 protons and correlated to C-2 and C-4 via ^2J correlations and to C-4a via a ^3J correlation, indicated C-3 to be a methylene carbon. The HMBC correlation of the methoxyl proton at δ 3.72 with δ 164.96 (C-5) confirmed its attachment at C-5. Hence, the remaining carbon, which is C-8, could be shown to be attached to a 3-hydroxy-2-methylbutanoyl moiety via HMBC correlations (see Fig.1). The C-2‴proton gave cross peaks with the C-2‴ methyl and C-3‴ methyl groups thus justifying its location. Meanwhile, a HMBC correlation was observed between the C-3‴ methine proton and the carbonyl carbon at position C-1‴. Thus, compound **1** was deduced to be benjaminin with the IUPAC name 7-hydroxy -8-(3-hydroxy-2-methylbutanoyl)-5-methoxy-6-(3-methylbut-2-enyl)-4-pentylchroman-2-one.

CONCLUSION

The detailed isolation work on the stem bark of *Calophyllum benjaminum* and *Calophyllum javanicum* have resulted in one new coumarin benjaminin (1), five xanthones fuscaxanthone C (2), β-mangostin (3), thwaitesixanthone (4), dombakinaxanthone (5), and caloxanthone A (6), together with four common triterpenes friedelin (7), β-sitosterol (8), lupeol (9) and stigmasterol (10). There was no report available on this particular plant before.

REFERENCES

Cottiglia, F., Dhanapal, B., Sticher, O., & Heilmann, J. (2004). New Chromanone Acids with Antibacterial Activity from *Calophyllum brasiliense. Journal of Natural Product*, *67,* 537-541.

Dahanayake, M., Kitagawa, l., Sornanathan, R., & Sultanbawa, U. M. S. (1974). Chemical Investigation of Ceylonese Plants, Part V1I. l. Extractives of *Calophyllum thwaitesii* Planch and Triana and *Calophyllurn walkerri* Wight (Guttiferae). *Journal of the Chemical Society, Perkin Transactions 1*, 2510 – 2514.

Ee, G. C. L., Mah, S. H., Teh, S. S., Rahmani, M., Go, R., & Taufiq-Yap, Y. H. (2011). Soulamarin, a New Coumarin from Stem Bark of *Calophyllum soulattri. Molecules*, *13*(10), 956-960.

Guilet, D., Se´raphin, D., Rondeau, D., Richomme, P., & Bruneton, J. (2001). Cytotoxic coumarins from *Calophyllum dispar. Phytochemistry*, *58*, 571–575.

Iinuma, M., Tow, H., Tanaka, T. and Yonemori, S. (1994). Two Xanthones From Root

Bark of *Calophyllum Inophyllum. Phytochemistry*, *35*, 527-532.

Ito, C., Itoigawa, M., Takakura, T., Ruangrungsi, N., Enjo, F., Tokuda, H., Nishino, H., & Furukawa, H. (2003). Chemical Constituents of *Garcinia fusca*: Structure Elucidation of Eight New Xanthones and Their Cancer Chemopreventive Activity. *Journal of Natural Product*, *66,* 200-205.

Pertanika J. Trop. Agric. Sci. 38 (1): 1 - 6 (2015)

5

Itoigawa, M., Ito, C., Tan, H. T.W., Kuchide, M., Tokuda, H., Nishino, H., Furukawa, H. (2001). Cancer chemopreventive agents, 4-phenylcoumarins from *Calophyllum inophyllum*. *Cancer Letters*, *169*, 15-19.

Mahabusarakam, W. & Wiriyachitra, P. (1987). Chemical Constituents of *Garcinia mangostana*. *Journal of Natural Product*, *50,* 474-478.

Morel, C., Se´raphin D., Oger, J-M., Litaudon, M., Se´venet, T., Richomme, P., & Bruneton J. (2000). New Xanthones from *Calophyllum Caledonicum*. *Journal of Natural Product, 63,* 1471-1474.

Ranjith, H., Dharmaratne, W., Nishanthi, W. M., & Wuesinghe M. (1997). A Trioxygenated Diprenylated Chromenxanthone from *Calophyllum Moonii*. *Phytochemistry, 46*, 1293-1295.

Yimdjo, M. C., Azebaze, A. G., Nkengfack, A. E., Meyer, A. M., Bodo, B. and Fomum, Z. T. (2004). Antimicrobial and cytotoxic agents from *Calophyllum inophyllum*. *Phytochemistry*, *65*, 2789–2795.

Sea Cucumber (Echinodermata: Holothuroidea) Species Richness at Selected Localities in Malaysia

Kamarul Rahim Kamarudin[1]*, Gires Usup[2], Ridzwan Hashim[3] and Maryam Mohamed Rehan[1]

[1]Food Biotechnology, Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM), 71800 Nilai, Negeri Sembilan, Malaysia
[2]Marine Science Programme, School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[3]Department of Biomedical Science, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, Jalan Istana, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

ABSTRACT

This study aimed at documenting species richness and distribution of sea cucumbers (Phylum Echinodermata: Class Holothuroidea) in Malaysia. Sea cucumber collections were conducted from August 2004 until November 2011 from several study sites in Peninsular Malaysia and Sabah. A total of fifty two morphospecies of sea cucumbers from four orders comprising of 12 genera were documented. Thirty eight species were recorded for Sabah, followed by 24 species for Peninsular Malaysia, and 10 species were recorded in both regions. However, nine species required further taxonomic works for their identification. Of the 15 *Actinopyga* species recorded, 14 species were from Sabah and one species was from Peninsular Malaysia. The order Aspidochirotida in general, and genus *Holothuria* in particular were the dominant taxa. *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835) is the dominant species in Malaysia as it was observed and documented at all collection sites. Future studies on the species richness of sea cucumbers are required in Sarawak marine waters and the molecular phylogeny of the sea cucumbers in order to obtain a better understanding of the evolutionary relationships between the sea cucumbers of Malaysia.

INTRODUCTION

Malaysia is one of the 12 mega diverse countries in the world that houses many marine taxa including sea cucumbers (Phylum Echinodermata; Class Holothuroidea). Biogeographically, Malaysia lies within the Oriental Region (Huggett, 1998) but Sabah and Sarawak are situated adjacent to the Wallacea Region, with the former being closer. According to Mohsin and Ambak (1991), southern Thailand, southern Indo-China, Sumatra, Java, Peninsular Malaysia and Borneo were connected by the Sunda Platform during the Pleistocene Epoch. The lowering of sea level forming the Sunda Platform is speculated to have caused the unique biogeographic distribution patterns of flora and fauna, including the sea cucumbers, especially throughout the Malaysian marine waters (Cannon et al., 2009).

The sea cucumber is considered as a marine heritage of Malaysia and it is estimated that more than 80 species of sea cucumbers inhabit the marine and coastal waters of Malaysia (Kamarul Rahim et al., 2010). Local names for the sea cucumber include *timun laut*, *gamat*, *bat*, *balat*, and *brunok*. The sea cucumber is also referred to as *hoi sum* or *hai shen* by the Malaysian Chinese community, which is translated to sea ginseng due to its healing properties. The local name *gamat* is the accepted name among Malaysians and it is used as reference for all the sea cucumber species within the family Stichopodidae. There are two genera within the Stichopodidae family found to date in Malaysia, i.e.

Stichopus and *Thelenota*. The *gamat* species includes *Stichopus horrens* (Selenka, 1867) (Dragonfish) and *Thelenota anax* (Clark, 1921) (Amberfish). *Gamat* is well known in the traditional medicine industry, especially in the Langkawi and Pangkor islands. *Stichopus horrens* is the most popular and the most well-studied *gamat* species, where it has been commercially exploited for its body fluid extracts (*air gamat*), lipid extracts (*minyak gamat*) and also for the *gamat*-based dietary supplements or health food products produced via modern technologies. In Sabah, sea cucumbers are exploited for the food industry and contribute to the state's economy.

The diversity and commercial values of Malaysia's sea cucumbers have made the marine animals the focus of various studies to date. Early studies in Malaysia on the presence and distribution of sea cucumbers were conducted by Ridzwan and Che Bashah (1985), George and George (1987), Ridzwan (1987), Kaswandi et al. (1990), Ridzwan (1993), Ridzwan and Kaswandi (1995), Ridzwan et al. (1995), Kaswandi et al. (1995), Ridzwan et al. (1996), Ridzwan et al. (1998a), and Ridzwan et al. (1998b). The above studies were mainly focused in Sabah, while the other studies were carried out in Langkawi Island (Kedah), Balik Pulau (Penang), Pangkor Island (Perak), Tioman Island (Pahang), Besar Island and Aur Island (Johor) in Peninsular Malaysia, as well as in Brunei (Baine & Forbes, 1998; Forbes & Ilias, 1999; Siti et al., 1999a; Siti et al., 1999b; Zulfigar & Tan, 1999; Zulfigar et al., 2000; Lane et al., 2000; Zainuddin &

Forbes, 2000; Zaidnuddin, 2002; Kamarul Rahim & Ridzwan, 2005; Zulfigar *et al*., 2007; Zulfigar *et al*., 2008; Sim *et al*., 2008; Sim *et al*., 2009; Kamarul Rahim *et al*., 2009; Kamarul Rahim *et al*., 2010). Among the sea cucumbers that were reported from the previous studies, some were not identified to the species level.

Thus, the aim of this study was to document the sea cucumber species and their distributions in several localities of Peninsular Malaysia and Sabah. Updates on species identification from previous studies including that of Kamarul Rahim *et al*. (2009, 2010) are also reported.

MATERIALS AND METHODS

Study Sites and Collection Method

Several localities in Peninsular Malaysia and Sabah were selected based on the known distributions of the sea cucumbers (Fig.1). Sea cucumber collection was conducted for approximately seven years, i.e. from August 2004 until November 2011. The researchers sought the assistance of professional divers and employed local residents, and there were no fixed sampling hours allocated. Global Positioning System (GPS) was utilised to record each sampling site (not specifically shown in Fig.1). The
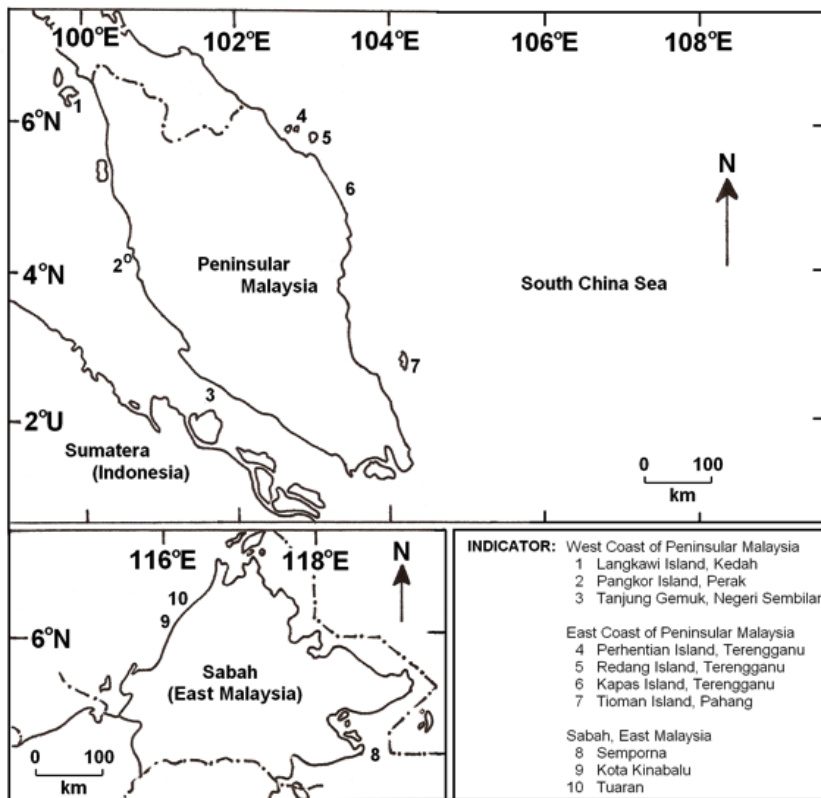
Fig.1: Sea cucumber collection sites in Malaysia (Adapted from Kamarul Rahim *et al*., 2009)

sea cucumber specimens of sea cucumber were observed, collected, photographed, measured and released, or collected after an official permission as reference specimens. The collection of sea cucumbers was conducted during low tide.

Species Identification

Sea cucumber species identification was done by referring to the experts [(namely, Assoc. Prof. Alexander M. Kerr from Marine Laboratory, University of Guam, USA) and the participants of NSF PEET Holothuroid Systematics Workshop held on 7-16 June 2010 at the Marine Laboratory, UOG, USA)], Ridzwan (2011), the World Register of Marine Species database at http://www.marinespecies.org/index.php, and also through the information given by local residents. The undetermined species were temporarily designated as local names. Several updates to the undetermined sea cucumber taxa recorded from previous studies were also done. The collected specimens were placed at the laboratories of the Department of Museum Malaysia, Universiti Kebangsaan Malaysia (UKM) Bangi, and at the International Islamic University Malaysia (IIUM), Indera Mahkota Campus in Kuantan.

Storage and Preservation

For short-term storage, live and dead specimens of sea cucumbers were kept in ice boxes while in the laboratory, sea cucumber specimens were transferred into -20°C or -80°C freezers for long-term storage or preserved in 70% ethanol. All the

sea cucumber specimens were catalogued accordingly.

RESULTS AND DISCUSSION

The present study recorded 52 morphospecies of sea cucumbers from 4 orders comprising 12 genera (see Table 1, Fig.2-Fig.53). Of the total number of sea cucumber species collected, 16 species (i.e. 31%) were from the genus *Holothuria*, followed by *Actinopyga* (15 species; 29%), *Stichopus* (8 species; 15%), *Bohadschia* (4 species; 8%) and *Synapta* (2 species; 4%). Meanwhile, the genera *Pearsonothuria*, *Thelenota*, *Molpadia*, *Polycheira*, *Leptosynapta*, *Thyone*, and *Cercodemas* comprised 1 species each. The species richness of *Actinopyga* from the present study was higher than that recorded by Ridzwan (1993), Baine and Forbes (1998), and Forbes and Ilias (1999). *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835), which is commonly known as *bat puntil or white threads fish* is the most dominant species in Malaysia, as it was observed and documented at all collection sites. Kamarul Rahim *et al*. (2009) also reported that *H. leucospilota* is the most dominant species in Malaysia. It is estimated that there are more than 80 species of sea cucumbers present in the marine and coastal waters of Malaysia (Kamarul Rahim *et al*., 2010).

Thirty eight species of sea cucumbers were collected from Sabah while 24 species were collected from Peninsular Malaysia, with 10 species overlapping [namely, *H. leucospilota*, *Holothuria*

TABLE 1
Presence, richness and distribution of sea cucumbers in Malaysia. PM=Peninsular Malaysia, S=Sabah.

No.	Species	Local Name	PM	S
	Order Aspidochirotida			
	Family Holothuriidae			
1	*Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835)	*Bat puntil / White threads fish*	x	x
2	*Holothuria (Mertensiothuria) hilla* Lesson, 1830	*Bat / Tiger tail sea cucumber*	x	
3	*Holothuria (Metriatyla) scabra* Jaeger, 1833	*Bat putih / Sandfish*		x
4	*Holothuria (Metriatyla) ocellata* Jaeger, 1833	*Bat*	x	
5	*Holothuria (Metriatyla) lessoni* Massin, Uthicke, Purcell, Rowe & Samyn, 2009	*Bat putan / Golden sandfish*		x
6	*Holothuria (Halodeima) atra* Jaeger, 1833	*Bat hitam / Lollyfish*	x	x
7	*Holothuria (Halodeima) edulis* Lesson, 1830	*Bat senjata anjing / Pinkfish*	x	x
8	*Holothuria (Microthele) nobilis* (Selenka, 1867)	*Bat susu / White teatfish*		x
9	*Holothuria (Microthele) fuscopunctata* Jaeger, 1833	*Bat / Elephant trunkfish*		x
10	*Holothuria (Acanthotrapeza) coluber* Semper, 1868	*Bat sumping / Snakefish*	x	x
11	*Holothuria (Lessonothuria) pardalis* Selenka, 1867	*Bat*	x	
12	*Holothuria (Theelothuria) notabilis* Ludwig, 1875	*Bat*	x	
13	*Holothuria (Thymiosycia)* aff. *impatiens*	*Bat*	x	
14	*Holothuria (Panningothuria) forskali* Delle Chiaje, 1823	*Bat*	x	
15	*Holothuria* sp. - 3	*Bat brown*		x
16	*Holothuria* sp. - 6	*Bat kasut*		x
17	*Bohadschia argus* (Jaeger, 1833)	*Bat / Leopardfish / Tigerfish*		x
18	*Bohadschia vitiensis* (Semper, 1868)	*Bat nangka / Brown sandfish / Bat sawa*	x	x
19	*Bohadschia* sp. - 1	*Bat*	x	
20	*Bohadschia* sp. - 2	*Bat*	x	
21	*Actinopyga lecanora* (Jaeger, 1833) - 1	*Bat puyuh / Stonefish*		x
22	*Actinopyga lecanora* (Jaeger, 1833) - 2	*Bat puyuh / Stonefish*		x
23	*Actinopyga lecanora* (Jaeger, 1833) - 3	*Bat puyuh / Stonefish*		x
24	*Actinopyga lecanora* (Jaeger, 1833) - 4	*Bat puyuh / Stonefish*		x
25	*Actinopyga lecanora* (Jaeger, 1833) - 5	*Bat puyuh / Stonefish*		x
26	*Actinopyga lecanora* (Jaeger, 1833) - 6	*Bat puyuh / Stonefish*		x
27	*Actinopyga lecanora* (Jaeger, 1833) - 7	*Bat puyuh / Stonefish*		x
28	*Actinopyga lecanora* (Jaeger, 1833) - 8	*Bat puyuh / Stonefish*		x
29	*Actinopyga lecanora* (Jaeger, 1833) - 9	*Bat puyuh / Stonefish*		x
30	*Actinopyga lecanora* (Jaeger, 1833) - 10	*Bat puyuh / Stonefish*		x
31	*Actinopyga lecanora* (Jaeger, 1833) - 11	*Bat puyuh / Stonefish*		x
32	*Actinopyga lecanora* (Jaeger, 1833) - 12	*Bat puyuh / Stonefish*		x
33	*Actinopyga lecanora* (Jaeger, 1833) - 13	Bat puyuh / Stonefish		x
34	*Actinopyga lecanora* (Jaeger, 1833) - 14	Bat puyuh / Stonefish		x

TABLE 1 (*continue*)

No.	Species	Local Name	PM	S
	Order Aspidochirotida			
	Family Holothuriidae			
35	*Actinopyga lecanora* (Jaeger, 1833) - 15	*Bat puyuh / Stonefish*	x	
36	*Pearsonothuria graeffei* (Semper, 1868)	*Bat / Flowerfish / Blackspotted sea cucumber*		x
	Family Stichopodidae			
37	*Stichopus rubermaculosus* Massin, Zulfigar, Hwai & Boss, 2002	*Gamat*		x
38	*Stichopus chloronotus* Brandt, 1835	*Talifan varieti hitam / Greenfish*	x	x
39	*Stichopus horrens* Selenka, 1867	*Gamat / Dragonfish*	x	x
40	*Stichopus ocellatus* Massin, Zulfigar, Hwai & Boss, 2002	*Gamat*	x	x
41	*Stichopus herrmanni* Semper, 1868	*Gamat / Curryfish*	x	
42	*Stichopus vastus* Sluiter, 1887	*Gamat batu / Gamat kiulu*	x	x
43	*Stichopus* sp. - 1	*Kumbatas*		x
44	*Stichopus* sp. - 2	*Kambatan*		x
45	*Thelenota anax* H.L. Clark, 1921	*Bat / Amberfish*		x
	Order Molpadiida			
46	*Acaudina molpadioides* (Semper, 1867)	*Bat hati / Beronok /Brunok*	x	x
	Order Apodida			
47	*Synapta maculata* (Chamisso & Eysenhardt, 1821)	*Taliaga*		x
48	*Synapta* sp. - 1	*Taliaga*		x
49	*Polycheira rufescens* (Brandt, 1835)	*Bat*	x	
50	*Leptosynapta* sp. - 1	*Bat*	x	
	Order Dendrochirotida			
51	*Thyone* sp. - 1	*Bat*	x	
52	*Cercodemas anceps* (Selenka, 1867)	*Bat*	x	

(Halodeima) atra (Jaeger, 1833); *Holothuria (Halodeima) edulis* (Lesson, 1830); *Holothuria (Acanthotrapeza) coluber* (Semper, 1868); *Bohadschia vitiensis* (Semper, 1868); *Stichopus chloronotus* (Brandt, 1835); *S. horrens*; *Stichopus ocellatus* (Massin, Zulfigar, Hwai & Boss, 2002); *Stichopus vastus* (Sluiter, 1887), and *Acaudina molpadioides* (Semper, 1867)]. There were two undetermined species of

Holothuria, *Bohadschia* and *Stichopus*, while one species was undetermined from *Synapta*, *Leptosynapta* and *Thyone* (Table 1). Interestingly, 14 out of the 15 *Actinopyga* species recorded were from Sabah, while only one was recorded from Redang Island (east coast of Peninsular Malaysia). Nine species required further species identification (based on ossicle characters, behaviour and molecular phylogeny).

Order Aspidochirotida (45 species)

Family Holothuriidae (36 species)

Genus *Holothuria* (16 species – Fig.2 through Fig.17)
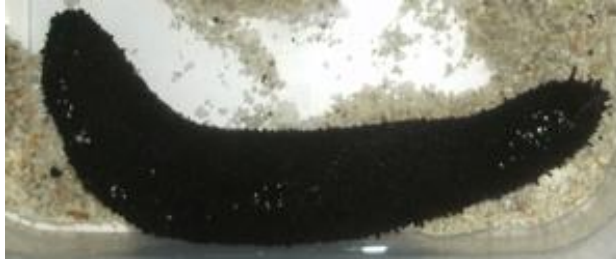
Fig.2: *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835)
(Photo source: Kamarul Rahim Kamarudin)

Fig.3: *Holothuria (Mertensiothuria) hilla* Lesson, 1830 (Photo source: Kamarul Rahim Kamarudin)

Fig.4: *Holothuria (Metriatyla) scabra* (Jaeger, 1833). Left photo = dorsal view, right photo = ventral-dorsal view (Photo source: Ridzwan Hashim)

Fig.5: *Holothuria (Metriatyla) ocellata* (Jaeger, 1833). Left photo = dorsal view, right photo = ventral view (Photo source: Department of Museums Malaysia)
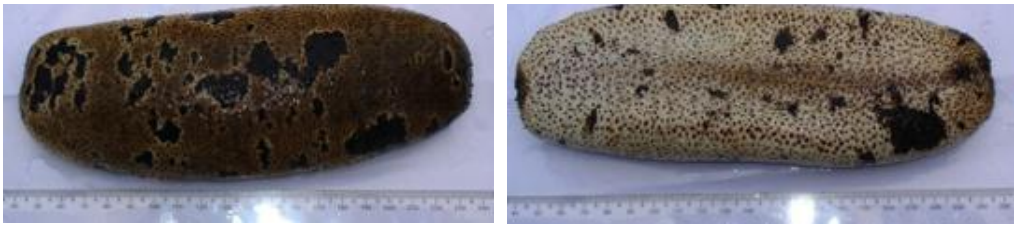
Fig.6: *Holothuria (Metriatyla) lessoni* (Massin, Uthicke, Purcell, Rowe & Samyn, 2009). Left photo = dorsal view, right photo = ventral view (Photo source: Ridzwan Hashim)
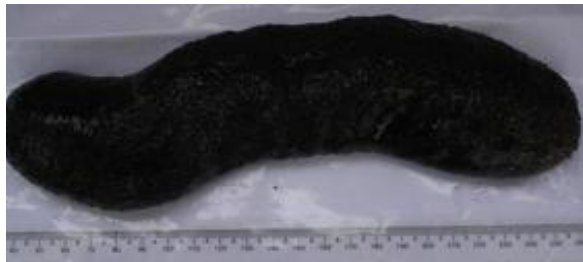
Fig.7: *Holothuria (Halodeima) atra* (Jaeger, 1833) (Photo source: Kamarul Rahim Kamarudin)

Fig.8: *Holothuria (Halodeima) edulis* (Lesson, 1830). Left photo = dorsal view, right photo = ventral view (Photo source: Department of Museums Malaysia)

Fig.9: *Holothuria (Microthele) nobilis* (Selenka, 1867). Left photo = dorsal view, right photo = ventral view (Photo source: Ridzwan Hashim)
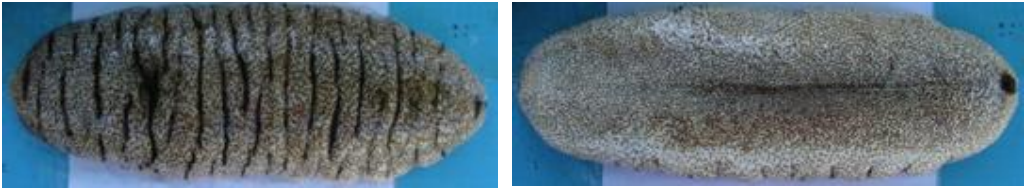
Fig.10: *Holothuria (Microthele) fuscopunctata* (Jaeger, 1833). Left photo = dorsal view, right photo = ventral view (Photo source: Ridzwan Hashim)

Fig.11: *Holothuria (Acanthotrapeza) coluber* (Semper, 1868) (Photo source: Ridzwan Hashim)

Fig.12: *Holothuria (Lessonothuria) pardalis* (Selenka, 1867) (Photo source: Kamarul Rahim Kamarudin)

Fig.13: *Holothuria (Theelothuria) notabilis* Ludwig, 1875. Left photo = dorsal view, right photo = ventral view. Photo source: Department of Museums Malaysia

Fig.14: *Holothuria (Thymiosycia)* aff. *impatiens* (Photo source: Kamarul Rahim Kamarudin)

Fig.15: *Holothuria (Panningothuria) forskali* (Delle Chiaje, 1823)
(Photo source: Kamarul Rahim Kamarudin)
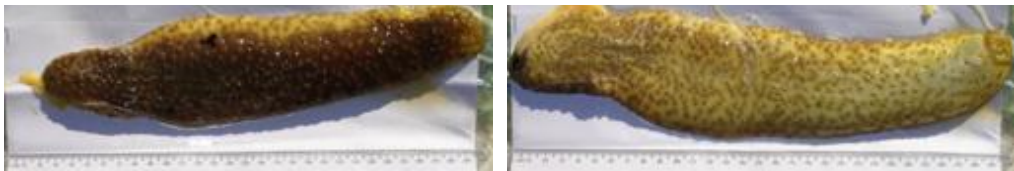
Fig.16: *Holothuria* sp. – 3; Left = dorsal view, right = ventral view (Photo source: Ridzwan Hashim)

Fig.17: *Holothuria* sp. – 6; Left = dorsal view, right = ventral view (Photo source: Ridzwan Hashim)

The undetermined species in Peninsular Malaysia were from the genera *Bohadschia* (2 taxa), *Leptosynapta* (1 taxon) and *Thyone* (1 taxon), while the undetermined species in Sabah were from the genera *Holothuria* (2 taxa), *Stichopus* (2 taxa) and *Synapta* (1 taxon). The higher species richness and distribution of the sea cucumbers in Sabah as compared to Peninsular Malaysia were probably related to its proximity

to the Wallace's line and better marine water quality. Further studies, however, are required especially to determine the phylogenetic relationships of the Peninsular Malaysia's species and the Borneo species so as to further understand their distribution and adaptive radiation.

According to Ho (1992), the coral reef in Sabah within the Sulawesi Sea that includes the Bodgaya and Ligitan group of islands is well developed and provides suitable habitats for the sea cucumbers. The abundant organic matters in the coral reef areas in the tropical region (for example, seagrass detritus, coral mucus and algal remnants) are food for sea cucumbers and coupled with the multitude habitats, provide rich feeding areas for the soft-bodied marine-living echinoderms (Ridzwan, 1993). The above two factors may have contributed to the diverse species of sea cucumbers in the marine waters of Semporna, Maiga Island (part of Bodgaya group) and Mabul Island (part of Ligitan group). Approximately 139 tonnes of sea cucumbers were landed in Sabah (Annual Fisheries Statistics, Sabah, 2000–2005), as estimated by the Sabah Fisheries Department. This places the state as the most significant for sea cucumber fisheries in Malaysia. A wide variety of species are targeted mainly for the food industry of beche-de-mer in Sabah such as *Holothuria (Metriatyla) scabra* (Jaeger, 1833), *Holothuria (Halodeima) atra* (Jaeger, 1833), *Holothuria (Halodeima) edulis* (Lesson, 1830), *H. leucospilota*, *Stichopus herrmanni* (Semper, 1868), *Stichopus chloronotus* (Brandt, 1835), and *Thelenota*

ananas (Jaeger, 1833) (Choo, 2008). Sound management practices of the marine parks in Sabah (for example, the Tunku Abdul Rahman Park, Kota Kinabalu) may have contributed to the higher species richness in Sabah (Kamarul Rahim *et al.*, 2009). Besides that, recreational and reserve zones are being implemented in the marine waters of Manukan Island, Sabah, in the effort to retain the sea cucumber gene pool while developing eco-tourism in the state.

There is paucity of literature on the sea cucumbers of Sarawak. Ridzwan (1993) mentioned the use of *brunok* (Order: Molpadiida) as fishing bait among Sarawak residents. *Brunok* or *Beronok* is also known as *bat hati* in Sabah (Table 1). Ho (1992) stated that the growth of coral reefs surrounding Talang-Talang Besar, Talang-Talang Kechil, Sampadi, Satang Besar and Satang Kechil Islands in the marine waters of Sarawak is limited due to high turbidity and influx of freshwater from inland rivers. The condition has worsened due to tourism activities and also the close proximity of the islands to the mainland. These factors may have brought about the low species diversity of not only sea cucumbers but also other marine organisms in the Sarawak marine waters. Meanwhile, 14 morphospecies of sea cucumbers are known from Brunei Darussalam marine waters (Lane, 2005) and the species richness may be similar to those of Sarawak. *Holothuria atra*, *H. edulis*, and four species from the genus *Bohadschia* are among the sea cucumbers present in the marine waters of Brunei Darussalam. Lane (2005) stated that two of the four

Bohadschia species collected were possibly new species. Unlike Sabah, sea cucumbers in Brunei Darussalam have not been fully explored and are relatively unexploited (Lane, 2004).

Direct exploitation, introduced species, extinction cascades, habitat loss and degradation are among the factors responsible for species decline and extinction (Gaston & Spicer, 2004). In relation to these, the regular import of sea cucumbers from Adang, Thailand, during the 1990s (Baine and Sze, 1998) could possibly indicate the decreasing stocks of sea cucumbers in the marine waters of Peninsular Malaysia. Ho (1992) stated that the patchy distributions of coral reefs as habitat and food, as well as high water turbidity along the west coast of Peninsular Malaysia are the possible factors affecting species richness of the sea cucumbers. Forbes and Ilias (1999) reported that *S. horrens* was rare in Langkawi, which is one of the main traditional fishery sites in the West Coast of Peninsular Malaysia, beside Pangkor Island, thus supporting the possibility of stock decline of local sea cucumbers, more so the *gamat* species. *Gamat* has become popular as the main ingredient in the traditional medicine industry, as well as in the health food industry in Malaysia. *Stichopus horrens* (the golden *gamat*) has been exploited for its body fluid extracts (*air gamat*) and lipid extracts (*minyak gamat*). Baine and Sze (1998) suggested a three-pronged approach to help maintain sea cucumber stocks for trade in Langkawi: through sea cucumber restocking initiatives, imports from Thailand

in a trade agreement and from Pangkor Island, as part of a managed fishery.

The present study recorded nine species of *gamat* comprising eight *Stichopus* species and one *Thelenota* species (Table 1) [*Stichopus rubermaculosus* (Massin, Zulfigar, Hwai & Boss, 2002) (see Fig.38), *Stichopus chloronotus* (Brandt, 1835) (see Fig.39), *S. horrens* (Fig.40), *Stichopus ocellatus* (Massin, Zulfigar, Hwai & Boss, 2002) (see Fig.41), *Stichopus herrmanni* (Semper, 1868) (see Fig.42), *Stichopus vastus* (Sluiter, 1887) (see Fig.43), *Stichopus* sp. - 1 (see Fig.44), *Stichopus* sp. - 2 (see Fig.45) and *Thelenota anax* (Clark, 1921) (see Fig.46). Two unidentified specimens, labelled as *Stichopus* sp. 1 and *Stichopus* sp. 2, were collected in Sabah. *Thelenota ananas* (Jaeger, 1833) was listed as one of Malaysia's commercial species (Choo, 2008), increasing the total number of Malaysia's *gamat* species to date to 10 taxa. Sabah shows higher species richness of the *gamat* species as compared to Peninsular Malaysia. Four species (namely, *S. chloronotus*, *S. horrens*, *S. ocellatus*, and *S. vastus*) were recorded in both Peninsular Malaysia and Sabah (Table 1), suggesting a low species overlap.

Fifty two species of Asia's sea cucumbers are commercially exploited as food, with most being tropical and sub-tropical species from the Holothuriidae and Stichopodidae (Choo, 2008). This suggests high species richness of sea cucumbers in the marine waters of the Asian region benefitting countries in terms of their economy. Indonesia is the world's

top producer of Holothuroidea from its capture fishery with 35 commercial species, followed by China (27), the Philippines (26), Malaysia (19), Japan and Vietnam (11 each) and Thailand (8). No commercial species were recorded for Singapore and Brunei Darussalam (Choo, 2008); thus, supporting the findings of Lane (2004) with regards to non-exploitation of sea cucumbers in Brunei Darussalam. Malaysia, Thailand, Indonesia, the Philippines, and Vietnam have five species of sea cucumbers exploited as food; these are *Holothuria (Metriatyla) scabra* (Jaeger, 1833), *H. atra*, *H. edulis*, *H. leucospilota*, *Stichopus herrmanni* (Semper, 1868), *Stichopus chloronotus* (Brandt, 1835) and *Thelenota ananas* (Jaeger, 1833). Therefore, continuous studies on the sea cucumbers in Malaysia, with reference to their species presence and richness, distribution and stock assessment, are important for their sustainability, not only for the gene pool but also as food and medicinal resources.

Genus *Bohadschia* (4 species – see Fig.18 to Fig.21)

Fig.18: *Bohadschia argus* (Jaeger, 1833); Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim).

Fig.19: *Bohadschia vitiensis* (Semper, 1868); Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Fig.20: *Bohadschia* sp. – 1; Left = dorsal view, right = ventral view
(Photo source: Department of Museums Malaysia)

Fig.21: *Bohadschia* sp. – 2; Left = dorsal view, right = ventral view
(Photo source: Kamarul Rahim Kamarudin)

Genus *Actinopyga* (15 species – Fig.22 to Fig.36)

Fig.22: *Actinopyga lecanora* (Jaeger, 1833) – 1; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Fig.23: *Actinopyga lecanora* (Jaeger, 1833) – 2; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Fig.24: *Actinopyga lecanora* (Jaeger, 1833) – 3; Left = dorsal view, right = ventral-dorsal view
(Photo source: Ridzwan Hashim)
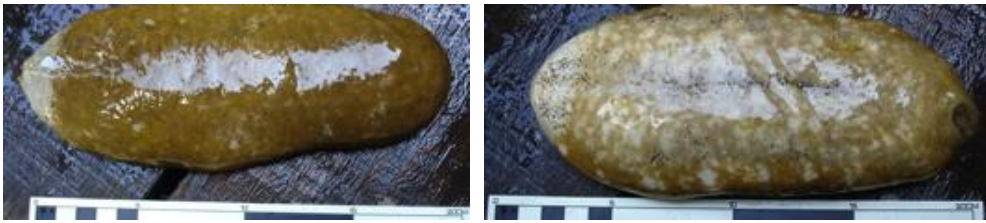
Fig.25: *Actinopyga lecanora* (Jaeger, 1833) – 4; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)
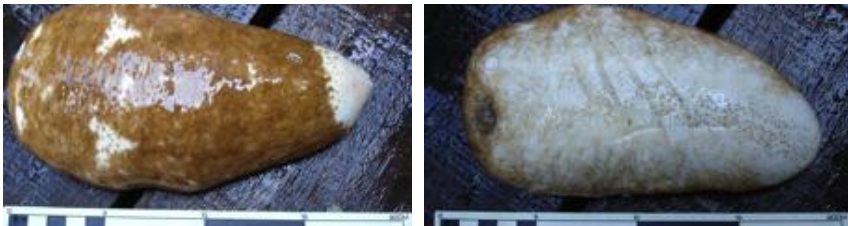
Fig.26: *Actinopyga lecanora* (Jaeger, 1833) – 5; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Fig.27: *Actinopyga lecanora* (Jaeger, 1833) – 6; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Fig.28: *Actinopyga lecanora* (Jaeger, 1833) – 7; Left = dorsal view, right = ventral-dorsal view (Photo
source: Ridzwan Hashim)

Fig.29: *Actinopyga lecanora* (Jaeger, 1833) – 8; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Fig.30: *Actinopyga lecanora* (Jaeger, 1833) – 9; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)
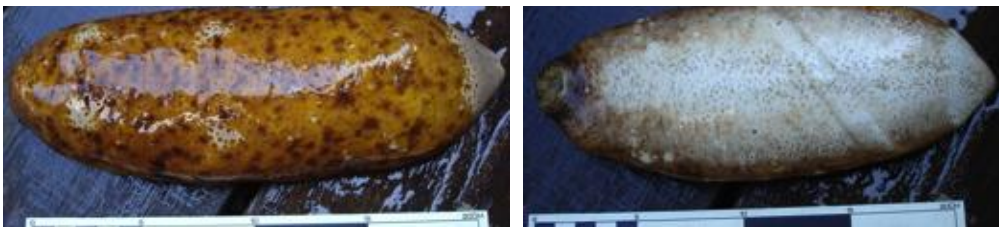
Fig.31: *Actinopyga lecanora* (Jaeger, 1833) – 10; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Fig.32: *Actinopyga lecanora* (Jaeger, 1833) – 11; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Fig.33: *Actinopyga lecanora* (Jaeger, 1833) – 12; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)
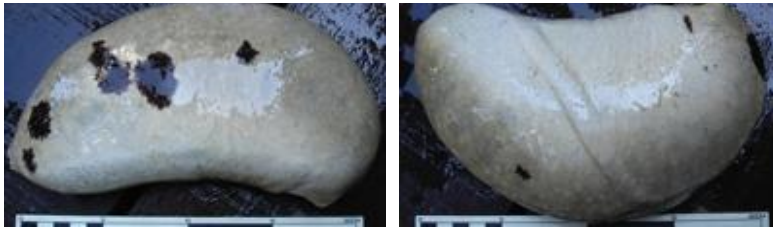
Fig.34: *Actinopyga lecanora* (Jaeger, 1833) – 13; Left = dorsal view, right = ventral-dorsal view
(Photo source: Ridzwan Hashim)
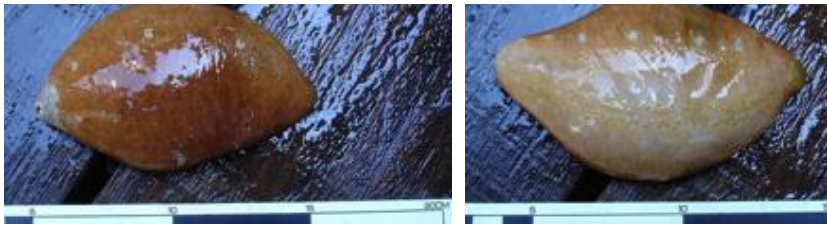
Fig.35: *Actinopyga lecanora* (Jaeger, 1833) – 14; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Fig.36: *Actinopyga lecanora* (Jaeger, 1833) – 15; Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Genus *Pearsonothuria* – 1 species
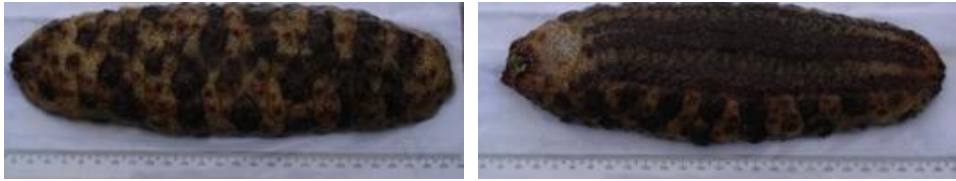
Fig.37: *Pearsonothuria graeffei* (Semper, 1868); Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Family Stichopodidae (9 species)

Genus *Stichopus* – 8 species – Fig.38 until Fig.45

Fig.38: *Stichopus rubermaculosus* **(**Massin, Zulfigar, Hwai, & Boss, 2002); Left = dorsal view, right =
ventral view (Photo source: Ridzwan Hashim)
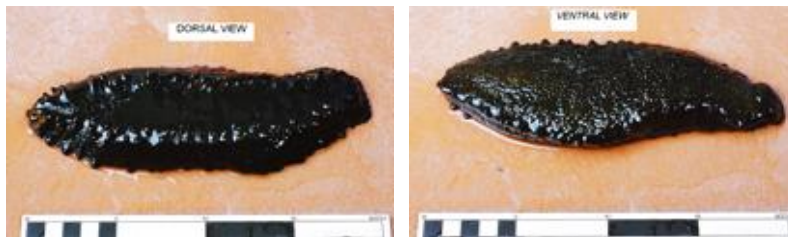
Fig.39: *Stichopus chloronotus* (Brandt, 1835); Left = dorsal view, right = ventral view
(Photo source: Department of Museum, Malaysia)

Fig.40: *Stichopus horrens* (Selenka, 1867); Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)
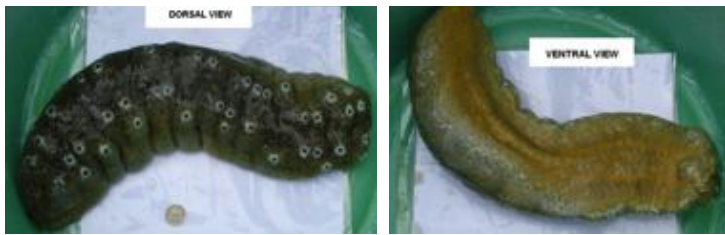
Fig.41: *Stichopus ocellatus* (Massin, Zulfigar, Hwai, & Boss, 2002); Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)
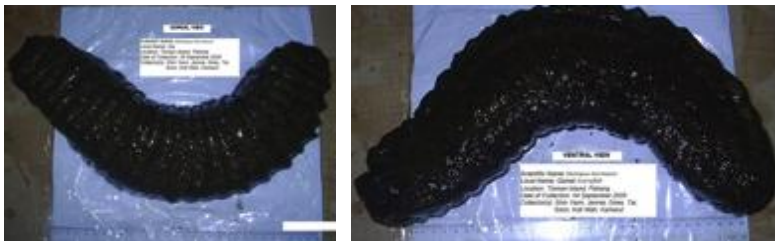
Fig.42: *Stichopus herrmanni* (Semper, 1868); Left = dorsal view, right = ventral view
(Photo source: Kamarul Rahim Kamarudin)

Fig.43: *Stichopus vastus* (Sluiter, 1887); Left = dorsal view, right = ventral view
(Photo source: Department of Museum, Malaysia)

Fig.44: *Stichopus* sp. – 1; Left = dorsal view, right = ventral view (Photo source: Ridzwan Hashim)
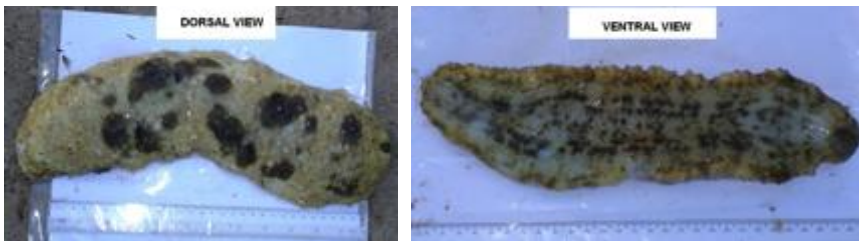
Fig.45: *Stichopus* sp. – 2; Left = dorsal view, right = ventral view (Photo source: Ridzwan Hashim)

Genus *Thelenota* – 1 species

Fig.46: *Thelenota anax* (Clark, 1921); Left = dorsal view, right = ventral view
(Photo source: Ridzwan Hashim)

Order Molpadiida (1 species)

Genus *Acaudina* - 1 species

Fig.47: *Acaudina molpadioides* (Semper, 1867) (Photo source: Department of Museum, Malaysia)

Order Apodida (4 species)

Genus *Synapta* – 2 species

Fig.48: *Synapta maculata* (Chamisso & Eysenhardt, 1821) (Photo source: Ridzwan Hashim)

Fig.49: *Synapta* sp. - 1 (Photo source: Ridzwan Hashim)

Genus *Polycheira* – 1 species

Fig.50: *Polycheira rufescens* (Brandt, 1835) (Photo source: Kamarul Rahim Kamarudin)

Genus *Leptosynapta* – 1 species

Fig.51: *Leptosynapta* sp. – 1 (Photo source: Kamarul Rahim Kamarudin)

Order Dendrochirotida (2 species)

Genus *Thyone* – 1 species

Fig.52: *Thyone* sp. - 1 (Photo source: Department of Museum, Malaysia)

Genus *Cercodemas* – 1 species

Fig.53: *Cercodemas anceps* (Selenka, 1867); Left = dorsal view, right = ventral view (Photo source: Department of Museum, Malaysia)

CONCLUSION

Fifty two morphospecies of sea cucumbers were recorded from the present study. Four orders comprising 12 genera with 38 species were found in Sabah, 24 species in Peninsular Malaysia and 10 species that were overlaps were recorded. With regard to the *Actinopyga* genus, 14 species were found to be from Sabah and one species from Peninsular Malaysia. Further species identification was required for the nine species. High species richness was recorded from the order Aspidochirotida and within the genus *Holothuria Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835), locally known as *bat puntil*, which is suggested as the most dominant species in Malaysia. Sabah showed higher diversity of sea cucumbers as compared to Peninsular Malaysia. More study sites including from Sarawak and molecular phylogeny may lead to a better understanding of the distribution of sea cucumbers in Malaysia.

ACKNOWLEDGEMENTS

REFERENCES

Annual Fisheries Statistics, Sabah. (2000–2005). Department of Fisheries, Sabah, Kota Kinabalu, Sabah.

Baine, M., & Forbes, B. (1998). The taxonomy and exploitation of sea cucumbers in Malaysia. *SPC Beche-de-mer Information Bulletin, 10,* 2-7.

Baine, M., & Sze, C. P. (1999). Sea cucumber fisheries in Malaysia: towards a conservation strategy. *SPC Beche-de-mer Information Bulletin, 12,* 6-10.

Cannon, C. H., Morley, R. J., & Bush, A. G. B. (2009). The current refugial rainforests of Sundaland are

unrepresentative of their biogeographic past and highly vulnerable to disturbance. *PNAS, 106,* 11188–11193.

Choo, P. S. (2008). Population status, fisheries and trade of sea cucumbers in Asia. In Sea cucumbers. A global review of fisheries and trade. V. Toral-Granda, A. Lovatelli, & M. Vasconcellos (Eds). *FAO Fisheries and Aquaculture Technical Paper*. No. 516.

Forbes, B., & Ilias, Z. (1999). The taxonomy and ecology of sea cucumbers in Malaysia. *SPC Beche-de-mer Information Bulletin, 12,* 5.

George, J. D., & George, J. (1987). The coral reefs of the Bodgaya Islands (Sabah: Malaysia) and Pulau Sipadan. 4. Macroinvertebrates. *Malayan Nature Journal, 40,* 225-260.

Ho, S. L. (1992). *Coral reefs of Malaysia*. Kuala Lumpur:Tropical Press Sdn. Bhd., pp.3-11, 117-119.

Huggett, R. J. (1998). *Fundamentals of biogeography*. London: Routledge Fundamentals of Physical Geography, pp.10-12.

Kamarul Rahim, K., Aisyah, M. R., Ahmad Lutfi, L., Hajar Fauzan, A., Mohd Hanafi, A., Noor Faizul Hadry, N., Ridzwan, H., Rosnah, H., & Gires, U. (2009). Coral Reef Sea Cucumbers in Malaysia. *Malaysian Journal of Science, 28,* 171-186.

Kamarul Rahim, K., & Ridzwan, H. (2005). Distribution and Taxonomic Revision of Sea Cucumbers (Echinodermata: Holothuroidea) in Several Populations of Malaysia. In *International Conference on Biogeography and Biodiversity: Wallace in Sarawak- 150 Years Later.* Sarawak: Institute of Biodiversity and Environmental Conservation (IBEC) and Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS), 13-15 July 2005. p.215.

Kamarul Rahim, K., Ridzwan, H., & Gires, U. (2010). Phylogeny of Sea Cucumber (Echinodermata: Holothuroidea) as Inferred from 16S

Mitochondrial rRNA Gene Sequences. *Sains Malaysiana, 39,* 209-218.

Kaswandi, M.A., Nor 'Aini, A. G., & Ridzwan, B. H. (1990). Profil protein dinding timun laut. *Malaysian Biochemical Society Conference, 15,* 132-134.

Kaswandi, M. A., Ridzwan, B. H., Mohd Nor, I., Zainab, A., Azman, S., & Rozali, M. B. O. (1995). Timun Laut: Analisis Semasa. In *Simposium Sumber Alam Kebangsaan Pertama FSSA*, 3: pp.17-32, Sabah: UKM Kampus Sabah.

Lane, D. J. W. (2005). Sea cucumber diversity and resources in Brunei, Borneo Island. *SPC Beche-de-mer Information Bulletin, 22,* 57-58.

Lane, D. J. W. (2004). Sea cucumber diversity and resources in shallow waters of Brunei, Borneo Island. *SPC Beche-de-mer Information Bulletin, 19,* 62.

Lane, D., Marsh, L. M., Vandenspiegel, D., & Rowe, F. W. E. (2000). Echinoderm fauna of the South China Sea: an inventory and analysis of distribution patterns. *The Raffles Bulletin of Zoology Supplement, 8,* 459-493.

Mohsin, A. K. M. & Ambak, M. A. (1991). *Ikan Air Tawar Di Semenanjung Malaysia*. Kuala Lumpur: Dewan Bahasa dan Pustaka, pp.6-7.

Ridzwan, B. H. (2011). *Timun Laut Warisan Malaysia*. Kuala Lumpur, Malaysia: Research Management Centre, International Islamic University Malaysia.

Ridzwan, B. H. (1993). *Sumber Makanan Persisiran Laut Sabah*. Malaysia: Dewan Bahasa dan Pustaka, pp. 309.

Ridzwan, B. H. (1987). Kajian holothurian di perairan cetek Sabah: nilai pemakanan, kandungan lumen usus dan tumbesaran. In *Biologi Untuk Industri*. M. Noraini *et al*. UKM, Bangi, Selangor, Malaysia: UKM Publisher. pp.339-369.

Ridzwan, B. H., & Che Bashah, C. C. (1985). Tinjauan awal taburan dan penilaian holothurian

di beberapa kawasan perairan Sabah sebagai sumber pemakanan. *SUMBER, I,* 143-155.

Ridzwan, B. H., & Kaswandi, M. A. (1995). Hidupan marin intertidal: makanan tambahan penduduk daerah Semporna. *Malaysian Journal of Nutrition, 1,* 105-114.

Ridzwan, B. H., Haniza, B. H., Dayang Fredalina, B., Kaswandi, M. A., Norlida, A. L., Suhaina, S., Zali, B. I., & Mohd. Nor, H. (1996). A brief insight into current research on sea cucumber of Malaysia. In *Joint seminar by UKM-Universitas Hassanudin, Dinamika Perkembangan Kebaharian Masyarakat Indonesia dan Malaysia*, Ujung Pandang, Sulawesi, 10-12 September 1996.

Ridzwan, B. H., Shukri, H. I., Fadhil, M. H., Noor Ibrahim, M. S., Kaswandi, M. A., Hawa, I., Jamaludin, M., Rozali, M. B. O., Mohd. Nor, B. H., & Zali, B. I. (1998a). The species distribution of sea cucumbers in Malaysia. In *Malaysia Science and Technology Congress '98*, Kota Kinabalu, Sabah, 23-25 November 1998.

Ridzwan, B. H., Kaswandi, M. A. Dyg. Fredalina, B., & Haniza, B. (1995). A Brief Look Into Current Research On Sea Cucumbers in Malaysia. In *JSPS-VCC Joint Seminar On Marine Science*: Universiti Putra Malaysia (UPM), 5-8 December 1995.

Ridzwan, B. H., Noor Ibrahim, M. S., Nihayah, M., Shukri, H. I., Zali, I., Mohd. Nor, H., Awang, H., Nasir, B., & Basri, I. (1998b). A preliminary observation of the possibility of rearing sea cucumbers in cages. In *Malaysia Science and Technology Congress '98*, Kota Kinabalu, Sabah, 23-25 November 1998.

Sim, Y. K., Tan, S. H., & Zulfigar, Y. (2009). The Diversity and Abundance of the Sea Cucumber (Echinodermata: Holothuroidea) from the Archipelagos of Beting Patinggi Ali, Beting Raja Jarom and Pulau Layang–Layang, South China Sea. In *6th IMT-GT UNINET Conference 2008: Sustaining Natural Resources towards Enhancing the Quality of Life within the IMT-GT Zone.* Penang: The Gurney Resort Hotel, 25-30 August 2008. pp.357-360.

Sim, Y. K., Tan, S. H., Zulfigar, Y., Wong, W. S., Chai, J., Ng, B. W., & Luar, L. (2008). The Diversity and Distribution of Holothuroidea in Pulau Aur, Johor. In *Research and Information Series of Malaysian coast 2.* Pusat Penyelidikan Ekosistem Marin (EKOMAR), UKM. pp.13-22.

Siti, Z. R. S., Zulfigar, B. Y., & Aileen, T. S. H. (1999a). Species of sea cucumber found in Pulau Besar, Johor, with special emphasis on the genus *Stichopus*. *SPC Beche-de-mer Information Bulletin, 12*, 4.

Siti, Z. R. B., Zulfigar, Y., & Tan, S. H. (1999b). Distribution of Sea cucumber Species at Pulau Besar, Johor with special emphasis on the *Stichopus* genus. In *Sea Cucumber International Conference.* Kuala Lumpur. pp.37-41.

Zaidnuddin, I. (2002). Sea cucumber species diversity and abundance from the south–western waters of Balik Pulau, Penang. In *Workshop on the impact of development on the coastal fisheries off south–west Penang Island, Malaysia.* P.S. Choo, I. Ismail, P. E. Chee, & Chuah, T. T. Fisheries Research Institute, Penang, Malaysia. pp.57–62.

Zaidnuddin, I., & Forbes, R. (2000). Sea cucumbers of Pulau Payar, Kedah Marine Park Islands. In *Proceedings of National Symposium on Pulau Payar Marine Park.* Ali, A., Arshad, M. A., Ilias, Z. and Mohd Sah, S. A. (Eds.) (pp.174–177).

Zulfigar, Y., & Tan, S. H. (1999). Biodiversity of sea cucumber in the lower part of The South China Sea. In *The Joint Seminar on Marine and Fisheries Sciences.* Malaysia: Melaka, 1-3 December 1999. pp.73-82.

Zulfigar, Y., Sim, Y. K., & Tan, S. H. (2007). The Distribution of Sea Cucumbers in Pulau Aur, Johore, Malaysia. In *Publications of the Seto Marine Biological Laboratory, Special*

Publication Series Volume 8 (from Selected Paper of NaGISA World Congress 2006), Japan: Seto Marine Biological Laboratory, Field Science Education and Research Centre, Kyoto University. pp.73-86.

Zulfigar, Y., Sim, Y. K., Tan, S. H., & Shirayama, Y. (2008). *Field guide to the Echinoderms (Sea cucumber and Sea Stars) of Malaysia*. The Nippon Foundation, Japan: Kyoto University Press. p.103.

Zulfigar, Y., Tan, S. H., Fujita, T., & Terazaki, M. (2000). The Distribution of sea cucumber associated with seagrass beds found in The South China Sea. *In the 11th JSPS Joint Seminar on Marine Science*, pp.362-368.

Inherent Variability in Growth and Development of Two Ecotypes of Sesame Plant (*Sesamum* sp.) in Malaysia

Tan, S. P., Rosli, B. M., Adam, B. P. and M. M. Morshed*

Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Differences in the growth and development of sesame ecotypes growing sporadically as uncultivated plant in dissimilar agro-ecological and habitat conditions of Serdang and Port Dickson of Malaysia have not been studied. The purpose of the study was to compare the inherent variations in growth parameters and degree of relationships between the two ecotypes over time. The quantitative growth parameters of the plants' height, leaf number per plant, total leaf area, fresh and dry weight of leaves, fresh and dry weight of roots were measured at 2, 4, 8 and 12 weeks after planting under the same growing conditions. Results indicated that the two ecotypes manifested almost similar growth patterns and the variations on growth parameters between the ecotypes were statistically insignificant. Regression analysis showed that all the growth parameters increased exponentially over time, and the growth parameters were positively correlated between the ecotypes. Thus, this study suggested that a high homology in the growth patterns between the two sesame ecotype could exist when grown under the same growing conditions, although both have adapted to different habitat conditions.

Keywords: Ecotypes, growth parameters, morphological characters, sesame, Malaysia

INTRODUCTION

Sesame (*Sesamum* sp.) is an ancient annual oilseed crop that belongs to the *Pedaliaceae* family (Kobayashi, 1991). It is a short duration crop grown widely in tropical and subtropical areas as a source of high quality edible oil and dietary supplement (Koca *et al.*, 2007). There are approximately 38 species of the genus *Sesamum* worldwide,

and most of them are wild (Bisht *et al.*, 1998). However, most of the wild species are located in Africa, which subsequently spread to India and China in very early times (Ashri, 1989; Bisht *et al.*, 1998). Similar to other crops that have been domesticated for a long time, different varieties of *Sesamum indicum* were found to have considerable variability in plant size, shape, growth habit, corolla colour, seed size, colour, and composition (Weiss, 2000). In fact, different cultivars (landraces) can develop very differently under the same conditions, and the same cultivars can develop very differently under different conditions (Langham, 2007). According to IPGRI (2004), there are both intra- and inter-specific diversities in a sizeable number of morphological traits of sesame as revealed in germplasm characterization and evaluation studies. However, the variations in the growth and development of sesame plants have major effects on how plants respond to yield potential and on yield quality (Langham, 2008). On the other hand, plant's growth and development affect its characteristics and processes, as well as the magnitude of their responses to environmental influence and/or production inputs (Groot *et al.*, 1986).

Although Malaysia is endowed with favourable ecologies for sesame cultivation, planting of commercial sesame is still lacking in Malaysia due to the insufficient information of the local species/cultivars. Over the past few years, uses of sesame as cooking oil and industrial solvent, as well as in food and drug manufacture, have remarkably increased in Malaysia. This

was substantiated by the trend of sesame seed import from 2003-2007 in Malaysia during which the imported quantity was of 7,584 tons, with an increase of 9,131 tons in 2007 (FAO, 2003-2007). Due to the rise of consumption volume, sesame has received increasing interest in Malaysian agriculture, with an aim to cultivate commercially to meet the domestic demands of this excellent source of oil with antioxidant constituents (Weiss, 1983). It was reported that two sesame species *Sesamum radiatum* (with black seeds) and *Sesamum indicum* (with pale white seeds) have been sporadically growing as uncultivated (wild) plant in some agro-ecological areas of Malaysia such as Malacca, Port Dickson, Serdang and Langkawi (Yao, 2010). In the previous study by Khairi (2011), two ecotypes identified as *Sesamum radiatum* were seen growing widely in dissimilar habitats of Serdang and Port Dickson. It has been recognized that some of the morphological (quantitative) traits between the two ecotypes significantly varied with leaf length, stem circumference and branching, even though they produced seeds of basically similar physical characteristics (length, width and thickness). However, information regarding these two ecotypes with respect to their growth and development is still limited. The evaluation of growth and the development of the ecotypes are important since it directly translates into seed production in sesame cultivation. This study was, therefore, carried out to obtain information necessary to draw comparisons of growth and development of the two sesame ecotypes collected from Port Dickson and Serdang in Malaysia.

MATERIAL AND METHODS

Source of Seed and Plant's Habitat Conditions

Sesame seeds of two ecotypes (*Sesamum radiatum*) were collected from Peninsular Malaysia: Serdang, Selangor (2.9992°N, 101.7078°E) and Port Dickson, Negeri Sembilan (2.5167°N, 101.8008°E). Although sesame plants of both populations were found to grow in open areas with other plants, some different habitat and soil conditions were observed in relation to their growth and development.

The Serdang population (Fig.1) grows in several spotted clumps (about 10 m² each) on less attended cattle grazing paddock, while the Port Dickson population (Fig.2) grows individually and distributed within a certain distance along the river and road. On the other hand, the soil where the serdang population grows is dark in colour having pH4.79 with nutrient contents of 2.6%N, 0.004% P, 0.01% K, 0.01% Ca, and 0.002% Mg, whereas the soil in the Port Dickson is reddish with pH3.97, and nutrient contents of 10% N, 0.003% P, 0.006% K, 0.02% Ca, and 0.001% Mg (Khairi, 2011).

Fig.1: Serdang population

Fig.2: Port Dickson population

Morphological Characteristics of the Serdang and Port Dickson Ecotypes

The range of variations among morphological (quantitative and qualitative) characteristics from the Serdang and Port Dickson populations showed the following descriptions and scores (see Table 1) (Khairi, 2011).

Seeds Processing

The collected seeds were sieved to remove extraneous plant or floral materials. The sieved seeds were divided into light and heavy fractions by using the seed blower (Model AMPS 1). The heavy fraction, which represents the fully developed seeds, was used in raising seedlings (Clewis *et al.*, 2007). The seeds were then surface sterilized with 0.01% $HgCl_2$ solution for one minute and subsequently rinsed several times with distilled water. This was followed by blotting the seeds with filter paper and drying them at room temperature (25-30^{0}C).

Raising of Seedling

Seeds of each ecotype were broadcast in a separate plastic tray of 5 L size filled with 4 kg sandy loam soil. The plastic tray was kept at the nursery shed at Field 2 of Universiti Putra Malysia. Immediately after sowing, light watering was given to ensure good germination. Further watering was carried out regularly until the seedlings were fully established.

Experimental Design and Set Up

The experiment was conducted as a completely randomized design (CRD) with five replications. Forty sesame seedlings of each ecotype at 4 weeks old were hand transplanted in 3:2:1 (top soil: peat: sand) ratio of soil mixture in polybags (14 x 16 inches). Meanwhile 0.5 g (25 kg/ha) fertilizer each of N, P_2O_5 and K_2O per polybag was made as basal applications. Polybags containing one plant in each were placed in the field with a spacing of 30 cm between the rows and 15 cm between the plants. During the study period, the weather parameters at the experimental location were favourable for sesame growth. Temperature was in a range of 25.6 to 32.2^{0}C, and average relative humidity was 70%. The plants were irrigated during the growth period and pesticides were applied whenever necessary.

Measurement of Growth Parameters

Growth parameters of both the sesame ecotypes were measured on three randomly selected plants, out of the eight plants under each replication. The quantitative growth parameters measured were plant height, leaf number, total leaf area, fresh and dry weight of leaves, as well as fresh and dry weight of roots. Plant height and leaf number were recorded twice in a month (every 2 weeks). In addition, two plants from each replicate were harvested at 4, 8, and 12 weeks after planting so as to measure fresh and dry weight of roots and leaves as well as total leaf area. The leaf area was measured using a leaf area meter (LI-3100, USA). For dry

TABLE 1
The morphological characteristics of Serdang and Port Dickson ecotypes

No	Quantitative characteristics	Descriptors and scores	
		Serdang	Port Dickson
1	Plant height	Tallest = 102 cm; Shortest = 45 cm	Tallest = 126 cm; Shortest = 57 cm
2	Circumference of stem *	Largest = 6.5cm; Smallest = 3 cm	Largest = 11.5 cm; Smallest = 2 cm
3	No. of branches *	Highest = 6; Lowest = 1	Highest = 10; Lowest = 3
4	No. of leaves on main stem	Highest = more than 300; Lowest = less than 100	Highest = more than 300; Lowest =less than 100
5	No of leaves with serrate margin on main stem	Highest = more than 50; Lowest = less than 50	Highest = more than 50; Lowest = less than 50
6	Leaf length*	Highest = 5.39 cm; Lowest = 1.86 cm	Highest = 9.47 cm; Lowest = 5.27 cm
7	Leaf width	Highest = 8.42 cm; Lowest = 2.49 cm	Highest = 2.9 cm; Lowest = 1.43 cm
8	Petiole length	Highest = 2.13 cm; Lowest = 0.49 cm	Highest = 2.12 cm; Lowest = 0.64 cm
	Qualitative characteristics		
9	Pubescences on stem	(1) Present	(1) Present
10	Stem colour	(1) Green	(1) Green to (3) brown
11	Degree of branches	Range from (1) more than 450, (2) lower than 450 or (3) equal 450	Range from (1) more than 450, (2) lower than 450 or (3) equal 450
12	Leaf colour	(1) Green	(1) Green
13	Leaf arrangement	(2) Alternate	(2) Alternate
14	Leaf apex shape	(2) Acute	(2) Acute
15	Leaf base shape	(2) Cuneate	(2) Cuneate
16	Leaf margin	(1) Entire to (2) Serrate	(1) Entire to (2) Serrate
17	Leaf texture	(2) Thick	(2) Thick
18	Type of primary vein	(1) Pinnate	(1) Pinnate
19	Type of secondary vein	(1) Brochidodromous	(1) Brochidodromous
20	Type of tertiary vein	(1) Reticulate	(1) Reticulate

* mean significant differences of the characters at $P<0.05$

weight measurements, the roots and leaves were oven dried for three to five days at 70-80°C.

Statistical Analysis

The data were analyzed by using analysis of variance (ANOVA). Mean separations were conducted by least significant difference (LSD) test at $P < 0.05$ level using Statistical Analysis System (SAS, 1998). Regression analysis was carried out to describe the nature and degree of relationship on various growth parameters between two ecotypes over time.

RESULTS

Data on the growth parameters of plants' height, leaf number per plant, root fresh weight, root dry weight, leaf fresh weight, leaf dry weight and total leaf area showed no significant difference between the Port Dickson and Serdang sesame ecotypes (Table 2).

Plant Height

Record on plant height indicated that it increased exponentially over time (Fig.3).

There was significant correlation between plant height and time. Regression analysis showed that the plant height of Port Dickson ($R^2 = 0.8488$) and Serdang ($R^2 = 0.9273$) ecotypes was positively correlated with time. However, there was no significant difference between the plant's height of the two ecotypes over the time until 12 weeks after planting. The plant height of Port Dickson (67.3 cm) showed a higher value compared to Serdang ecotype (61.05 cm) at 12 weeks after planting when the plants started flowering (Table 2).
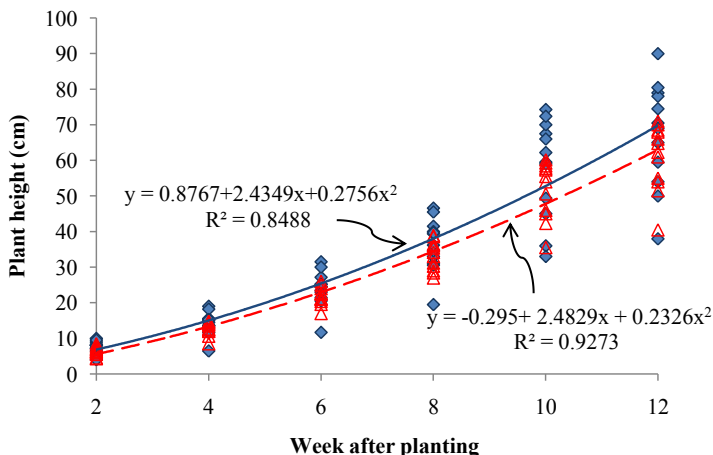
Fig.3: Plant height of Port Dickson (◆) and Serdang (△) sesame ecotypes from transplanting to 12 week after planting

In the figure: $y = 0.8767 + 2.4349x + 0.2756x^2$, $R^2 = 0.8488$ and $y = -0.295 + 2.4829x + 0.2326x^2$, $R^2 = 0.9273$

TABLE 2
Growth parameters of Port Dickson and Serdang sesame ecotypes at week 12 after planting

Ecotype	Plant height (cm)	Leaf number per plant -	Root fresh weight (g)	Root dry weight (g)	Leaf fresh weight (g)	Leaf Dry weight (g)	Total leaf area (cm^2)
Port Dickson	67.33	109.92	14.14	3.52	22.95	5.51	770.6
Serdang	61.05	89.00	11.99	2.72	23.14	5.26	794.7
LSD	10.375	27.21	3.0276	0.7687	6.0698	1.5899	224

LSD at ($P < 0.05$)

Leaf Number per Plant

Leaf number per plant also increased exponentially over time and the relationship between the leaf number of Port Dickson ($R^2 = 0.7247$) and Serdang ($R^2 = 0.8541$) and time was significantly correlated. The results showed that leaf number increased with age of both the genotypes but leaf production ability of Port Dickson was insignificantly greater than that of Serdang at all growth stages (Fig.4). At 2 weeks after planting, the leaf number per plant of Port Dickson and Serdang ecotypes was 16 and 10, respectively, whereas at 12 weeks after planting, the Port Dickson and Serdang ecotype was 110.92, and 89, respectively (Table 2).

Root Fresh Weight

Data subjected to regression analysis showed that root fresh weight of Port Dickson ($R^2 = 0.7001$) and Serdang ecotype ($R^2 = 0.8227$) was significantly correlated with times, and it increased positively over time (Fig.5). However, the root fresh weight for Port Dickson ecotype exhibited higher root fresh weight in comparison to Serdang ecotype at weeks 4 to 12 after planting, which was statistically insignificant (Table 2). The root fresh weight was 1.92 g and 1.61 g at week 4, which subsequently increased to 14.14 g and 11.99 g at week 12, after planting for the Port Dickson and Serdang ecotypes, respectively.

Root Dry Weight

Root dry weight of both ecotypes increased exponentially from week 4 to week 12 (Fig.6). The root dry weight of Port Dickson ($R^2 = 0.7022$) and Serdang ($R^2 = 0.8319$) ecotypes were significantly correlated with times. At week 4 after planting, the initial weight of Port Dickson and Serdang ecotype was 0.17 g and 0.13 g respectively. It increased to 1.49 g and 1.23 g at week 8, and continued to increase to 3.52 g and
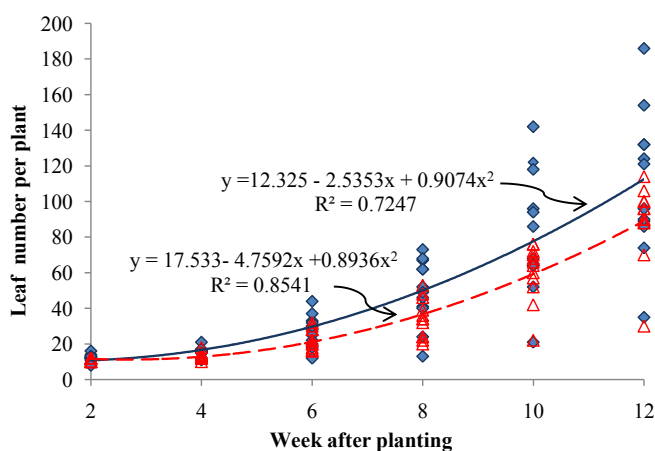
Fig.4: Leaf number per plant of Port Dickson (◆) and Serdang (△) sesame ecotypes from transplanting to 12 week after planting

2.72 g at week 12 after planting, for the Port Dickson and Serdang ecotypes, respectively (Table 2). However, there was no significant difference between the two ecotypes.

Leaf Fresh Weight

Leaf fresh weight of the sesame plants increased exponentially over time (Fig.7). Nonetheless, no significant differences were observed on the leaf fresh weight between the Port Dickson and Serdang ecotypes.
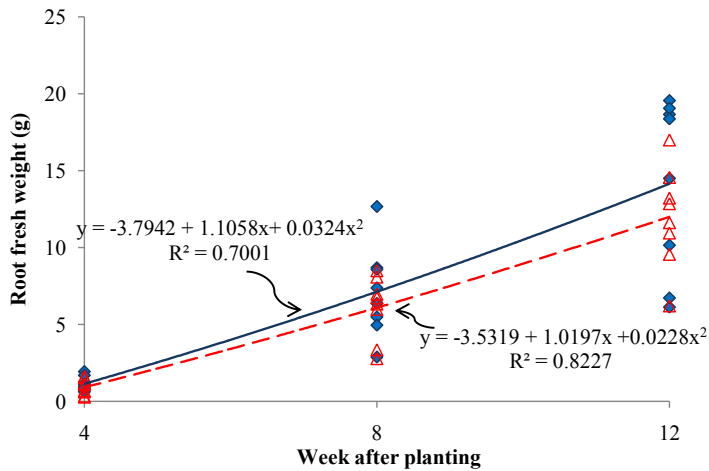
Fig.5: Root fresh weight of Port Dickson (◆) and Serdang (△) sesame ecotypes from transplanting to 12 weeks after planting
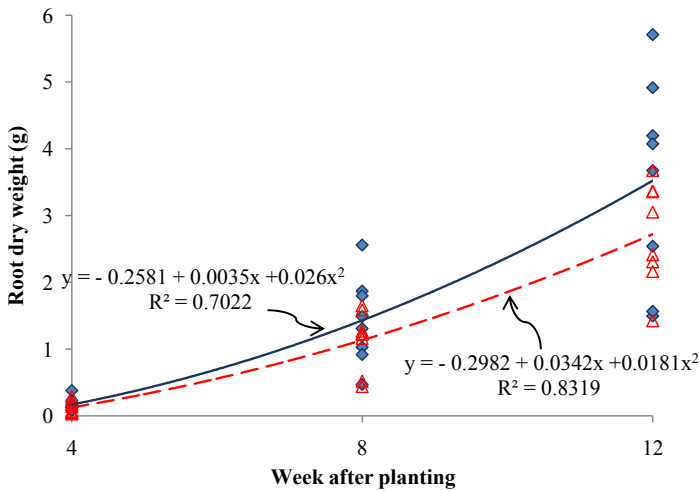
Fig. 6: Root dry weight of Port Dickson (◆) and Serdang(△)sesame ecotypes from transplanting to 12 week after planting.

The regression analysis showed that the leaf fresh weight of the two sesame ecotypes had strong relationship with time, which showed $R^2 = 0.7402$ for Port Dickson ecotype and $R^2 = 0.8169$ for Serdang ecotype, respectively. The leaf fresh weight of both ecotypes was almost the same from week 8 to week 12 after planting. It reached 22.95 g for Port Dickson and 23.14 g for Serdang ecotype at week 12 after planting (Table 2).

Leaf Dry Weight

The leaf dry weight of sesame plant also increased exponentially from week 4 to week 12 after planting (Fig.8). The increment was from 0.87 g to 5.51 g for Port Dickson ecotype and 0.78 g to 5.26 g for Serdang ecotype. The regression analysis showed that the leaf dry weight of Port Dickson ($R^2 = 0.7208$) and Serdang ecotype ($R^2 = 0.8191$) was correlated with time. However, no significant difference was observed for the leaf dry weight between the two ecotypes (Table 2). At week 12 after planting, the leaf dry weight for Port Dickson and Serdang ecotype was 5.51 g and 5.26 g, respectively.

Total Leaf Area

Total leaf surface area increased exponentially from week 4 to week 12 after planting (Fig.9). The regression analysis showed that the leaf surface area of Port Dickson ecotype ($R^2 = 0.7377$) and Serdang ecotype ($R^2 = 0.8012$) was generally correlated with time. There was also no significant difference in the total leaf area between Port Dickson and Serdang ecotypes over time. At the initial stage, the total leaf area of Port Dickson ecotype (147.59 cm^2) was higher than that of Serdang ecotype (96.80 cm^2). However, the total leaf area of Serdang ecotype increased more steadily after week 8, and it
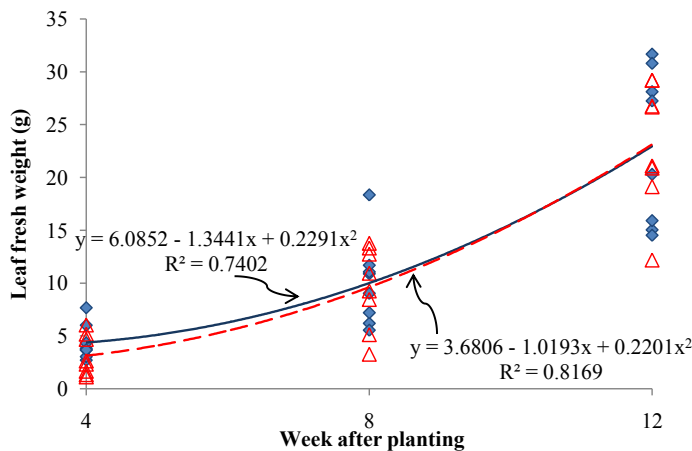
Fig.7: Leaf fresh weight of Port Dickson (◆) and Serdang (△) sesame ecotypes from transplanting to 12 week after planting
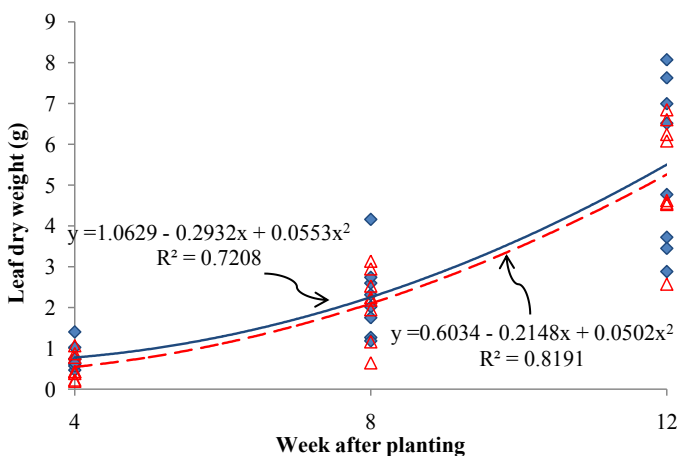
Fig.8: Leaf dry weight of of Port Dickson (◆) and Serdang (△) sesame ecotypes from transplanting to 12 weeks after planting
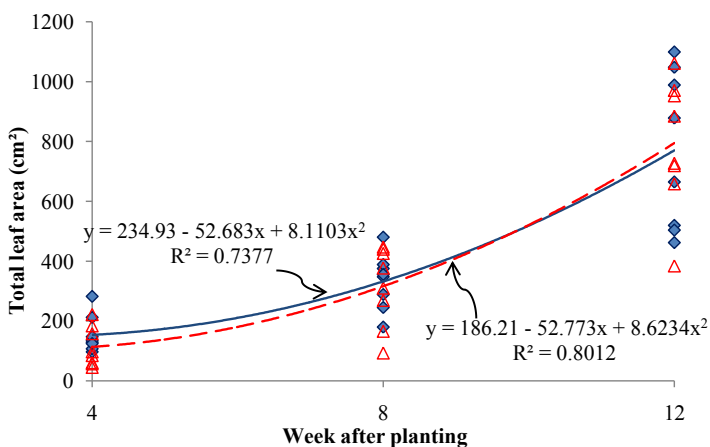
Fig.9: Total leaf area of Port Dickson (◆) and Serdang (△) sesame ecotypes from transplanting to 12 weeks after planting

was slightly higher (794.7 cm²) than that of Port Dickson ecotype (770.6 cm²) at week 12 after planting (Table 2).

DISCUSSION

The results obtained from the study showed that there were no statistically significant differences on the growth parameters between the two sesame ecotypes although both had adapted to different agro-ecological and habitat conditions. It was generally agreed that the phenotypic or morphological traits of a plant are the reflection of the interaction between the crop's genotype and the existing climatic and ecological factors (Akinyele & Osekita,

2006). Since the study had eliminated the environmentally induced variations by bringing the two ecotypes into cultivation under the same environmental factors, the growth parameters that showed insignificant variations between the two ecotypes were most likely due to the genotypic similarity as both belonged to the same species of *Sesamum radiatum*. This could be confirmed through morphological characterization and identification of both populations with different molecular tools. During the whole growth period, the Port Dickson plants recorded lower height, leaf number per plant and dry matter accumulation in shoot and root compared to Port Dickson ecotype. In Port Dickson ecotype, higher plant height, coupled with higher leaf number per plant, led to a higher photosynthetic activity that ultimately increased the partitioning of dry matter in the shoot and root at both early and late growth phase. Earlier observations on growth performances among the Indian sesame cultivars revealed by Basavaraj *et al*. (2000) confirmed the results of the present study.

In the regression analysis, all the growth factors of sesame such as plant height, leaf number, root fresh weight, leaf fresh weight, leaf dry weight, and total leaf area increased exponentially with time (note that this is expected, growth having a sigmoidal curve). Port Dickson and Serdang ecotypes had similar growth pattern when grown in the soil medium under the same environmental field. The exponential growth pattern with time of the growth factors showed conformity with the growth stages as described by Langham *et al*. (2008). The flattened graph for all the growth factors during the early growth period of 4 to 8 weeks after planting indicated slow growth, while the growth was faster as indicated by the steeper graph during the second phase (8 to 12 weeks after planting). These observations confirmed the results reported by Langham *et al*. (2008) on sesame as characterized by a slower growth rate in the first 30 days, when the roots grow faster than the leaves and stems. In the early growth period, sesame grows slowly because it uses its resources for the development of the root system to get proper moisture.

CONCLUSION

Since sesame plants have shown tremendous amounts of variability in the vegetative phases that ultimately affect its yield and quality, growth and development of sesame are therefore of great importance. In this study, two cultivated sesame ecotypes showed no significant differences in the growth parameters studied. On the basis of the present findings, it is found that even though the two ecotypes naturally habituated in different environmental conditions, similar growth patterns over various growth parameters of sesame plants were observed when they were grown under the same growing conditions.

REFERENCES

Akinyele, B. O., & Osekita, O. S. (2006). Correlation and path coefficient analyses of seed yield attributes in okra – *Abelmoschus esculentus* (L.) Moench. *Afr. J. Biotechnol., 5*(14), 1330-1336.

Ashri, A. (1998). Sesame breeding. In J. Janick (Ed.), *Plant breeding review* (pp. 179-228). John Wiley and Sons, Inc.

Basavaraj, B., Shetty, R. A., Patil, S. G., & Hunshal, C. S. (2000). Nutrient content and uptake by sesame varieties as influenced by fertilizer and population levels under summer irrigated conditions. *Karnataka Journal of Agricultural Sciences*, *13*(1), 141-143.

Bisht, I. S., Mahajan, R. K., Loknathan, T. R., & Agrawal, R. C. (1998). Diversity in Indian sesame collection and stratification of germplasm accessions in different diversity groups. *Gen. Res. Crop Evol., 43*, 325-335.

Clewis, S. B., Jordan, D. L., Wilcut, J. W. (2007). Influence of environmental factors on cutleaf eveningprimrose (*Oenothera laciniata*) germination, emergence, development, vegetative growth and control. *Weed Sci., 55*, 264-272.

FAO (Food and Agriculture Organization). (2003-2007). *Market for Sesamum Seeds: Import Analysis From Year 2003 to 2007*. FAO: Region for Asia and Pacific with UNCTAD - APEDA Agri Exchange, India.

Groot, J. J. R., Kropff, M., Vossen, F. J. H., Spitters, C. J. T., & Rabbinge, R., (1986). A decimal code for the developmental stages of maize and its relation to accumulated heat units. *Netherlands J. Agric. Sci., 34*, 67-73.

IPGRI (International Plant Genetic Resources Institute). (2004). *Descriptors for sesame* (*Sesamum* spp.). Rome, Italy. p. 63.

Khairi, I. H. K. (2011). *Seed physical properties and habitat analysis of two sesame ecotypes in Malaysia*. Project paper (PRT 4999, 2009/2010), Bachelor of Agricultural Science, Faculty of Agriculture, Universiti Putra Malaysia, Selangor D.E., Malaysia.

Kobayashi, T. (1991). Cytogenetics of sesame (*Sesamum indicum*). In T. Tsuchiya & R.K Gupta (Eds.), *Chromosome engineering in plants: Genetics, breeding, evolution* (pp. 581-592). Elsevier Science Publishing Company Inc. Amesterdam.

Koca, H., Bor, M., Ozdemir, F., & Turkan, I. (2007). The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. *Environmental and Experimental Botany, 60*, 344-351.

Langham, D. R. (2007). Phenology of Sesame. In J. Janick & A. Whipkey (Eds.), *Issues in new crops and new uses*. Alexandria, VA: ASHS Press.

Langham, D. R. (2008). *Growth and Development of Sesame*. Sesaco Corporation, USA.

Langham, D. R., Riney, J., Smith, G., & Wiemers. T. (2008). *Sesame Grower Guide*. Sesaco Corporation, San Antonio, TX.

SAS Institute Inc. (1998). *SAS User Guide*. Statistic SAS Institute Inc., Cary, NC.

Weiss, E. A. (1983). *Oilseed Crops*. Longman Group Limited. pp. 282-341.

Weiss, E. A. (2000). *Oilseed crops* (2nd edn.). Malden, MA: Blackwell Science.

Yao, T. L. (2010) Pedaliaceae. In R. Kiew, R. C. K. Chung, L. G. Saw, E. Soepadmo, & P. C. Boyce (Eds.), *Flora of Peninsular Malaysia, Seveie II: Seed Plants, Vol.1* (pp. 155-159). Malayan Forest Record No. 49, Forest Research Institute Malaysia, Kepong, Selangor D.E.

Growth Trajectory of the Nigerian Indigenous and Exotic Strains of Chicken Embryos during Incubation under Nigerian Condition

Oke, O. E.*, Obanla, L. O., Onagbesan, O. M. and Daramola, J. O.

Department of Animal Physiology, Federal University of Agriculture, Abeokuta, Nigeria

ABSTRACT

This study investigated embryonic growth and early growth rate in three strains of chicken. A total of nine hundred hatching eggs, 300 each from 3 strains of chicken, were used for this study. The strains of chicken used were Nigerian indigenous chicken (NIC), Isa Brown (IB) and Nera Black (NB) strains. Twenty eggs per strain were randomly selected for breakout at Embryonic Days (ED) 7, 11, 15, and 18 of incubation to determine embryonic weight, egg weight, egg weight loss and shell weight loss during incubation. Embryonic weight was monitored on ED 7, 11, 15, and 18. The results showed that the NIC had greater (P < 0.05) Haugh unit (71.78) than those of NB (53.23) and IB (52.36). Also, percentage egg yolk weight in NIC (28.74) was higher than that of NB (25.87) but similar to that of IB (26.55). Strain significantly (P < 0.05) affected the percentage shell weight from ED 0 until ED15, except at ED18, where they were similar. At ED7, the NB(6.12) showed significant weight loss compared to that of the IB (4.16) but the weight loss was similar to that of the NIC chickens. At ED7 and 18, there were significant differences (P < 0.05) in the embryo weight as percentage of egg weight between the strains. NIC showed a higher embryo weight as percentage of egg weight than the two other strains which were similar in value. It was concluded that the present incubation protocol is adequate for NIC as the embryo weight (expressed as percentage of egg weight) was superior to those of the exotic strains.

Keywords: Nigerian indigenous chicken, Isa Brown, Nera Black, incubation, embryonic development, egg traits

INTRODUCTION

The native chicken constitutes about 80 percent of the 120 million poultry birds found in Nigeria. These birds are also known for their adaptation superiority in terms of their resistance to endemic diseases

and other harsh environmental conditions (Nwakpu *et al.,* 1999). Adedeji *et al.* (2006) reported that these chickens adapt very well to the traditional small scale system of production that is prevalent in most areas of Nigeria. However, their characteristics of poor growth, small body size and small egg size have made them to be non-attractive and undesirable in a competitive economic situation (Ibe, 1990).

Studies have shown that the indigenous fowl possesses great potentials for improvement (Peters, 2000; Adedeji *et al.,* 2008, Adebambo *et al.*, 2009). This is due to the fact that they have some inherent advantages which include good fertility and hatchability, better flavour of meat and egg, high degree of adaptability to prevailing condition, high genetic variance in their performance, hardiness, disease tolerance, ease of rearing and ability to breed naturally (Adedeji *et al*., 2008).

The Nigerian local chickens have often been adjudged to be poor in performance with respect to egg laying, body weight, chick production and growth rate (IBe, 1990). Thus , most of the commercial layer chickens used for table egg and chick production are imported strains that have gone through several generations of selection and improvement. The Nigerian local chickens have not gone through such stringent improvement programmes. A direction of improvement is not even clear, that is, whether to improve for layer production or for broiler production. This requires a comparative study of the local chicken strain with the imported strains.

This study therefore attempted a comparison of the Nigerian local chicken (NIC) strain with two commercial strains of layer chickens (NB and *IB*) of high performance. The study evaluated hatching egg quality, incubation performance and hatchability in order to provide physiological basis for any production and economic differences between the indigenous and exotic strains in an effort to improve the performance of the native chicken breed.

MATERIALS AND METHODS

The experiment was conducted at the Teaching and Research Farm, Federal University of Agriculture Abeokuta (FUNAAB), Alabata, Ogun State, which is located within the rain forest zone of South Western Nigeria. The climate is humid with a mean annual rainfall of 1037 mm. The annual mean temperature and relative humidity are 34°C and 82%, respectively (Amujoyegbe *et al.,* 2008).

Egg collection and management

Hatching eggs (300 per strain) from two strains of exotic chickens (*IB* and NB) were collected from CHI, Ajanla Farm, Ibadan, and 300 hatching eggs from the Nigeria indigenous (NIC) normal feather chicken were collected from the Animal Breeding and Genetics Unit of the Federal University of Agriculture, Abeokuta, Nigeria. The eggs were stored for three days in the cold room at the temperature of 18°C prior to setting in the incubator. Before setting, the eggs were fumigated in the fumigation chamber for five (5) minutes using formaldehyde (40%)

and potassium permanganate crystals at a ratio of 2:1.

Incubation

The hatching eggs from each strain were replicated twice at 150 eggs per replicate. The eggs were numbered for identification and set within the same incubator. The eggs were positioned in the setting crates with broad end up to ensure ease of gas exchange (CO_2 and O_2) between the eggs and the environment.

Incubation Protocol

Single Stage Western® Incubator (Shandong, China) was used for setting of the eggs at a temperature of 37.8℃ and 60% relative humidity (R.H) with oxygen concentration of 20%. At Day 19, R.H. was increased to 70%, and towards day 21 or when the chicks were likely to be hatched, R.H. was reduced to 60%. This was to allow the chicks to dry off before being taken out of the hatcher.

Turning of eggs was automatically done by the incubator at an angle of 90^0 hourly until embryonic day (ED) 18. Turning ensures even distribution of nutrients and prevents adherent of embryo to eggshell. At ED 18, the eggs were candled in the dark room to remove unfertile eggs and the fertile eggs were transferred from setting crates to partitioned hatching trays.

Determination of Egg Quality Parameters

On the day of egg collection, the eggs from each strain were identified and weighed to ascertain the weight of the individual eggs. Ten eggs from each strain were randomly selected for breakout to take these measurements:

Egg weight, yolk weight and shell weight using Mettler top-loading weighing balance. The yolk and shell weights were subtracted from the corresponding egg weight to determine the albumen weight. Shell from the egg's equatorial region was used to measure shell thickness to 0.01 mm with the aid of micrometer screw gauge. Albumen height was measured using a spherometer to determine the Haugh unit of the individual eggs.

Haugh Units: Individual Haugh Unit (HU) score was calculated using the egg weight and albumen height (Haugh, 1937). The Haugh Unit value was calculated for individual eggs using the following formula:

$$HU = 100 \log_{10} (H + 7.5 - 1.7W^{0.37})$$

where:, H = Observed height of the albumen in mm, W = Weight of egg in grams

Determination of Incubation Phase Parameters

Twenty eggs per strain were randomly selected for breakout at ED7, 11, 15, and 18 of incubation to collect data on the following: Egg weight (to determine egg weight loss during incubation) and eggshell weight to determine the shell weight loss. The same weighing balance used in egg quality parameters was also used.

Determination of Unfertile Eggs, Embryo Weight and Embryo Mortality

At the egg breakout, the developing embryo was carefully separated from the thick

albumen and yolk. The embryo was weighed using a Mettler top-loading weighing. Unfertile egg, dead-in-germ (DIG) and dead-in-shell (DIS) embryos were examined on the days of breakout. After candling, the eggs that were not transferred to the hatcher at Day 18 of incubation and unhatched eggs were also broken to examine the unfertile eggs, DIG and DIS embryos.

Experimental Design

The experiment was a Completely Randomized Design (CRD). The model is shown below:

Model:
$Y_{ij} = \mu + T_i + \Sigma_{ij}$
Y_{ij} = Observed value of dependent variable
μ = Population mean
T_i = Effect of Ith strains of chicken
Σ_{ij} = Residual error

Statistical Analysis

All the data collected were analysed using the General Linear Model procedure of SAS (1999). Significantly different means were compared using Duncan Multiple Range Test (DMRT) (Duncan, 1955).

RESULTS

Comparison of the Egg Quality Parameters among Three Strains of Chicken

Effects of strains on egg parameters are shown in Table 1. Strain had significant effects (P < 0.05) on egg weight, shell weight, yolk weight, albumen weight and

Haugh unit. Eggshell thickness and albumen height were not significantly affected by strain. NB and IB were found to be similar in egg weight, yolk weight, albumen weight and percentage albumen weight, but greater compared to NIC. The shell weight of the *IB* was heavier compared to NIC, while NIC and IB showed greater values than NB strain for % shell weight. Whereas the NIC showed greater Haugh unit and percentage yolk weight compared to the other strains, *IB, which* showed intermediate value for percentage yolk weight.

The eggshell weight during incubation as affected by strain is presented in Table 2. Strain did not significantly (P>0.05) affect shell weight at ED (Embryonic Day) 7, 11, and 18. At ED15, shell weights differed significantly among the strains. At ED15, the *IB* egg shell weight was significantly higher than that of NIC, but similar to that of NB. However, the NIC egg shared similar eggshell weight with NB at ED15 of incubation. Strain significantly affected (P < 0.05) the shell weight as the percentage of egg weight from Day 0 to ED15, with the exception at ED18 where they were similar. Shell weight loss was higher in the eggs of the IB strain chicken compared to the egg of NB and NIC, while the eggs of NB had the lowest shell weight loss.

Comparison of Egg Weight Loss during Incubation

Table 3 shows the comparison of egg weight loss (%) during incubation. Strain showed significant (P <0.05) effect on weight loss at ED7 of incubation but not on other days

of measurements. At ED7, the NB showed a significant higher weight loss compared to that of the *IB* but the weight loss was similar to that of the eggs of the NIC. Weight loss by the NIC eggs was also similar (P>0.05) to that of the *IB* eggs during incubation. In effect, weight loss by the NIC eggs was intermediate between the NB and *IB* eggs. At ED18, egg weight loss was similar among the three strains.

Comparison of Egg Weight at Setting, Egg Weight and Embryo Weight during Incubation

Egg weight at setting, egg weight and embryo weight at different incubation periods for each of the three strains are shown in Table 4. At ED 7 and 15 of incubation, egg weights of NB and IB were significantly (P< 0.05) higher compared to NIC. The NB strain had greater (P< 0.05) egg weight than IB and NIC at ED11 and 18. NIC had similar weight with IB. As incubation days advanced, strain did not show significant (P>0.05) effect on embryo weights except on ED 18 of the embryo development, where NB had significantly (P< 0.05) higher embryo weight than the two other strains.

Comparison of Embryo Weight as the Percentage of Egg Weight (%) during Incubation

The embryo weight as the percentage of egg weight affected by strain is shown in Table 5. At ED7 and 18, there were significant differences (P < 0.05) in the embryo weight relative to the egg weight

between the strains. At ED 7, this observation was higher in NIC than those of IB and NB, while IB and NB were similar. Moreover, at ED18, the embryo weight as the percentage of egg weight was significantly higher in NIC than that of IB but similar to that of NB. However, there were no significant effects (P>0.05) of strain on the embryo weight as the percentage of egg weight at ED11 and 15.

DISCUSSION

In this study, strain was found to have significant effects on the absolute shell weight, percentage shell weight, absolute yolk weight, percentage yolk weight, absolute albumen weight, percentage albumen weight and the Haugh unit values. There were no significant effects of strain on the eggshell thickness and albumen height. Absolute yolk and albumen weights, with the exception of the absolute shell weight of these different strains of chicken, were directly proportional to the egg weights. This has previously been observed by Marion *et al*. (1964) who reported significant differences in egg weights among the lines of White Leghorns and that both egg yolk and shell weights were directly proportional to egg weight. The higher egg weights in the NB and IB strains of chicken when compared to that of NIC could be attributed to strain effects. The similarity in the eggshell thickness of these strains showed their similar ability to withstand losses due to cracks. In a corresponding study, smaller eggs had stronger shells than larger ones, as hens have a finite capacity to

TABLE 1
Comparison of the egg quality parameters among the three strains of chicken

Strain	N	Egg weight (g)	Eggshell thickness (mm)	Shell weight (g)	%Shell weight □	Yolk weight (g)	%Yolk weight □	Albumen weight (g)	%Albumen weight □	Albumen height (mm)	Haugh unit
NIC	10	48.82±3.54[b]	0.43±0.09	5.05±0.53[b]	10.37±1.03[a]	14.00±1.21[b]	28.74±2.31[a]	29.69±2.80[b]	60.74±1.77[b]	5.14±0.52	71.78±3.06[a]
NB	10	63.98±8.66[a]	0.36±0.03	5.09±0.63[b]	8.04±1.12[b]	16.41±1.59[a]	25.87±2.48[b]	42.48±7.40[a]	66.09±3.26[a]	4.50±1.30	53.23±4.41[b]
IB	10	63.47±6.07[a]	0.42±0.06	6.02±0.90[a]	9.55±1.55[a]	16.78±1.58[a]	26.55±2.42[ab]	40.67±5.30[a]	63.91±3.27[a]	4.59±1.08	52.36±5.73[b]

a,b Means(±SD) within a column with different superscripts differ significantly (P < 0.05).
N=Number of observations; □=As percentage of egg weight

TABLE 2
Comparison of the eggshell weight, %shell weight and shell weight loss during incubation

Strain	N	(ED0) Sw	(ED0) %sw □	(ED7) Sw	(ED7) %sw □	(ED11) Sw	(ED11) %sw □	(ED15) Sw	(ED15) %sw □	(ED18) Sw	(ED18) %sw □	(ED0-ED18) Swl(%)
NIC	10	5.05±0.53[b]	10.37±1.03[b]	5.24±0.59	11.00±0.7[a]	5.58±0.98	11.55±1.58[a]	5.04±0.52[b]	10.69±1.17[b]	4.54±0.73	10.84±2.02	10.09±0.03[b]
NB	10	5.09±0.63[b]	9.55±1.12[a]	4.96±0.57	8.75±0.60[c]	5.04±0.69	8.78±0.93[b]	5.30±0.63[ab]	9.49±0.75[b]	4.95±0.67	9.48±0.81	2.75±0.04[c]
IB	10	6.02±0.90[a]	8.04±1.55[b]	5.64±0.94	9.95±1.25[b]	5.86±0.62	10.92±1.08[a]	5.68±0.52[a]	10.70±0.6[a]	5.19±0.71	10.54±1.11	15.99±0.14[a]

a,b Means(±SD) within a column with different superscripts differ significantly (P < 0.05).
N=Number of observations; ED0= day0; Sw= Shell weight; Swl%=Shell weight loss %; □ = As the percentage of egg weight; ED= Embryonic day

TABLE 3
Comparison of egg weight loss (%) during incubation

Strain	N	ED 7	ED 11	ED 15	ED 18
NIC	10	4.69±1.36[ab]	7.76±2.57	10.89±5.90	12.15±2.72
NB	10	6.12±2.29[a]	9.26±1.72	13.71±3.62	16.09±5.10
IB	10	4.16±1.21[b]	8.57±2.73	11.26±2.47	15.64±4.44

a,b Means(±SD) within a column with different superscripts differ significantly (P < 0.05).
ED=Embryonic day; N=Number of observations

TABLE 4
Comparison of egg weight at setting, egg weight and embryo weight during incubation

Strain	ED7			ED11		ED15		ED18	
	Ews	Ew	Emw	Ew	Emw	Ew	Emw	Ew	Emw
NIC	48.82±3.54[b]	47.56±0.12[b]	0.77±0.12	48.27±4.27[b]	3.52±0.25	47.35±4.02[b]	11.41±1.45	42.71±7.25[b]	21.07±3.30[ab]
NB	63.98±8.66[a]	56.67±0.09[a]	0.67±0.10	57.62±7.95[a]	3.82±0.45	55.96±6.24[a]	11.76±2.54	52.24±5.99[a]	23.94±2.16[a]
IB	63.47±6.07[a]	56.63±0.27[a]	0.63±0.27	53.93±5.58[ab]	3.60±0.38	53.04±2.42[a]	11.57±0.59	49.28±5.20[ab]	20.27±2.76[b]

[a,b] Means(±SD) within a column with different superscripts differ significantly ($P < 0.05$).
N=Number of observations; Ews=Egg weight at setting; Ew=Egg weight; Emw=Embryo weight

TABLE 5
Comparison of embryo weight as the percentage of egg weight (%) during incubation

Strain	N	ED7	ED11	ED15	ED18
NIC	10	1.63±0.25[a]	7.36±0.96	24.18±3.17	49.93±7.66[a]
NB	10	1.18±0.10[b]	6.75±1.22	21.00±4.07	46.18±5.01[ab]
IB	10	1.10±0.43[b]	6.78±1.24	21.87±1.69	41.71±8.21[b]

[a,b] Means(±SD) within a column with different superscripts differ significantly ($P < 0.05$).
N=Number of observations

Pertanika J. Trop. Agric. Sci. 38 (1): 45 - 55 (2015)

51

deposit calcium in the shell, and as a result, the same amount of calcium is spread over a larger area (Butcher & Miles, 2003).

In the present study, the local strain of chicken (NIC) had the lowest egg weight compared to NB and IB , but had similar eggshell weight with the NB strain, with the exception of the IB strains. Contrary to this finding, a report by Silversides *et al*. (2006) described the effect of strain on shell quality of layer chickens, with the largest strains producing the heaviest eggshells and the lightest strains producing the smallest eggs with lighter eggshell weights. Although the mean shell weights obtained in this study were slightly higher than that by Oguike and Onykweodiri (1999) for Yaffa and Issa Brown layers, those of NIC and NB were similar to what Fayeye *et al*. (2005) had observed in the egg traits of the Fulani-ecotype chickens, but lower than that of the egg of the IB strain. The higher percentage in the shell weight of the eggs of the NIC and IB strains chicken than that of the NB strain chicken suggested that they had more mineralization (calcium,Ca and P, phosphorus) deposit that could be ushered into skeletal development than that of NB strain. Pageze *et al*. (1996) reported that chicken embryos source Ca in the shell for mineralization during skeletal development. Calcium so recovered not only mineralize a developing skeleton previously cartilaginous, it also calcifies yolk sac spheres (Roufosse, 1979).

The egg of the NIC had higher percent yolk weight than that of the NB and IB chicken. The dissimilarity noticed in the weights of the yolk and percent yolk weight before incubation might be influenced by strain (Wolanski *et al.,* 2007). The dissimilarity in the yolk weights before setting might have been altered at Day 18 (after incubation) by the effect of different egg component reduction causes (evaporation and metabolism) in the incubator. The similar albumen height in these strains suggests that these strains had similar limitation in gas diffusion, nutrient availability, and embryonic growth (Peebles *et al.,* 2000). Peebles *et al.* (2000) reported that thick albumen might slow gas diffusion, limit nutrient availability, and thus decrease embryonic growth. The higher percentage albumen weights of the eggs of NB and IB strains over NIC probably afforded the exotic strains' ability of providing more protein for assimilation into tissue than the NIC during embryonic development. Romanof and Romanof (1949) reported that albumen contains approximately 67% of the protein content of the egg and increased in mass more acutely than the total mass of the egg. It was observed that the eggs from the NIC had higher Haugh unit than the eggs of the NB and IB strains that had similar Haugh unit. It is generally accepted that the higher the Haugh unit value, the better the quality of the egg albumen (Curtis *et al.,* 2005). A negative relationship was reported between egg weight and Haugh unit (Kinney, 1970). In contrast, Emsley *et al.* (1997) demonstrated that heavy eggs had higher Haugh unit. However, the result of this study is in agreement with the report by Kinney *et al.* (1970).

The results of the percent shell weight from Day 0 until ED18 suggested the influence of genetic variation at different phases of embryonic development. During incubation, the reduction in the eggshell weights between ED0 and ED18 indicated that some calcium components might have been synthesised into providing skeletal development in the developing embryos (Pageze *et al.*, 1996). This result might suggest that the embryo of the IB strain might have converted more calcium component to skeletal formation than the embryos of the NIC and NB strains, respectively. Although the eggs of the NB and IB strain chickens were heavier than that of the NIC, they did not show any effect on egg weight losses (which were the same for all the strains) except at ED7.. The similarity observed on the overall egg weight losses at Day 18 of incubation might suggest that the three egg categories used for the study had similar proportion of pore area or pore diameter regardless of the egg size (Abiola *et al.*, 2008). Deeming (1995) indicated that eggs that lost less than 10% or over 20% of their initial weight were less likely to hatch. The author attributed this to a reflection of functional porosity of the shell and the initial mass of each egg.

Although the embryo weights were similar up to ED15, the embryo's relative weight to the egg weight of the NIC showed superiority in the embryo development compared to the embryos of the NB and IB strains at ED7 and 18.

The disparity in the embryo weights at Day 18 did not agree with the pattern of the differences in the initial egg size and hatch weight. Although the embryo weight of NIC at Day 18 did not agree with its initial egg weight, it had an intermediate value. This could be linked to the finding of Peebles *et al.* (2000) who reported that albumen height may be a factor in determining dry matter (DM) accumulation of chicken embryos. Wolanski *et al.* (2006) also reported that a great deal of variation exists in the conversion of egg contents into chick body mass among strains. This was shown from ED0 to 7 and ED11 to 15 where NIC had lower reduction rate of albumen than the two other strains, but similar embryo weight as other strains. The results of the embryo weight during incubation showed that the embryo of the NIC had higher conversion rate of albumen than those of the IB and NB strains at ED7 and 15. The IB strain had higher conversion rate at ED11 than the two other strains, while NB strain had an intermediate conversion rate at ED7, 11, and 15. Overall, these findings suggested that different strains may have different growth trajectory at different periods during embryonic development.

The difference observed in hatch weight may have been influenced by initial egg weight, as supported by Yannakopoulos and Tserveni-Gousi (1987). The higher hatch (day-old chick) weight observed in NB and IB strains than the NIC at hatch could be attributed to the differences in yolk content retention of different strains at hatch (Wolanski *et al.,* 2006). These authors suggested that some chicken strains utilised yolk reserves more efficiently than

others when incubated with a common incubation profile. However, the NB and IB strain embryos had a better development than that of NIC from Day 18 of embryo development to Day 21 (hatching day). This was observed in the hatch weights recorded. It was therefore suggested that multi-stage incubator be used between days 18 and 21 of incubation to ascertain the cause(s) of this dispersion in growth of the embryos at these stages of incubation.

Arising from the results of this study, using the currently used incubation protocols under a south-western Nigerian condition, it can be concluded that the reduction in the eggshell weights until ED18 suggested that some calcium components had been ushered into providing skeletal development in the developing embryos. The similarity observed on the overall egg weight losses at ED18 of incubation suggests that the three egg categories used for the study had similar proportion of pore area or pore diameter regardless of the egg size. The weight differences of the embryos at ED18 suggest ed the influence of genetic differences. Based on the findings from this study, it can be concluded that the incubation protocol used for the exotic strains of chicken is adequate for the Nigerian indigenous chickens.

REFERENCES

Abiola, S. S., Meshioye, O. O., Oyerinde, B. O., & Bamgbose M. A. (2008). Effect of egg size on hatchability of broiler chicks. *Archivos de Zootecnia, 57*(217), 83-86.

Adebambo, O. A., Ikeobi, C. O. N., Ozoje, M. O., & Adebambo, O. A. (2009). Variation in growth performance of pure and crossbred meat type chickens. *Nigeria Journal of Animal Production, 36*(2), 211- 227.

Adedeji, T. A., Adebambo, A. O., Ozojie, M. O., Ojedapo, L. O., & Ige, A. O. (2006). Preliminary results of short-term egg laying performance of pure and crossed bred progeny in a humid environment. *Journal of Animal and Veterinary Advances, 5*(8), 670-673.

Adedeji, T. A., Ojedapo, A. O., Ige, S. A., Akinwunmi, A. O., & Amao, S. R. (2008). *Genetic evaluation of growth performance of pure and crossbred chicken progenies in a derived savannah environment*. Proc. of the 13th Annual Conference of the Animal Science Association of Nigeria, (ASAN), Sept. 15-19. ABU Zaria: 8-12.

Amujoyegbe, B. J., Bamire, A. S., & Elemo, K. O. (2008). Agronomic analysis of fertilizer effect on maize/cowpea intercrop in Ile-Ife and Abeokuta, South-western Nigeria. *Asset Series A, 8*(1), 62-72.

Butcher, G. D., & Miles, R. D. (2003). *Egg Specific Gravity: Designing a Monitoring Program*. IFAS Extension, University of Florida.

Curtis, P. A., Kerth, L. K., & Anderson, K. E. (2005). *Quality and compositional characteristics of layer hens as affected by bird age*. XI European Symposium on the Quality of Eggs and Egg Products, pp. 214-219.

Deeming, D. C. (1995). Factors affecting hatchability during commercial incubation of ostrich(Struthio camelus) egg. *British Poultry Science, 36*, 51-65.

Duncan, D. B. (1995). Multiple Range and F tests. *Biometrics, 11*, 1-42.

Emsley, A., Dickerson, G. E., & Kashyap, T. S. (1977). Genetic parameters in progeny-test selection for field performance of strain- cross layer. *Poultry Science, 56*, 121-146.

Fayeye, T. R., Adeshiyan, A. B., & Olugbami, A. A. (2005). Egg traits, hatchability and early growth performance of the Fulani-ecotype chicken. *Livestock Research Rural Development, 17*, 8.

Haugh, R. R. (1937). The haugh unit for measuring egg quality. *US Egg Poultry Magazine, 43*, 552-555.

Ibe, S. N. (1990). *Utilizing Local Poultry Genetic Resources In Nigeria*. Proc.4th World Cong. On Genet Applied Livestock Peoduction. Edinburgh, Scotland.

Kinney, T. B. Jr., Bohren, B. B., Craig, J. V., & Lowe, P. C. (1970). Responses to individual, family or index selection for short-term rate of egg production in chicken. *Poultry Science, 49*, 1052-1064.

Marion, W. W., Nordskog, A. W., Tolman, H. S., & Forsythe, R. H. (1964). Egg composition as influenced by breeding, egg size, age and season. *Poultry Science, 43*, 255-264.

Nwakpu, P. E., Odo, B. I., Omeje, S. I., Akpa, M., & Edoga, C. C. (1999). *Hatching performance of three strains of layer-type chicken and their lines.* Proceedings of the 26th Annual Conference, NSAP, 21-25 March, 1999, Ilorin.

Oguike, M. A., & Onyekweodiri, E. O. (1999). *Eggshell quality of four commercial strains of layers.* Proceedings of the 26th Annual NSAP Conference, 21-25 March, 1999, Ilorin.

Pageze, L., Manesse, M., Navarro, M. Carretero, A., Arboix, M., Ruberte, J., & Sautet, J. (1996). The morphometric study of the 19-day chicken embryo skeleton (Gallus domesticus). *Veterinary Human Toxicology, 38*, 299–301.

Peebles, E. D., Gardner, C. W., Brake, J., Benton, C. E., Bruzual, J. J., & Gerard. P. D. (2000). Albumen height and yolk and embryo compositions in broiler hatching eggs during incubation. *Poultry Science, 79,* 1373–1377.

Peters S. O. (2000). *Genetic variation in the reproductive performance of indigenous chicken and the growth rates of its pure and half bred progeny.* (M.Sc. Thesis). Dept of Anim. Breeding and Genetics University of Agriculture. Abeokuta. Pp 120.

Romanof, A. L., & Romanof, A. J. (1949). *The Avian Egg*. Wiley, New York.

Roufosse, A. H. (1979). Identification of brushite in newly deposited bone miNBl from embryonic chicks. *Journal of Ultrastructural Research, 68*, 235–255.

SAS Institute. (1999). *SAS User's Guide*. Statistics Inc., Cary, N.C., Pp. 92

Silversides, F. G., Korver, D. R., & Budgell, K. L. (2006). Effects of strain of layer and age at photostimulation on egg production, egg quality, and bone strength. *Poultry Science, 85*, 1136–1144.

Wolanski, N. J., Renema, R. A., Robinson, F. E. Carney, V. L., & Fancher. B. I. (2006). Relationship between chick conformation and quality measures with early growth traits in males of eight selected pure or commercial broiler breeder strains. *Poultry Science, 85*, 1490–1497.

Wolanski, N. J., Renema, R. A., Robinson, F. E., Carney, V. L., & Fancher, B. I. (2007). Relationships Among egg characteristics, chick measurements, and early growth traits in ten broiler breeder strains. *Poultry Science, 86,* 1784-1792.

Yannakopoulos, A. L., & Tserveni-Gousi, A. S. (1987). Relationship of parent's age, hatching egg weight and shell quality to day-old chick weight as influence by oviposition time. *Poultry Science, 66*, 829-833.

Response to Foot and Mouth Disease (FMD) Vaccination among Local Malaysian Cattle of Various Vaccination Backgrounds from Endemic and Non-endemic FMD Areas

A. Abbo Hamad[1], L. Hassan[1]*, M. Z. Azmie[2], P. Loganathan[2], T. Jaafar[3], S. S. Arshad[1], J. Hashim[3], H. Amir[4], O. Norlida[5], M. A. Syarifah Asiah[5] and M. M. Salih[5]

[1]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang Selangor
[2]Department of Veterinary Services, Ministry of Agriculture and Agro-Based Industry, Wisma Tani, Block Podium, Lot 4G1, Precinct 4, Federal Government Administration Centre, 62630 Putrajaya, Malaysia
[3]Department of Veterinary Services, Jalan Raja Syed Alwi, 01000 Kangar Perlis, Malaysia
[4]Department of Veterinary Services, Jabatan Perkhidmatan Veterinar Negeri Kelantan, Kubang Kerian, 16150 Kota Bharu, Kelantan, Malaysia
[5]Animal Laboratory, Department of Veterinary Services, Jalan Kubang Kerian, 16150 Kota Bharu, Kelantan, Malaysia

ABSTRACT

A longitudinal study assessed the response to foot and mouth disease virus (FMDV) vaccination on sequential sera of local Malaysian Kedah-Kelantan cattle in two states of Peninsular Malaysia; Perlis, a foot and mouth (FMD) disease non-endemic state, and Kelantan, an endemic state for FMD. These cattle were from various vaccination backgrounds and some with unknown vaccination status. For the cattle in both states, the antibody against FMDV type O effectively increased to a strong protective level in the first week following vaccination, regardless of the vaccination background of the animals. In the endemic state, where vaccination was performed more routinely than the non-endemic state, the response had better magnitude and duration. In the non-endemic state, the antibody response level was good but appeared to last for a shorter period of time before it significantly declined. For naïve cattle with no evidence of infection or vaccination, the response was rapid and reached a strong level immediately by the first week. However, the level was not sustained and significantly declined thereafter. All the cattle stayed healthy and clinically FMD-

free throughout the study, even when there was a transient evidence of natural field infection detected among the cattle. A marked difference was observed in the patterns of antibody response between cattle in the FMD endemic and non-endemic areas. However, the level of antibodies generally rose to a strong protective level within the stipulated 7-14 days post-vaccination. The vaccine used was effective in eliciting immune response when naturally challenged by the local field FMD virus.

Keywords: FMD, cattle, vaccination, antibody response, structural-proteins, non-structural proteins

INTRODUCTION

Foot and mouth disease (FMD) is an important trans-boundary and re-emerging infection of the ungulates that can result in devastating economic and trade losses (Forman *et al.*, 2009). FMDV serotypes O, A, and Asia 1 were reported to be endemic to seven countries in mainland Southeast Asia (Cambodia, Laos, Malaysia, Myanmar, the Philippines, Thailand and Vietnam) (Gleeson, 2002). Three countries in this region (namely, Brunei, Indonesia and Singapore) are recognized by the OIE as free of the disease without vaccination. Part of the Philippines (Mindanao, Visayas, Palawan and Masbate) and part of Malaysia (Sabah and Sarawak) are also recognized internationally as being free of FMD without vaccination (Rweyemamu *et al.*, 2008b). FMD viruses, for example, Cathay 'O' and the Pan-Asia 'O' topotypes which have been described to originate in South

China, could have possibly joined by Asia 1 topotype and evolved in 2005 (Valarcher *et al.*, 2005), crossed into South-East Asia across the border into Vietnam, and then spread westwards into Cambodia, Laos and eventually Thailand (Rweyemamu *et al.*, 2008b). According to a recent report by the World Organization for Animal Health, only 36 of the 178 OIE member countries are FMD-free without vaccination (OIE, 2011). Several countries that had been free from FMD for decades have reported FMD outbreaks in the recent years (Bouma *et al.*, 2003; Ellis-Iversen *et al.*, 2011; Park *et al.*, 2003). However, many have regained disease freedom status, with or without the use of vaccines. The control and prevention of the disease may be achieved following the outbreak via stamping out or culling of all affected animals and herds (with or without emergency vaccinations), accompanied with various animal movement controls. However, in countries where the disease is endemic and widespread, vaccination accompanied by movement controls and zoo-sanitary measures are recommended as a more economically feasible mode of preventing and controlling the disease (Geering & Lubroth, 2002; Paton *et al.*, 2009). Many FMD-endemic countries have implemented strategic vaccination in an effort to control clinical FMD and as a step in the progressive phase of achieving disease-freedom (Gleeson, 2002; Rweyemamu *et al.*, 2008a; Windsor *et al.*, 2011).

The performance of most commercial FMD vaccines has been evaluated in countries where it is manufactured. Hence,

in situations and environments that differ, the vaccine may result in disparate levels of immunity and protection and should therefore be tested in the field situations where it is applied. Peninsular Malaysia, as one Southeast Asian country where the disease is generally endemic, has faced many outbreaks of FMD and used strategic vaccination and modified stamping out methods to control the disease, particularly in the northern part of the country. However, this policy was reviewed in the 1980's (Karuppanan & Naheed, 2000) due to the extensive nature of the FMD outbreaks and was discontinued in the early 1990s (Palanisamy *et al.*, 2000) when the disease became too widespread. Currently, FMD is endemic in many parts of the Peninsula. Only three serotypes are known to occur in Malaysia: serotype O, A and Asia 1. However, in the recent years, only the two former serotypes have been reported (Abbo *et al.*, 2010). As part of the national FMD prevention and control measures, vaccinations against the aforementioned serotypes using the FMD 'killed' (inactivated) vaccine (Merial Animal Health Limited) are performed regularly, although outbreaks have continued to occur (SEAFMD, 2007). It is important to note that even though the vaccine has been used for more than a decade, its performance based on local animal and field conditions has not been formally evaluated. Therefore, this study assessed the FMD antibody response levels following vaccination performed on the local Kedah-Kelantan cattle in the endemic and non-endemic FMD

areas of Peninsular Malaysia, with special emphasis on the challenges of performing vaccination in areas where recordings were sparse or poor.

MATERIALS AND METHODS

Study Design and Epidemiological Background

A longitudinal study was conducted where local cattle from Perlis and Kelantan, two states in the northern part of Peninsular Malaysia, were sampled between 17 November 2008 and 21st July 2009. The study was conducted in collaboration with, and under the approval of the Department of Veterinary Services Malaysia and was performed as part of the FMD vaccination and surveillance programme. The use of cattle in each farm was approved by the farm managers and owners. Although Perlis was not declared as FMD-free, based on the absence of FMD clinical outbreaks in the previous four years prior to the study (SEAFMD, 2007), it was considered as non-endemic for the purpose of this study. Meanwhile, Kelantan, which annually suffers outbreaks of the disease, was considered as FMD-endemic. The annual vaccination campaign for the states such as Perlis was aimed to vaccinate cattle at least once a year while for states such as Kelantan, twice a year. However, due to the various local cattle management styles and various degrees of knowledge of previous FMD vaccination, diverse vaccination backgrounds were anticipated in this study.

In order to determine the sample size, at least 70% of cattle were assumed to have

reached a strong protective antibody level if the vaccine was administered. At the desired precision of 10% and the confidence level of 95%, the number of animals required was 81 for each endemic and non-endemic state (Thrusfield, 2013). The study population consisted of local Kedah-Kelantan cattle (1.5-2.5 years) from: (1) eight villages in Perlis that were assembled in one large-scale Government beef farm (for the purpose of the study) and one small-scale beef farm, and (2) one large-scale Government cattle breeding farm in Kelantan. The cattle from the villages in Perlis were selected based on the permission by the animal owners and were temporarily placed in the government beef farm during the study period to facilitate animal management. Farms were selected based on the willingness of the farm managers to participate and the animals within the farms were initially selected using systematic sampling. All cattle were separated from the rest of the animals in the farm and remained as a closed unit until the study was completed.

Nonetheless, the researchers could not obtain any vaccination history from the animals that originated from the villages in Perlis as these animals were raised in an extensive management system. As for the other two farms, individual animal records were not accessible at the time of the study; however, it was reported that the last FMD vaccination was performed between 10-11 months prior to the study. Vaccination using inactivated highly purified trivalent vaccine containing serotype O_1 strain Manisa and O 3039 was performed as suggested by the vaccine manufacturer (Merial Animal Health Limited). This vaccine was the only approved vaccine in use in Malaysia for more than a decade. Blood from selected cattle was collected before the vaccine was given, after which each animal was serially sampled four times within a period of 4 months. The samples were collected from the coccygeal vein using 10 ml plain vacuum tubes at pre-vaccination day 0 (round 0; R0) and post-vaccination day 7-14 (R1), day 15-27 (R2), day 28-100 (R3) and day >100 (R4). The serum was separated and placed in Eppendorf Safe-Lock tubes on the same day of collection and stored at -20 Cº until further use.

Serological Analysis

All the serological analyses were performed at the National FMD Laboratory in Kota Bharu, Kelantan. Priocheck® FMDV type O Solid-Phase Blocking ELISA (SP-ELISA) was used to detect antibodies directed against the structural proteins (SP) of FMDV type O virus (Chénard *et al.*, 2003). This was followed by the Priocheck® FMDV Non-Structural Protein ELISA (NS- ELISA) to differentiate cattle infected with FMDV from those vaccinated via the detection of antibodies to one or more NS proteins (Sørensen *et al.*, 1998). These kits are presently manufactured by Prionics AG, Switzerland, and were formerly produced by Ceditest® Diagnostics. For both the tests, the optical density and the percentage inhibition (PI) of the reference and test sera at 450 nm were measured using an ELISA reader (Chénard *et al.*, 2003). In

this study, the levels of antibodies were categorised according to the SP, based on the work of Chénard et al. (2003), whereby PI > 90% (strong), 50-90% (weak) and < 50% (negative), and the levels of the antibodies against the NS virus proteins based on Brocchi *et al*. 2006), whereby PI ≥50% (positive) and <50 (negative). In addition, referring to the work of Westbury *et al*. (1988), Smitsaart *et al*. (1998) and Palanisamy *et al*. (2000), SP PI>90% was used as a guide for the protective level against FMD infection.

Data Analysis

A descriptive analysis was also performed in this work to examine the data in terms of normality, means and standard deviations. Then, Generalized Linear Model (GLM) two-way *repeated* ANOVA (Field, 2009) was used to evaluate the changes of the antibody response at different sampling times between the groups of cattle from Perlis and Kelantan. Mauchly's Test was used to test the assumptions of sphericity; if the assumption was not met, the Greenhouse-Geisser correction was then used for the analysis (Field, 2009). Meanwhile, pair-wise comparison between the sampling times was analyzed for each subgroup using Bonferroni test. All the analyses were performed at a significance level of α = 0.05 using SPSS ver. 19 (SPSS Inc, Chicago IL). Graphs were plotted using Microsoft Excel 2010 (Microsoft Office® 2010).

RESULTS

A total of 176 serum samples (88 each from Perlis and Kelantan) for each sampling time were analysed. Overall, at the pre-vaccination stage (R0), 92.6% (163) of the cattle from Perlis and Kelantan had some evidence of previous FMD vaccinations or virus exposure (PI > 50%) with 84.6% (149) cattle exhibiting a strong level of antibodies to SP (PI > 90%). Details on the antibody levels against SP for each sampling within groups of cattle are tabulated in Table 1. Cattle in Perlis responded rapidly to the vaccination, whereby an immediate increase in the proportion of cattle was detected with strong level of antibodies to SP (PI > 90%) was observed in the first week (R1), following vaccination (from 69% to 91%). However, the proportion waned in the subsequent weeks and by R4 only about 59.5% of animals had PI of >90%. The mean PI values increased from 85% to 96% by the 2nd week (R2) post-vaccination but then reduced to <90% by R3 and R4. All the cattle in Kelantan came into the study with the presence of a strong baseline level of antibodies to the SP. Vaccination increased the level of antibodies by R2, which then slightly declined by day 100 (R4). However, the mean PI remained very high (>95%) throughout the study period (Table 1).

The repeated measures ANOVA using the Greenhouse-Geisser correction determined that the PI values differed significantly between the sampling times ($F_{(3.1, 534.0)}$ = 12.3, P=0.001) and between the endemic and non-endemic states ($F_{(1,}$

$_{174)} = 122.7$, P=0.001). The Bonferroni pair-wise comparisons revealed that vaccination response varied significantly between sampling times only for Perlis (non-endemic FMD state), whereby the antibody increased significantly from R0 (84.8± 1.7) to R1 (95.8±0.8, P=0.001) and R2 (94.7±0.6; P=0.001), followed by a significant decline from the peak response by R3 (89.5±1.0; P=0.015) and beyond (87.5± 1.1; P<0.001). The cattle in Kelantan maintained higher antibody levels compared to cattle in Perlis and had a more gradual non-significant (P>0.05) incline from R0 (98.3±1.67) to R1 (99.1 ± 0.78) and R2 (99.6±0.61) and a slight non-significant (P>0.05) decline in R3 (98.5±1.05) and R4 (97.8±1.05) from the peak response (Fig.1).

The data were further stratified based on the background antibody levels to the SP detected at the start of the study and found 13 (7.4%) cattle (all from Perlis) were naïve for vaccination and natural infection, 14 (7.9%) had weak and 149 (84.6%) had strong baseline levels of antibodies. Fig.2 depicts the pattern of the responses based on the categories of cattle. The vaccination response significantly varied given sampling times and the initial background levels of antibodies against the SP ($F_{(5.6, 484.7)} = 59.6$, P=0.001). The 13 naïve cattle had a PI of 39% in R0 (39.0 ± 1.1), which then increased significantly and rapidly (95.3 ± 1.9; P=0.001) by R1, then gradually waned by R2 (94.4 ± 1.7; P=0.1) and significantly diminished from their peak by R3 (84.9± 2.9; P=0.04) and R4 (83.7± 2.9; 0.03). Among the cattle with weak baseline antibodies to the SP (71.2 ± 1.1),

the antibody level significantly increased in R1 (88.9± 1.9; P=0.01) and R2 (93.4 ± 1.6; P=0.001), before it insignificantly (P>0.05) declined in R3 (92.4 ± 2.8) and R4 (86.1± 2.8). Among the cattle with strong baseline antibodies to the SP (PI > 90%), vaccination insignificantly (P>0.05) increased the response from R0 (98.0 ± 0.3) to R1 (98.4± 0.5) and thereafter, insignificantly (P>0.05) declined in R2 (97.8± 0.5), R3 (94.9± 0.8) and R4 (94.1 ± 0.8).

Twenty-eight cattle came into the study with positive antibodies to NS proteins, indicating a prior natural FMDV field infection or exposure. However, none manifested clinical signs as they also possessed high levels of vaccination antibodies.

Sequential sampling at various points within the course of the study detected antibodies against the NS virus proteins in a proportion of cattle in both Perlis and Kelantan indicating exposure or infection to the field FMDV (Table 2). At every sampling, all the samples that were NS-positive were also SP-positive. The proportion of the infected cattle was consistently higher in Kelantan compared to Perlis. This coincides with the endemicity of FMD in Kelantan, where the animals are more likely to be exposed to field viruses. Nevertheless for most cases, antibodies against the NS proteins cleared up by the next sampling and none of the cattle manifested clinical FMD, which strongly indicated that the vaccine was efficacious in preventing the development of clinical disease following a natural field viral exposure.

Response to FMD Vaccination among Local Malaysian Cattle

TABLE 1
Percent inhibition (PI) of FMD Solid Phase Blocking ELISA (strong positive, weak positive, negative, mean PI and SD) on serum samples from cattle in Perlis and Kelantan

Sampling Rounds (days)	Perlis				Kelantan			
	Strong positive	Weak positive	Negative	Mean PI±SD	Strong positive	Weak positive	Negative	Mean PI±SD
R0(0)	61 (69.3)	14 (15.9)	13 (14.8)	84.76±22.01	88(100)	0	0	98.30±2.16
R1(7-14)	80 (90.9)	8(9.1)	0	95.87±10.26	88(100)	0	0	99.10±1.76
R2(15-27)	68 (78.2)	19 (21.8)	0	94.74±8.03	88(100)	0	0	99.66±1.37
R3(28-100)	64 (72.7)	24 (27.3)	0	89.48±13.87	88(100)	0	0	98.51±1.37
R4(>100)	50 (59.5)	34 (40.5)	0	87.50±13.75	88(100)	0	0	97.83±2.45

Strong +ve SPB-ELISA PI > 90%; Weak +ve SPB-ELISA PI 50-90; -ve SPB-ELISA PI 0-49

TABLE 2
Percent inhibition (PI) of FMD Non-Structural (NS) Proteins ELISA of serum samples from cattle in Perlis and Kelantan

Sampling Rounds (days)	Perlis		Kelantan	
	No Positive (%)	No Negative (%)	No Positive (%)	No Negative (%)
R0 (0)	6 (6.9)	81 (93.10)	22 (25.0)	66 (75.0)
R1 (7-14)	9 (10.2)	79 (89.8)	39 (45.3)	47 (54.4)
R2 (15-27)	6 (6.8)	82 (93.2)	37 (42.0)	51 (58.0)
R3 (28-100)	18 (20.5)	70 (79.5)	13 (14.9)	74 (85.1)
R4 (>100)	10 (11.4)	78 (89.6)	24 (27.6)	63 (72.4)

Positive PI ≥ 50, Negative PI < 50
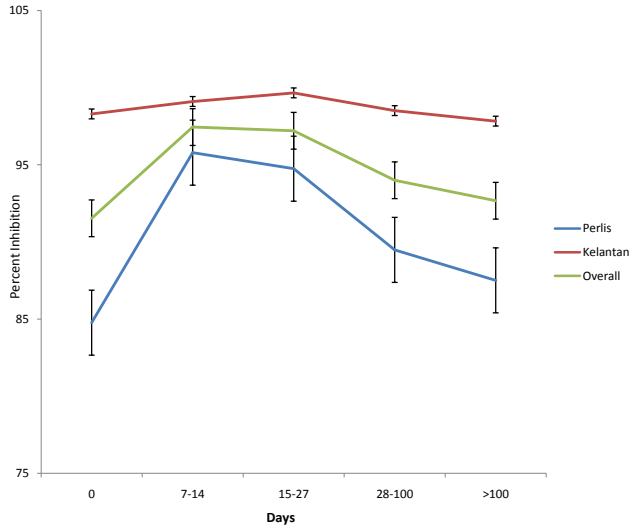
Fig.1: Percent inhibition (PI) of structural proteins SP-ELISA type O FMD throughout the study period for cattle in Kelantan (endemic), cattle in Perlis (non-endemic) and cattle from both states. Vertical lines indicate the 95% confidence interval
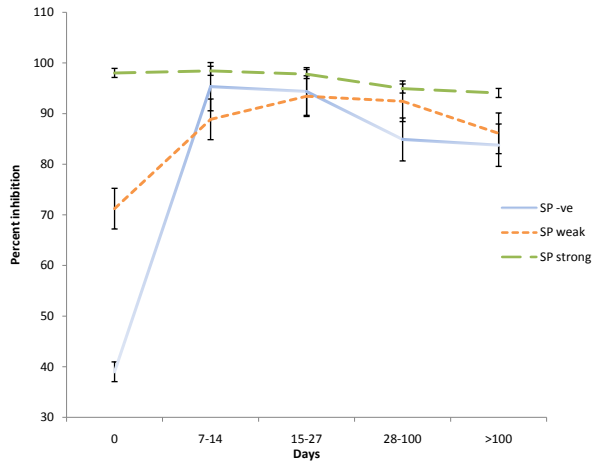
Fig.2: The antibody response patterns following FMD vaccination for groups of cattle with existing strong antibodies to structural proteins (SP), SP strong (PI >90%); weak antibodies, SP weak (PI=50-90%) and negative antibodies; SP –ve (PI<50) at the start of study. Vertical lines indicate the 95% confidence interval.

64

Pertanika J. Trop. Agric. Sci. 38 (1) 57 - 69 (2015)

DISCUSSION

In this study, the ELISA kit that detected the SP of FMDV type O was used to assess the level of protection against the most common serotype affecting local animal populations. The baseline antibodies against these proteins indicated that 149 (84.7%) sera were strongly positive even before the vaccine was administered and only 13 (7.4%) were negative. The antibodies were generally due to previous vaccination as only 28 (15.9%) were also positive for the antibodies to NS virus proteins. We found this interesting because the last reported vaccination for each farm (excluding the cattle from villages in Perlis) was performed between 10 to 11 months before the study began. The finding suggests that the vaccine is efficient and effective in conferring the animals' immunity against FMD infection, which is consistent with Doel's review that suggests repeated vaccine will elicit prolonged protective immunity even without annual booster doses (Doel, 2003). However, since recording is rather poor for individual animals involved in this study, we refrained from drawing any conclusion about the duration of immunity conferred from previous vaccinations. Our study found that a single dose of FMD vaccine was generally adequate to protect the animals against FMDV type O ideally until the administration of the second annual vaccine dose in the FMD-endemic areas. This was apparent as the majority of vaccinated animals had antibodies against the vaccination proteins with PI>90%, even

4 months post-vaccination in Kelantan. Maintenance of strong antibody response is vital to ensure that animals remain protected from clinical disease especially in areas where FMD is highly endemic. At the end of the sampling period, the antibody levels among the cattle in Kelantan remained strong (>90% PI), while the level significantly dropped for the cattle in Perlis. The observation in Kelantan is consistent with the report of routine biannual FMD vaccinations, which may have contributed to the lasting antibody response (Doel, 1999, 2003).

When the data were stratified based on the pre-existing levels of vaccination antibodies detected at the start of the study (R0), a significant difference was found in the response pattern between the groups. The pattern of response from the 13 naïve cattle (see Fig.1) is similar to those suggested by Doel (1999, 2003), where the response reached a strong protective level within less than 7 days and maintained at that level for about 4-5 weeks before declining. This response is considered adequate for a primary dose in naïve populations after which a booster dose (at 4-5 weeks following the primary dose) was suggested to be administered to stimulate more sustainable antibody production (Doel, 1999) to protect susceptible animals in highly-endemic FMD areas. All the 13 cattle remained free from natural FMD infection (NS proteins -ve) until the end of the study. The group of cattle with the initial weak antibodies to SP responded to the vaccination similarly by a rapid antibody increase and then a gradual

decline towards the end of the study. These findings support that the vaccine confers immediate immunity against clinical FMD within less than 1 week post-vaccination (McCullough *et al.*, 1992). It also indicated that the application of the vaccine would effectively prevent disease in emergency situations, especially among herds that have not been vaccinated regularly or have never been vaccinated. In addition, it emphasized the need for a booster dose in naïve populations so as to ensure that the herd remained protected from clinical FMD because the level of immunity would decline rapidly rendering the animal susceptible to a re-infection (McCullough *et al.*, 1992).

Evidence of field exposure to the FMD virus was detected transiently throughout the study. According to Sørensen *et al.* (1998), the detection of antibodies to the FMD virus NS-proteins is the most reliable index of infection in vaccinated animals. In this study, a higher proportion of NS protein-positive was detected in the cattle in Kelantan and the proportion appeared to fluctuate over the study period, which is consistent with the endemic status of FMD in Kelantan. In Perlis, although the disease had not been reported in the previous 4 years prior to this study, the evidence of natural infection was detected, albeit at a lower proportion than that of Kelantan. This is consistent with the fact that Perlis borders Thailand, which is highly endemic for FMD. None of the animals succumbed to the infection and for the majority of animals, the antibodies were not sustained for more than one sampling interval, indicating that the vaccine was effective and efficacious in protecting the animals against natural virus challenge even when the antibody response diminished to PI < 90%. Most FMD vaccines available do not confer sterile immunity, which prevents infection and carrier status (Barnett *et al.*, 2004) and even with protective immunity some levels of viral replication occur in vaccinated cattle upon exposure to field viruses (Barnett & Carabin, 2002; Golde *et al.*, 2005). In addition, cattle protected by vaccination can become transient FMD virus carriers or even become persistently infected without ever showing any clinical symptoms (Alexandersen *et al.*, 2003; Barnett & Carabin, 2002; Doel *et al.*, 1994). In this study, if the subclinical cattle were to be moved to a naïve herd, they could potentially become the source of a new FMD outbreak.

CONCLUSION

In this study, the sample of the local cattle from two large cattle farms in Perlis and Kelantan and several villages in Perlis may not be representative of the cattle population in Malaysia. However, our study indicated that the FMD vaccine used was effective in conferring immunity towards the virus and efficacious in preventing clinical FMD even when naturally challenged by field FMDV. Nonetheless, the magnitude and sustainability of the immunity elicited were significantly affected by the background levels of vaccination. Therefore, record keeping is pertinent to determining the precise vaccination status of individual animals within a herd so that vaccination

can be tailored to suit the population where it is being administered. The success of any vaccination programmes not only depends on use of effective vaccines but also on the vaccination coverage rates whereby at least 80% (Barteling *et al.*, 2004) of the cattle within a population must be vaccinated to ensure herd-level immunity. The coverage rates of vaccination in Peninsular Malaysia over the past decade have not achieved the suggested target rate (Abbo, 2010). Furthermore, the potency of a vaccine also depends on other factors such as vaccine cold-chain management, storage conditions of the vaccine prior to use, vaccine administration route, the level of skill and training of the vaccinators in terms of dose, rate and technique, vaccine preparation prior to use, animal species to be vaccinated and their health status, usage of expired vaccines and animal vaccinating related-problems (Merial, 2008). Moreover, adhering to handling and storage recommendations can be challenging in many tropical countries; non-adherence typically results in the compromising of the vaccine's quality and potency (ASEAN, 2009).

The findings of the current study have provided more knowledge and better understanding of the response of cattle towards the FMD vaccine in a tropical field situation where animal vaccination and infection status or backgrounds may be uncertain. These findings may be used to improve the way vaccines are administered and in making decisions about vaccination strategies.

REFERENCES

Abbo, H. A. (2010). *The epidemiology of foot and mouth disease and assessment of vaccination response among local cattle in Perlis and Kelantan states in Malaysia*. University Putra Malaysia (UPM).

Abbo, H. A., Hassan, L., Arshad, S. S., Azmie, M. Z., Loganathan, P., Norlida, O., Syarifah, A. M. A., & Salih, M. M. (2010). *Outbreaks of foot and mouth disease in cattle in Peninsular Malaysia between 1996 and 2007*. In The 22nd VAM Congress and 4th International ASZWM Meeting. Veterinary Association of Malaysia.

Alexandersen, S., Zhang, Z., Donaldson, A., & Garland, A. (2003). The pathogenesis and diagnosis of foot-and-mouth disease. *Journal of comparative pathology, 129*, 1-36.

ASEAN (2009). *Manual of Asean code of practice for the commercial storage, transportation and handling of animal vaccines*. Association of South East Asian Nations (ASEAN).

Barnett, P. & Carabin, H. (2002). A review of emergency foot-and-mouth disease (FMD) vaccines. *Vaccine, 20*, 1505-1514.

Barnett, P., Keel, P., Reid, S., Armstrong, R., Statham, R., Voyce, C., Aggarwal, N., & Cox, S. (2004). Evidence that high potency foot-and-mouth disease vaccine inhibits local virus replication and prevents the 'carrier'state in sheep. *Vaccine, 22*, 1221-1232.

Barteling, S. J., Yadin, H., & Sutmoller, P. (2004). *Discussion paper on guidelines for control of Foot-and-Mouth Disease (FMD) vaccine quality and performance in the field Food and Agricultural Organization (FAO), Rome, Italy*.

Bouma, A., Elbers, A. R., Dekker, A., de Koeijer A, Bartels, C., Vellema, P., van der Wal, P., van Rooij, E. M., Pluimers, F. H., & de Jong, M. C. (2003). The foot-and-mouth disease epidemic in The Netherlands in 2001. *Preventive Veterinary Medicine, 57*, 166.

Brocchi, E., Bergmann, I., Dekker, A., Paton, D., Sammin, D., Greiner, M., Grazioli, S., De Simone, F., Yadin, H., & Haas, B. (2006). Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus. *Vaccine, 24*, 6966-6979.

Chénard, G., Miedema, K., Moonen, P., Schrijver, R. S., & Dekker, A. (2003). A solid-phase blocking ELISA for detection of type O foot-and-mouth disease virus antibodies suitable for mass serology. *Journal of Virological Methods, 107*, 89-98.

Doel, T. (1999). Optimisation of the immune response to foot-and-mouth disease vaccines. *Vaccine, 17*, 1767-1771.

Doel, T. (2003). FMD vaccines. *Virus Research, 91*, 81-99.

Doel, T., Williams, L., & Barnett, P. (1994). Emergency vaccination against foot-and-mouth disease: rate of development of immunity and its implications for the carrier state. *Vaccine, 12*, 592-600.

Ellis-Iversen, J., Smith, R. P., Gibbens, J. C., Sharpe, C. E., Dominguez, M., & Cook, A. J. C. (2011). Risk factors for transmission of foot-and-mouth disease during an outbreak in southern England in 2007. *Veterinary Record, 168*, 128.

Field, A. (2009). *Discovering statistics using SPSS*. Sage publications.

Forman, S., Le Gall, F., Belton, D., Evans, B., François, J., Murray, G., Sheesley, D., Vandersmissen, A., & Yoshimura, S. (2009). Moving towards the global control of foot and mouth disease: an opportunity for donors. *Revue scientifique et technique-Office international des épizooties, 28*, 883-896.

Geering, W. A., & Lubroth, J. (2002). *Preparation of foot-and-mouth disease contingency plans (No. 16) (pp. 91)*. Food and Agricultural Organization (FAO), Rome Italy.

Gleeson, L. (2002). A review of the status of foot and mouth disease in South-East Asia and approaches to control and eradication. *Revue scientifique et technique-Office international des épizooties, 21*, 465-472.

Golde, W. T., Pacheco, J. M., Duque, H., Doel, T., Penfold, B., Ferman, G. S., Gregg, D. R., & Rodriguez, L. L. (2005). Vaccination against foot-and-mouth disease virus confers complete clinical protection in 7 days and partial protection in 4 days: use in emergency outbreak response. *Vaccine, 23*, 5775-5782.

Karuppanan, P., & Naheed, M. (2000). *Introduction and use of ELISA based technologies for the diagnosis and monitoring of foot-and-mouth disease in Malaysia*. Retrieved from http://www.iaea.org/inis/collection/NCLCollectionStore/_Public/31/031/31031691.pdf

McCullough, K., De Simone, F., Brocchi, E., Capucci, L., Crowther, J., & Kihm, U. (1992). Protective

immune response against foot-and-mouth disease. *Journal of virology, 66*, 1835-1840.

Merial (2008). *Merial's Foot and Mouth Disease Vaccines*. In M. Limited (Ed.). England and Wales.

OIE (2011). *Animal Health in the World: Official Disease Status*. International Office of Epizootics (OIE), Paris, France.

Palanisamy, K., Daud, Z., & Masran, M. (2000). *Immunity status of foot-and-mouth disease in the border districts of Peninsular Malaysia. Use of immunoassay technologies for the diagnosis and control of foot and mouth disease in Southeast Asia*. Proceedings of final research co-ordination meeting organized by joint FAO/IAEA division of nuclear techniques in food and agriculture, pp. 85. Animal Production and Health Section International Atomic Energy Agency, 22-26 February, 1999, Phnom Penh, Cambodia.

Park, J., Park, J., Kim, Y., Oem, J., Lee, K., Kye, S., & Joo, Y. (2003). Vaccination as a control measure during the outbreak of foot-and-mouth disease in 2000 in Korea. *Developments in biologicals, 119*, 63-70.

Paton, D. J., Sumption, K. J., & Charleston, B. (2009). Options for control of foot-and-mouth disease: knowledge, capability and policy. *Philosophical Transactions of the Royal Society B: Biological Sciences, 364*, 2657-2667.

Rweyemamu, M., Roeder, P., MacKay, D., Sumption, K., Brownlie, J., & Leforban, Y. (2008a). Planning for the Progressive Control of Foot and Mouth Disease Worldwide. *Transboundary and Emerging Diseases, 55*, 73-87.

Rweyemamu, M., Roeder, P., Mackay, D., Sumption, K., Brownlie, J., Leforban, Y., Valarcher, J. F., Knowles, N., & Saraiva, V. (2008b). Epidemiological Patterns of Foot and Mouth Disease Worldwide. *Transboundary and Emerging Diseases, 55*, 57-72.

SEAFMD (2007). *The Malaysia-Thailand-Myanmar (MTM) Peninsular Campaign for FMD Freedom Meeting of the MTM Tristate Commission for FMD Freedom*. Kota Bahru, Malaysia, 5 – 6 June, 2007.

Smitsaart, E., Zanelli, M., Rivera, I., Fondevila, N., Compaired, D., Maradei, E., Bianchi, T., O'Donnell, V., & Schudel, A. (1998). Assessment using ELISA of the herd immunity levels induced in cattle by foot-and-mouth disease oil vaccines. *Preventive Veterinary Medicine, 33*, 283-296.

Sørensen, K., Madsen, K., Madsen, E., Salt, J., Nqindi, J., & Mackay, D. (1998). Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. *Archives of Virology, 143*, 1461-1476.

Thrusfield, M. (2013). *Veterinary epidemiology (3rd Edn.)*. Blackwell Science.

Valarcher, J., Knowles, N., Ferris, N., Paton, D., Zakharov, V., Sherbakov, A., You-jun, S., Zaixin, L., Xiang-tao, L., & Sanyal, A. (2005). Recent spread of FMD virus serotype Asia 1. *Veterinary Record, 157*, 30-30.

Westbury, H., Chamnanpood, P., Tangchaitrong, S., & Doughty, W. (1988). Single dilution ELISA for detection of serum antibody to foot-and-mouth disease virus in cattle. *Veterinary microbiology, 18*, 273-283.

Windsor, P., Freeman, P., Abila, R., Benigno, C., Verin, B., Nim, V., & Cameron, A. (2011). Foot and Mouth Disease Control and Eradication in the Bicol Surveillance Buffer Zone of the Philippines. *Transboundary and Emerging Diseases, 58*, 421-433.

PERTANIKA
JOURNALS

Performance, Haematological Parameters and Faecal Egg Count of Semi-intensively Managed West African Dwarf Sheep to Varying Levels of Cassava Leaves and Peels Supplementation

Fasae, O. A.*, Amos, A. O., Owodunni, A. and Yusuf, A. O.

Department of Animal Production and Health, Federal University of Agriculture, P.M.B. 2240, Abeokuta, Nigeria

ABSTRACT

Thirty West African dwarf (WAD) sheep aged 18-24 months with average weight of 17.96±0.89kg, managed under semi-intensive system, were used in a 56-day experiment to investigate the effects of varying levels of cassava leaves (CL) and cassava peels (CPL) supplementation on the performance, haematological indices and faecal egg count of sheep. Animals were subjected to 6 dietary treatments of 100% CL, 100% CPL, 75% CL/25% CPL, 25% CL/75% CPL, 50% CL/50% CPL, and natural pasture (0% CL/0% CPL) in a complete randomized design. Results showed that weight gain (g/day) varied (P<0.05) across treatments from 26.25 to 44.64 with sheep supplemented with 75CL/25CPL having the highest (P < 0.05) growth rate and the least values observed in sheep on control treatment. The dry matter and crude protein digestibilities were least (P<0.05) in sheep supplemented with 100%CPL relative to other treatments. The white blood cells and total protein of sheep varied (P<0.05) across treatments, while supplementary diets of CL reduced faecal egg count in sheep. It was therefore concluded that the performance of sheep managed under the semi-intensive system, grazing natural pasture could further be enhanced through the supplementation of cassava leaves and peels with dietary levels of 75% CL/25% CPL recommended for sheep's optimum performance.

Keywords: Cassava leaves, cassava peels, faecal egg count, haematology, performance, sheep

INTRODUCTION

Sheep are important sources of animal protein in Nigeria. They adapt to a wide range of environments and utilize a variety of plant species. However, the productivity of these animals which are usually managed

under the semi–intensive system is low due to various factors including poor feeding. The unavailability of traditional diets for sheep which consist mainly of grasses especially during the dry season (Sodeinde *et al.* 2007) has prompted farmers to search for alternative, readily available by-products.

With Nigeria being the largest producer of cassava worldwide (Daniels *et al.,* 2011), cassava harvesting and processing produce large amounts of leaves and peels and are generally considered to contribute significantly to environmental problems when left in the surroundings of processing plants or carelessly disposed off. Cassava peels, which account for 10-13% of the tuber weight, are left to rot away or burnt off to create space for the accumulation of new generation of waste heaps, emitting carbon dioxide and producing a strong offensive smell (Aro *et al., 2010*).

However, cassava leaves and peels, if well processed, can be beneficial to livestock. To a limited extent, these by-products have been successfully used as livestock feeds, particularly in ruminant diets (Alli Balogun *et al.,* 2003; Fasae *et al.,* 2007). The peels of cassava are rich in protein oil and ash than the peeled portion. On a dry matter basis, cassava leaves have protein content that ranges between 14 and 69%, are fair in lysine content, marginal in tryptophan and isoleucine but deficient in methionine (Eruvbetine, 1995), whereas tannin in cassava leaves has been found to reduce faecal egg counts in ruminant animals (Netpana *et al.,* 2001).

This experiment therefore investigated into the effect of varying levels of cassava leaves and peels supplementation on the growth rate, haematology and faecal egg count of the semi-intensively managed West African Dwarf sheep.

MATERIALS AND METHODS

Experimental Site

The experiment was conducted at the Small Ruminant Experimental Unit of the Teaching and Research Farm Directorate Farms, Federal University of Agriculture, Abeokuta, Ogun State, South Western Nigeria. It has prevailing tropical climate with the mean annual rainfall of about 1037mm. The mean ambient temperature ranges from 28°C in December to 36°C in February, with a yearly average humidity of about 82%.

Preparation of the Cassava Leaves and Peels

Cassava leaves and peels were obtained from TMS 30572 variety of *Manihot esculenta.* Cassava peels were collected fresh from a gari processing plant after processing and sun dried on a flat concrete floor for three to four days, while cassava leaves were obtained from established plots and wilted for 24 hours so as to reduce hydrocyanic acid content present in the leaves. The Cassava leaves and peels were ground and mixed together at varying proportions before feeding to the animals.

Experimental Animals and Management

A total of thirty West African dwarf sheep aged 18 -24 months managed under semi-intensive system and with average weight of 17.96±0.89kg sourced from the flock of sheep of the Teaching and Research farms of the University were used for the experiment. They were randomized based on their initial weight and allotted to six dietary treatments in a completely randomized design: 100% CL; 100% CPL; 75% CL and 25% CPL; 50% CL and 50% CPL, 25% CL and 75% CPL and natural pasture (control treatment) for treatments 1 to 6, respectively. The animals were allowed to graze on natural pasture between 0900h and 1400h and were later confined into individual pens for the remaining hours of the day, during which the supplementary diets of cassava leaves and peels were fed at varying levels at 4% body weight per day on DM basis, while water was given *ad libitum*.

Feed Intake and Weight Gain Measurement

Data on feed intake by individual animals were recorded daily and the leftover feed was weighed and subtracted from the total quantity offered to determine the feed intake. The sheep were weighed initially at the start of the experiment and on a weekly basis thereafter until the termination of the experiment. Meanwhile, weight gained by the animals was calculated by subtracting the initial weight of the animals from the final weight.

Digestibility Trial

At the end of the 56-day growth trial, each animal on the supplementary treatments was transferred into individual metabolic crates and offered varying levels of cassava leaves and peels. Water was given *ad libitum.* Each digestion trial involved an adjustment period of 14 days to allow the animals to adjust to the diets, followed by a collection period of 7 days. Faecal output from each animal was weighed and DM determined daily. The dried faeces collected was bulked and stored in cellophane bag at room temperature for 2 weeks before analysis. The nutrient digestibility of the feed was determined by subtracting faecal nutrient output from feed nutrient intake.

Collections of Faecal and Blood Samples

Faecal samples of about 2 – 4 grams were obtained directly from the rectum of each animal on the first and the last three days of the growth experiment and taken to the laboratory to determine the faecal egg count using the Mcmaster technique.

At the termination of the digestibility experiment, 10ml of blood was drawn from each animal through the jugular vein using hypodermic needle and syringe. Five millimetres of the blood samples were released into the sample bottles containing ethylene diamine tetra acetic acid (EDTA) as anti coagulant and the bottles were shaken to ensure proper mixing of the blood with the EDTA acid to prevent coagulation. The remaining 5ml of blood samples were collected into bottles without

anti coagulant to harvest serum. The EDTA bottles containing the blood samples were kept in an ice – pack and taken to a standard laboratory for the analysis.

Chemical Analysis

The proximate compositions of cassava leaves, peels and faecal samples were determined by using the method of AOAC (1995). The DM was determined by oven drying at 100^0C for 24 hours, Crude protein (CP) by Kjedhal method and fat by Soxhlet fat analysis. The concentration of neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin both in the feed and faecal samples were also determined by using the method of Van Soest and Robertson (1985). The hydrocyanic (HCN) contents of the cassava leaves in the feed and faeces were also determined as described by Bradbury *et al.* (1999). Analyses of tannin were carried out by using the method described by Makkar *et al.* (2003).

The blood samples were taken on the same day to the hematological laboratory for the determination of some haematological indices: packed cell volume, white blood cell, red blood cell, haemoglobin, total protein and blood urea nitrogen (AOAC, 1995). The samples of faeces were analyzed using the Modified McMaster counting technique (Sloss *et al.,* 1994).

Statistical Analysis

All data generated were subjected to one-way analysis of variance (SAS, 1999), with significant means separated using the Duncan's Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

The chemical compositions of the cassava leaves and peels fed as supplements to sheep are shown in Table 1. The proximate values for the cassava peels (CPL) fell within the range reported (Fasae *et al.,* 2007). However, lower values (with the exception of the CP contents) were reported (Omotoso *et al.* 2010). The CP content of the CL reported is comparable to the range 29.8 - 33.7% protein (dry basis) in the leaves of different cassava cultivars (Oni *et al.* 2012), but higher than that reported by Alli-Ballogun *et al.* (2003) for cassava foliage. The DM contents of CL reported in this study were lower compared to those reported in the earlier literatures (Fasina *et al.,* 2008; Fasae & Olatunji, 2011) but fell within the range reported by Alli-Ballogun *et al.* (2003). Other researchers have shown variations in the chemical composition among leaves of different cassava varieties (Ngi *et al.,* 2006; Oni *et al.,* 2012).

The proportion of NDF in CL and CPL was congruent to the concentration of 600g/kg considered as safe for acceptable intakes of forages (Meissner *et al.* 1991). The performance indices of the semi intensively managed West African dwarf sheep supplemented with cassava leaves and peels are shown in Table 2. The DM intake (g/day) differed significantly (P < 0.05) across the treatments, with the highest DM intake observed in sheep fed 75% CL

and 25% CPL, similar to intake values obtained for the sheep supplemented with 50%CL/50%CPL diet. This might be due to the high energy content of the mixed diets, resulting in high degradability of cassava leaves and peels at these levels of mixture in the rumen (Smith, 1992). In addition, tannin contents in diets containing CL may even have been responsible for enhanced efficiency of utilizing the CP of the supplements by sheep. The tannin component of the supplementary diets, which is at the concentration level recommended for sheep (Pawelek *et al.*, 2008) could have impacted some qualities of ruminal undegradable protein thereby

TABLE 1
Chemical composition (g/kg) of cassava leaves and peels supplemented to semi intensively managed West African dwarf sheep

Composition	Cassava leaves	Cassava peels
Dry matter	286.0	255.0
Crude protein	200.6	42.6
Ether extract	83.9	16.5
Ash	160.7	52.3
Neutral detergent fibre	411.7	560.1
Acid detergent fibre	300.2	351.2
Acid detergent lignin	91.0	121.0
Energy (MJ/kg/DM)	14.25	18.17
Crude protein (g/kg)	200.65	42.61
Metabolizable energy (MJ/kg)	14.25	18.17
Tannin	32.4	17.0
Hydrocyanic acid (mg/kgDM)	352.2	285.5

TABLE 2
Effects of varying levels of cassava leaves and peels supplementation on the performance characteristics of semi-intensively managed West African dwarf sheep

Parameters	100% CL	100% CPL	75% CL/ 25%CPL	50%CL/ 50% CPL	25%CL/ 75% CPL	0%CL/ 0%CPL	SEM
Feed intake (g/day)	378.33[b]	341.67[c]	392.00[a]	388.67[a]	380.33[b]	-	3.83
Metabolic feed intake (g/KgW$^{0.75}$)	85.78[a]	79.44[b]	88.09[a]	87.54[a]	86.12[a]	-	1.76
Crude protein intake (g/day)	75.91[a]	14.53[c]	63.18[a]	47.30[b]	31.16[b]	-	1.52
ME intake (MJ/day)	5.38[b]	6.18[a]	6.04[a]	5.88[b]	5.76[b]	-	0.41
Initial weight (kg)	17.33	18.33	18.25	17.95	18.15	18.00	0.91
Final weight (kg)	19.03[b]	19.80[b]	20.75[a]	20.18[a]	20.13[a]	19.30[b]	0.96
Cumulative weight gain (kg)	1.70[b]	1.47[c]	2.50[a]	2.23[a]	1.98[b]	1.30[c]	0.35
Daily weight gain (g/day)	30.36[b]	26.25[c]	44.64[a]	39.82[a]	35.36[ab]	23.21[c]	1.63
Metabolic weight gain (KgW$^{0.75}$)	12.93[bc]	11.60[c]	17.27[a]	15.85[b]	14.50[b]	10.57[c]	0.77

[a,b,c] : Means in the same row with different superscript are significantly different (P<0.05)
CL: Cassava leaves; CPL: Cassava peels; ME: Metabolizable energy; SEM: Standard error of mean

Pertanika J. Trop. Agric. Sci. 38 (1): 71 - 81 (2015)

75

enhancing the utilization of its protein due to a potentially higher amino acid flow to the small intestine (Meissner *et al.,* 1991). The lowest feed intake observed in the sheep supplemented with 100%CPL diets corroborates the reports of Ahamefule *et al.* (2001) in the same breed of sheep fed with varying levels of cassava peel in poultry waste diets.

However, the intakes of diet (79.44 −87.54g/KgW$^{0.75}$) were remarkable and similar to 87 to 108g/KgW$^{0.75}$ reported by Adegbola *et al.* (1988) and Bawala *et al.* (2007), though higher than 59 to 68g/KgW$^{0.75}$ reported by Tual *et al.* (1992) when CPL was fed with adequate amount of nitrogen to WAD sheep.

The crude protein intake (g/day) differed significantly (P < 0.05) across the treatments with the highest (P < 0.05) CP intake observed in sheep fed with 100% CL and 75% CL/25%CPL. The CP intakes seemed to be reflections of the CP contents of the dietary supplements and their corresponding DM intake levels. This corroborates the reports of Mtenga and Shoo (1990) of a positive correlation between crude protein intake and dry matter intake. Moreover, the metabolizable energy intake (MJ/day) was the highest (P < 0.05) in sheep fed with 100% CPL and 75% CL/25%CPL.

The cumulative weight gain (kg) and daily weight gain in sheep supplemented with CL and CPL differed significantly (P < 0.05) across the treatments, with sheep fed all the supplementary diets gained weight. Sheep supplemented with a mixed diet of CL and CPL containing diets 75% CL/25%CPL

and 50%CL/50%CPL had higher (P < 0.05) gains, which could be attributed to the amount of protein, minerals, vitamins and energy levels in cassava leaves which had been reported to increase weight gain in sheep (Alli-Balogun *et al.,* 2003; Fasae & Olatunji, 2011).

Sheep supplemented with 100% CPL with the lowest (P>0.05) weight gain, which ranked the same with sheep on the control treatment, could be indicative of the deficiency of rumen degradable nitrogen in the CPL that led to poor interaction of ruminal microorganism in the diet. Earlier studies carried out on sheep and goats showed weight loss after consuming only cassava peels (Baah *et al.,* 1999; Fasae *et al.,* 2007).

The average daily weight gain (g/day) values reported in this study (23.21 – 44.64) were comparable to some earlier reports (Anaeto *et al.,* 2013) on the same breed of sheep fed cassava peels supplemented diets while the metabolic weight range of 10.57 to 17.27KgW$^{0.75}$ is higher than 6.14 to 6.70KgW$^{0.75}$ in sheep fed with cassava peels (Tual *et al*., 1992).

Table 3 shows the dry matter and nutrient digestibility coefficient of WAD sheep fed CL and CPL. The diets did not have any significant (P>0.05) effect on the fat and ash digestibility coefficients, while DM, crude protein and fibre fractions digestibility coefficients differed significantly (P<0.05). The dry matter digestibility (DMD) coefficient of CL and CPL in this study is similar to that obtained by Fasae and Olatunji (2011) when sheep

were subjected to different cassava leaves processed under different methods, but higher than those obtained by Ukanwoko *et al.* (2009) in WAD bucks fed with cassava peel-cassava leaf meal based diets. This might be as a result of the difference in the feed composition as well as the breed of animals used.

The high CP digestibility observed with higher levels of inclusion of CL could be due to the high protein and essential amino acids in CL (Eruvbetine, 1995), as well as the energy contents of CPL mixture, which were more degradable by the rumen microbes. The high intake resulting in higher digestibility might be connected with the nature of the diet. Higher CP and energy in diets have been considered as important factors that enable high intake of feed.

The low DMD value of sheep supplemented with 100% CPL was probably due to non inclusion with CL which probably reduced acceptability, palatability and digestibility, while on the other hand, the high values of DMD of diets containing CL might therefore be due to the high intake as a result of palatability of the diets. This is in consonance with the findings of Van Soest and Robertson (1985) that increased protein supplementation tends to improve intake by increasing nitrogen supply to the rumen microbes. This has a positive effect by increasing microbial population and also improves the rate of breakdown of digesta. Therefore, high DMD is an evidence of high palatability and acceptability of the diet.

Table 4 presents some haematological parameters of the sheep supplemented with varying levels of cassava leaves and peels. The data showed that white blood cell count (WBC) and total protein (TP) differed significantly (P < 0.05) across the treatments but fell within recommended range for healthy sheep. The values obtained for red blood cell counts, the packed cell volume and the haemoglobin concentration in this study were within the normal physiological range for sheep (Jain, 1993)

TABLE 3
Apparent nutrient digestibility (g/Kg DM) of varying levels of cassava leaves and peels in West African dwarf sheep

Parameters	100% CL	100% CPL	75% CL/ 25% CPL	50%CL/ 50% CPL	25%CL/ 75% CPL	SEM
Dry matter	63.83[a]	51.78[b]	65.14[a]	64.30[a]	61.99[a]	0.83
Crude protein	63.01[a]	40.10[c]	69.30[a]	64.40[a]	54.40[b]	0.91
Ether extract	68.06	58.85	67.41	60.38	66.40	0.75
Ash	65.50	66.05	65.92	66.36	68.12	0.63
Neutral detergent fibre	60.11[a]	49.17[b]	61.08[a]	58.45[a]	51.03[b]	0.61
Acid detergent fibre	63.41[a]	43.64[c]	60.67[a]	57.77[ab]	51.82[b]	0.57
Acid detergent lignin	57.24[a]	45.05[b]	56.49[a]	55.12[a]	49.56[ab]	0.55

[a,b,c] : Means in the same row with different superscripts are significantly different (P<0.05)
CL - Cassava leaves; CPL - Cassava peels; SEM - Standard error of mean

and comparable to those previously reported (Egbe Nwiyi *et al.,* 2000) for apparently healthy sheep. The WBC values reported in the sheep supplemented with CL indicate high immunity status of the animals to fight against infection (Aikhuomobhogbe & Orheruata, 2006).

The values for TP obtained in the CL supplemented sheep are similar to 59.27 – 74.00g/l (Taiwo & Ogunsanmi, 2002). Deficiency of protein impairs both humoral and cell mediated immunity, thus predisposing an animal to disease (Titgemeyer & Loest, 2001).

Table 5 shows the effects of varying level of cassava leaves and peels supplementation on the faecal egg counts (FEC) of sheep managed semi-intensively. There is an evidence of positive effect of anthelmintic activity of cassava foliage on the basis of observed reductions in animals fed the supplementary diets. This could be attributed to phytochemicals, particularly the complex phenolics which are generally referred as tannins present in cassava leaves. Earlier findings in sheep as well as in grazing cattle and buffaloes respectively show much lower EPG after feeding cassava leaf supplements

TABLE 4
Haematological parameters of semi intensively managed West African Dwarf Sheep supplemented with varying levels of cassava leaves and peels

Parameter	100% CL	100% CPL	75% CL/ 25% CPL	50%CL/ 50% CPL	25%CL/ 75% CPL	0%CL/ 0%CPL	SEM
Packed Cell Volume (%)	35.00	30.00	35.00	33.00	36.00	35.00	0.45
Haemoglobin (g/dl)	11.80	11.20	12.00	10.10	10.10	11.90	0.23
White Blood Cells(x10$^{9/l}$)	8.80[b]	13.80[a]	9.11[b]	9.30[b]	9.50[b]	11.20[a]	0.20
Red Blood Cell X10$^{12/l}$	12.01	11.89	10.55	10.70	12.45	12.13	0.21
Total Protein(g/l)	81.20[a]	52.20[c]	80.40[a]	79.10[a]	69.20[bc]	60.00[c]	0.56
Blood Urea Nitrogen (mg/dl)	23.40	18.40	19.20	22.20	18.90	20.55	0.31

[a,b,c] : Means in the same row with different superscripts are significantly different (P<0.05)
CL - Cassava leaves; CP - Cassava peels; SEM - Standard error of mean

TABLE 5
Faecal Egg Count (Egg per gram) of West African Dwarf sheep before and after supplementation with cassava leaves and peels

Treatments	Pre-treatment	Post –treatment	Percentage Reduction
100%CL	1620	310	80.86
100%CPL	1640	1670	-1.7
75%CL	1580	340	78.48
50%CL	1490	410	70.59
25%CL	1530	720	52.94
0%CL/0%CPL	1580	1690	-6.96
Average EPG	1573.33	856.77	-

CL - Cassava leaves; CP - Cassava peels

(Seng Sokenya & Rodriguez, 2001; Netpana *et al.,* 2001).

However, the results of this study further showed that FEC in sheep reduced with an increase with the CP content of the supplementary feed, suggesting the positive effects of high dietary CP in reducing FEC. This corroborates well with earlier reports (Brunsdon, 1964; Osoro *et al.*, 2007) that a high level of dietary protein benefited animals with established parasitic infections resulting in an improvement in their clinical condition, reduction in faecal egg count and increased resistance to re-infection.

CONCLUSION

This study showed that the response of WAD sheep managed semi intensively to supplementation of cassava leaves and peels resulted in significantly higher performance in terms of feed intake, weight gain and nutrient digestibilities. The optimum inclusion levels of cassava leaves and peels at 75CL/25CPL resulted in the best performance characteristics and this can be adopted in sheep production for improving the nutritional status, growth rate and reducing faecal egg count for maximum performance.

REFERENCES

Adegbola, A. A., Smith, O. B., & Okeudo, N. J. (1988). Responses of West African Dwarf sheep fed cassava peel and poultry manure based diets. In PANESA/ARNAB. *Utilization of research results on forage and agricultural by-product materials as animal feed resources in Africa*. Proceedings of first joint workshop held in Lilongwe, Malawi, 5-9 December, 1988.

PANESA/ARNAB, Addis Ababa, Ethiopia, 357-366.

Ahamefule, F. O., Ibeawuchi, J. A. Ibemere, J. C., & Okpara M. (2001). Digestibility and nitrogen balance studies in West African Dwarf sheep fed fortified cassava peel meal – poultry waste diets. *Journal of Applied Chemistry and Agricultural Research, 7*, 69-74.

Aikhuomobhogbe, P. U. & Orheruata, A.M. (2006). Haematology and blood biochemical indices of West African Dwarf Goats vaccinated against *Pestes des petit* ruminants *African Journal of Biotechnology, 5*, 743-748.

Alli Balogun, J. K., Lakpini, C. A. M, Alawa, J. P., Mohammed, A., & Nwanta J. A. (2003). Evaluation of cassava foliage as a protein supplement for sheep. *Nigerian Journal Animal of Production, 30*, 37-46.

Anaeto, M., Sawyerr A.F., Alli, T. R., Tayo, G. O., Adeyeye, J. A., & Olarinmoye, A. O. (2013). Cassava leaf silage and cassava peel as dry season feed for West African dwarf Sheep. *Global Journal of Science Frontier Research Agriculture and Veterinary Sciences, 13*, 2.

AOAC, Association of Analytical Chemist. (1995). *Official Methods of Analysis* (15th edition). Washington, D.C., USA, 69-88.

Aro, S. O., Aletor, V. A., Tewe, O. O., & Agbede, J. O. (2010). Nutritional potentials of cassava tuber wastes: A case study of a cassava starch processing factory in south-western Nigeria. *Livestock Research Rural Develpoment, 22*, 11.

Baah, J., Tait, R. M., & Tuah, A. K. (*1999).* The effect of supplementation with ficus leaves on the utilization of cassava peels by sheep. *Bioresource Technology, 67*, 47-51.

Bawala, T. O., Adegoke, E.O., Ojekunle, A. O., Adu, I. F., & Aina, A. B. J. (2007). Utilization of cassava peel and rumen epithelial waste diets by West African Dwarf Sheep. *Asset Series A, 7*, 168-180.

Pertanika J. Trop. Agric. Sci. 38 (1): 71 - 81 (2015)

79

Bradbury, M. G., Egan, S. V., & Bradbury, J. H. (1999). Determination of all form of cyanogens in cassava roots and cassava products using picrate paper kits. *Journal Science Food Agriculture, 79*, 593-601.

Brunsdon, R. B. (1964). The effect of nutrition on the establishment of persistence of Trichostrongyle infestation. *New Zealand Vet. J., 12*, 108-111.

Daniels, A., Udah A., Elechi, N., Oriuwa, C., Tijani G., & Sanni A. (2011). *Report on cassava value chain analysis in the Niger Delta.* Report of PIND cassava value hain assessment validation workshop held on the 20th of July 2011 at Cyprian Hotel Warri. Nigeria, 1.

Duncan, D. B. (1955). New Multiple Range Test. F test, *Biometrics, 11*, 1-42.

Egbe-Nwiyi, T. N, Nwaosu, S. C., & Salam, H. A. (2000). Haematological values of apparently healthy sheep and Goats as influenced by age and sex in arid zone of Nigeria. *African Journal Biomedicine, 1*, 109-115.

Eruvbetine, D. (1995). The availability of phosphorus in rapeseed meal for broiler chicken. *Journal of Science Research Development, 2*, 65-74.

Fasae, O. A, Adegoke, H. B., Ogunmekan, K. O., & Adu, I. F. (2007). Improving the feed utilization of cassava peels in smallholder goat production. *Nigerian Journal Animal of Production, 34*, 251-257.

Fasae, O. A. & Olatunji, J. A. (2011). Effect of simple processing methods of cassava leaves on hydrocyanic acid content and utilization by sheep. *Agricultural Tropical et Sub tropica, 42*, 15-18.

Fasae, O. A., Adu I. F., Aina A. B. J., & Dipeolu M. A. (2011). Growth performance, carcass characteristics and meat sensory evaluation of West African dwarf sheep fed varying levels of maize and cassava hay. *Tropical Animal Health and Production, 43*, 503-510.

Fasina, O. E., Ijaduola, O. O., Bello, A. I., & Fayinminu, O. O. (2008). Utilization of whole cassava plant (TMS 0581) by West African dwarf sheep. In O. A. Adeyemi, A. M. Ogunbgesan, A. O. Dada, O. O. Eniolorunda, H. A. Awojobi, D. B. Oke, & J. A. Agunbiade (Eds.), Proceedings of the 33rd Annual Conference of Nigerian Society of Animal Production, Olabisi Onabanjo University, Yewa campus, Ayetoro, Ogun State, Nigeria, March 18th 2008, 622–624.

Jain, N. C. (*1993).* Physiology of *blood* with some comments on response to disease. *International Journal of Animal Science, 8*, 195-231.

Makkar, H. P. S. (2003). Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research, 49*, 241-256.

Meissner, H. H., Koster, H. H., Nieuwoudt, S. H., & Coertze, R. J. (1991). Effect of energy supplementation on intake and digestion of early and mid season ryegrass and panicum/smuts finger hay on *in sacco* disappearance of various forage species. *South African Journal of Animal Science, 21*, 22-42.

Mtenga, L. & Shoo, R. (1990). Growth rate, feed intake and feed utilization of Small East African goats supplemented with *Leucaena leucocephala.* *Small Ruminant Research*, *3*, 9-18.

Netpana, N., Wanapat, M., Poungchompu, O., & Toburan, W. (2001). Effect of condensed tannins in cassava hay on fecal parasitic egg counts in swamp buffaloes and cattle. In Proceedings International Workshop on Current Research and Development on Use of Cassava as Animal Feed (editor T. R Preston, B. Ogle and M. Wanapat). Khon Kaen University, Thailand. July 23-24, 2001. http://www.mekarn.org/procKK/netp.htm

Ngi, J., Ayoade, J. A., & Oluremi, O. I. A. (2006). Evaluation of dried cassava leaf meal and maize offal as supplements for goats fed rice straw

in dry season. *Livestock Research for Rural Development, 18*, 9.

Omotoso, O. M, Babayemi, O. J., Afolabi, K. O., & Fabowale, A. A. (2010). Evaluation of wheat offal, cassava starch residue and cassava peels as supplement in fattening white Fulani cattle. Proceedings of the 15th Annual Conference of Animal Science Association of Nigeria, 13 -15 September, 2010, University of Uyo, Nigeria, 579-582.

Oni, A. O., Onwuka, C. F. I, Arigbede, O. M, Anele, U. Y, Oduguwa, O. O, Onifade O. S, & Tan, Z. L. (2010). Chemical composition and nutritive value of four varieties of cassava leaves grown in South-Western Nigeria. *Journal of Animal Physiology and Animal Nutrition, 5*, 25-32.

Osoro, K., Benito-Peña, A., P. Frutos, P., García, U., Ortega-Mora, L. M., Celaya, R., & Ferre. I. (2007). The effect of heather supplementation on faecal egg counts of gastrointestinal nematodes and live weight changes in Cashmere and local Celtiberic goats on pasture. *Small Rumin. Res., 67,* 184–191.

Pawelek, D. L., Muir, J. P., Lambert B. D., & Wittie, R. D. (2008). *In sacco* rumen disappearance of condensed tannins, fibre, and nitrogen from herbaceous native Texas legumes in goats. *Animal Feed Science Technology, 140*, 225-240.

Puls, R. (1988). *Mineral levels in animal health: diagnostic data* (2nd Ed.). Sherpa International, Clear Brook, Canada, 304.

Sokerya, S. & Rodriguez, L. (2001). Foliage from cassava, *Flemingia macrophylla* and bananas compared with grasses as forage sources for goats: effects on growth rate and intestinal nematodes. *Livestock Research for Rural Development, 13*, 2.

Smith, O. B. (1992). *A review of ruminant responses to cassava-based diets.* Proceedings of a Workshop on cassava as livestock feed in Africa held at Ibadan, Nigeria. IITA/ILCA, 39-52.

SAS (Statistical Analysis system). (1999). *SAS User's guide: SAS Institute Inc.*, Cary North Carolina, USA.

Sloss, M. W., Kemp, R. L., & Zajac, A. M. (1994). *Veterinary Clinical Parasitology* (6th Ed.). Iowa State Press, Ames, Iowa, p. 9.

Sodeinde, F. G., Akinlade, J. A., Asaolu, V. O., Oladipo, M. A., Amao, S. R., & Alalade, J. A. (2007). A survey of some dry season feed materials for small ruminants in Ogbomoso, Nigeria. *Journal of Animal and Veterinary Advances, 6*, 142-145.

Taiwo, V. O. & Ogunsanmi, A. O. (2002). Haemaology, plasma, whole blood and erythrocyte biochemical values of clinically healthy captive-reared grey duiker (*sylvicapra grimmia*) and West African dwarf sheep and goats in Ibadan, Nigeria, *Israel Journal of Veterinary Medicine, 58*, 2-3.

Tual, A. K., Orskov, E. R., Obese, F. Y., Okai, D. B., & Greenhalgh, J. F. D. (1992). The effect of supplementation of cassava peel diets with graded levels of palm kernel cake on the performance of growing Djallonke sheep. Proceedings of the 2nd Bienniel Conference of the African Small ruminant Research Network, AICC, Arusha, Tanzania, 7-11 December, 1992.

Titgemeyer, E. C. & Loest, C. A. (2001). Amino acid nutrition: Demand and supply in forage-fed ruminants. *Journal of Animal Science, 79*(Suppl.), E 180-189.

Ukanwoko, A. I., Ahamefule, F. O., & Ukachukwu, S. N. (2009). Nutrient intake and digestibility of West African dwarf bucks fed cassava peel-cassava leaf meal based diets in South Eastern Nigeria. *Pakistan Journal of Nutrition, 8,* 983-987.

Van Soest, P. J. & Robertson, J. B. (1985). Analysis of forages and fibrous foods. AS 613 manual, Department of Animal Science, Cornell University, Ithaca, 105-106.

Development of Fertilizer Recommendation for Aquatic Taro (*Colocasia esculenta*) in Grey Terrace Soil

S. Noor[1], M. R. Talukder[2], M. K. R. Bhuiyan[3], M. M. Islam[1]*, M. A. Haque[2] and S. Akhter[2]

[1]*Tuber Crops Research Centre, Bangladesh Agricultural Research Institute, Gazipur-1701, Bangladesh*
[2]*Soil Science Division, Bangladesh Agricultural Research Institute, Gazipur-1701, Bangladesh*
[3]*Farm Division, Bangladesh Agricultural Research Institute, Gazipur-1701, Bangladesh*

ABSTRACT

A field experiment was conducted to find out optimum doses of N, P, K and S for yield maximization of aquatic taro (*Colocasia esculenta*) during the summer seasons of 2008-2009 and 2010-2011 in Grey Terrace Soil (poorly drained, grey, and silty) of Gazipur. There were four levels of nitrogen (0, 75, 100 and 125 kg ha^{-1}), four levels of phosphorus (0, 30, 45 and 60 kg ha^{-1}), four levels of potassium (0, 80, 100 and 120 kg ha^{-1}) and four levels of sulphur (0, 10, 20 and 30 kg ha^{-1}). The experiment was laid out in a randomized complete block design with three replications. The yield attributes and yield of aquatic taro were significantly increased by the application of NPKS fertilizers. The highest stolon yields (25.60 and 28.16 t ha^{-1} for 2008-09 and 2010-11, respectively) were found in $N_{100}P_{45}K_{100}S_{20}$ kg ha^{-1} combination. From the regression analysis, it could be concluded that around 110-50-105-24 kg ha^{-1} N-P-K-S was the optimum dose for the production of aquatic taro in Grey Terrace Soil of Gazipur.

Keywords: Colocasia esculenta, fertilizers, optimum dose, yield

INTRODUCTION

Panikachu (*Colocasia esculenta* L.) is one of the most common aroids. It is grown in low lying and swampy areas of the Philippines (Pardales & Villancieva, 1984; Onwueme, 1999), Southern China (Matthews, 2000; Yongping *et al*., 2003), Bangladesh (Rana & Adhikary, 2005) and India (Sen *et al*., 1998; Saud & Baruah, 2000). Rhizome and stolon (trailing suckers) are edible, and though they

are primary for stolon, the rhizomes which are not of good quality may be considered for some other uses. The stolon emerging continuously from the base of the developing sucker corms are highly acceptable as vegetable due to its non-acridity and taste (Sen *et al.*, 19998). Panikachu (cv Latiraj) is famous for the production of good quality stolon. It is highly nutritious and palatable. Stolon contains 1.12 g iron, 38 mg calcium, 500 IU vitamin A, 38 mg vitamin C and 35 Kilocalorie food energy under 100 g edible portion (Bhuiyan *et al.,* 2008). It is also a promising crop for exporting to the foreign countries. In Bangladesh, stolon producing Panikachu occupies an area of about 6,886 ha, with a total production of 38,502 tonnes of stolon, and an average yield of 5.6 tonnes per hectare in 2009-10 (BBS, 2010). Generally, it is harvested throughout the kharif season when vegetables are deficit in the market. So, it can easily meet up the demand of vegetables at that time. It can grow easily with less care and input. Moreover, disease and insect infestations are less in case of Latiraj. Thus, there is a great opportunity to improve its production and quality through nutrient management.

Fertilizer management is one of the major determining factors to get the maximum yield of any crop. Nasreen and Islam (1989) stated that the major causes of low yield in crops may be either due to lack of high yielding varieties or poor fertility management. Judicious and proper use of manures and fertilizers are essential to get good yield (Sadhu, 1993). There is no fertilizer recommendation for Latiraj yet.

So, the experiment was initiated to study the response of Latiraj to added nutrients and to find out the optimum doses of nitrogen (N), phosphorus (P), potassium (K) and sulphur (S) so as to maximize the yield and develop a package of fertilizer for recommendation.

MATERIALS AND METHODS

Experimental Site and Soil Characteristics

The experiment was conducted at Tuber Crops Research Centre, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh during the summer seasons of 2008-09 and 2010-11. The experimental site is located at the centre of the Madhupur Tract at about $24^0 23'$ north latitude and $90^0 08'$ east longitude. The experimental plot was medium low land having silty clay soil. The soils are poorly drained, grey and silty and overlie heavy, grey, little-altered, deeply weathered Madhupur or Piedmont clay. The major part of the subsoil is an E-horizon (FAO, 1988; Brammer, 1996). The soil was slightly acidic (pH of 6.4) and low in exchangeable K (0.14 c mol kg^{-1}) and total N (0.10%). The soil boron (B) and zinc (Zn) contents were at par with critical level, while P and S were above the critical levels (Critical levels of P and S were 14 and 14 ppm, respectively and that of K was 78.2 ppm).

Treatment Details, Fertilizer and Manure Application

The treatment combinations were: T1=$N_0P_{45}K_{100}S_{20}$ kg ha^{-1}, T2=$N_{75}P_{45}K_{100}S_{20}$ kg ha^{-1}, T3=$N_{100}P_{45}K_{100}S_{20}$ kg ha^{-1}, T4=$N_{125}P_{45}K_{100}S_{20}$ kg ha^{-1}, T5=$N_{100}P_0K_{100}S_{20}$

kg ha^{-1}, T6=N$_{100}$P$_{30}$K$_{100}$S$_{20}$ kg ha^{-1}, T7=N$_{100}$P$_{60}$K$_{100}$S$_{20}$ kg ha^{-1}, T8=N$_{100}$P$_{45}$K$_0$S$_{20}$ kg ha^{-1}, T9=N$_{100}$P$_{45}$K$_{80}$S$_{20}$ kg ha^{-1}, T10=N$_{100}$P$_{45}$K$_{120}$S$_{20}$ kg ha^{-1}, T11=N$_{100}$P$_{45}$K$_{100}$S$_0$ kg ha^{-1}, T12=N$_{100}$P$_{45}$K$_{100}$S1$_0$ kg ha^{-1}, T13=N$_{100}$P$_{45}$K$_{100}$S3$_0$ kg ha^{-1} and T14=Native nutrient (control).

The experiment was laid out in a randomized complete block design (RCBD) with three replications. Urea, triple superphosphate (TSP), muriate of potash (MoP) and gypsum were used as the sources of N, P, K and S, respectively. The entire quantities of phosphorus, potassium and sulphur were applied before planting and mixing into the soil. Nitrogen was to-dressed at 30, 50 and 70 DAP (days after planting).

Planting and Harvesting

Aquatic taro (*Colocasia esculenta*) variety of Latiraj was used as a test crop. The unit plot size was 3 m × 4.5 m. Seedlings of aquatic taro were planted with a spacing of 60 cm × 45 cm on 1st and 5th April of 2008 and 2010, respectively. The stolon was harvested at 60 DAP and harvesting continued 10 days interval throughout the growing season up to October.

Intercultural Operation

The field was intensively kept free from weeds for the first three months. Standing water of 8-10 cm was maintained in the field. Sometimes, the standing water was shaken and drained out. Dead leaves were removed regularly from the field. In some areas of the field, leaf blight disease was found to have occurred, and this was treated by spraying Ridomyl gold @ 2 gL^{-1} water for 3 times at 30-days interval.

Soil Sampling and Chemical Analysis

Soil samples were collected, dried and ground for chemical analysis. Bulk density was determined by using core sampler Method (Blake, 1965), soil pH by glass electrode pH meter (1:2.5) and organic carbon by wet oxidation method (Walkley & Black, 1935). Total N content of soil was determined by using the Kjeldahl method (Jackson, 1973), whereas available P, exchangeable K and available S contents by using 0.5M NaHCO$_3$ (pH 8.5), NH$_4$OAc and CaCl$_2$ extraction methods, respectively, as outlined by Page *et al*. (1982).

Data Collection

Yield attributes (plant height, number of leaves, number of stolon and stolon length) were recorded from 10 randomly selected plants. Stolon yield per plot was recorded and based on that, per hectare yield was calculated.

Statistical Analysis

The analysis of variance for yield attributes and yield was done following the ANOVA (analysis of variance) test and the mean values were compared by DMRT (Steel & Torre, 1960). One-way ANOVA table was used to perform this analysis. Computation and preparation of graphs were done using Microsoft EXCEL 2003 programme.

RESULTS AND DISCUSSION

Effects of Fertilizers on the Growth Parameters of Aquatic Taro

Plant height, number of leaves, number of stolon and stolon length were significantly influenced by the application of fertilizers. Plant height ranged from 60.6 to 79.9 cm and 63.1 to 87.9 cm in 2008-09 and 2010-11, respectively. The highest plant height (79.9 and 87.9 cm for 2008-09 and 2010-11, respectively) was found in T3 ($N_{100}P_{45}k_{100}S_{20}$ kg ha^{-1}), followed by T4 ($N_{125}P_{45}K_{100}S_{20}$ kg ha^{-1}). Treatments T3, T4, T7 and T10 showed statistically similar plant height. The lowest plant height (60.6 and 63.1 cm for 2008-09 and 2010-11, respectively) was in the control (Table 2). Mehla *et al*. (1997) reported that the plant height of *Colocasia esculenta* increased significantly after the applications of 150 kg N and 50 kg P per hectare at Haryana, India. The fertilizers had significant effect on the number of leaves. In particular, T3 showed the highest number of leaves (4.00 and 4.40 for 2008-09 and 2010-11, respectively), followed by T4 (3.87 and 4.09). The lowest number of leaves (3.00 and 3.30 in 2008-09 and 2010-11, respectively) was in the control. Mehla *et al*. (1997) observed the maximum number of leaves plant^{-1} of *Colocasia esculenta,* with 100 kg N ha^{-1} and 50 kg P ha^{-1}. Meanwhile, Verma *et al*. (1996) reported the maximum number of leaves plant^{-1} with 80 kg N ha^{-1} and 120 kg K ha^{-1}, and the results corroborated with the findings of the current work. The number of stolon plant^{-1} was also significantly variable among the different treatment combinations. The highest number of stolon plant^{-1} (29.7 and 32.7 in 2008-09 and 2010-11, respectively) was found in T3, and this was followed by T7 (28.0 and 30.8 for 2008-09 and 2010-11, respectively). The lowest number of stolon (17.4 and 19.2 for 2008-09 and 2010-11, respectively) was in the control (Table 2). It was found that the number of stolon increased with the increase in nitrogen levels. A similar result was also observed by Sen *et al*. (1998) in swamp taro. The fertilizers showed significant effects on stolon length. The maximum stolon length (100.7 and 110.8 cm in 2008-09 and 2010-11, respectively) was obtained in T3, and this was followed by T4 (95.8 and 99.0 cm for 2008-09 and 2010-11, respectively). The minimum stolon length (63.7 and 70.1 cm for 2008-09 and 2010-11, respectively) was in the control (Table 2). In 2008-09, T3, T4, T7, T10 and T11 showed statistically similar stolon lengths. Alam *et al*. (2010) reported that the highest stolon length was found in 125-36-125 kg ha^{-1} of N-P-K, which is in agreement with the finding of our study.

Effects of Fertilizer on the Stolon Yield

Stolon yield was significantly influenced by the fertilizers. In more specific, stolon yields ranged from 8.80 to 25.6 t ha^{-1} and 7.68 to 28.2 t ha^{-1} for 2008-09 and 2010-11, respectively. The highest stolon yield (25.6 and 28.2 t ha^{-1} in 2008-09 and 2010-11, respectively) was found in T3 ($N_{100}P_{45}K_{100}S_{20}$ kg ha^{-1}), which was significantly higher than the other treatments. Meanwhile, T4 ($N_{125}P_{45}K_{100}S_{20}$ kg ha^{-1}) showed the second highest yield (22.05 and 24.92 t ha^{-1} for 2008-09 and 2010-11, respectively), and

TABLE 1
Initial fertility status of soils of the experimental field

Soil properties	pH	OM %	Ca	Mg	K	Total N %	P	S	B	Cu	Fe	Mn	Zn
			meq/100g							µg g⁻¹			
2008-09	5.5	1.10	4.2	4.1	0.18	0.11	15	17	0.2	6	112	35	2.2
2010-11	5.6	1.08	4.1	4.2	0.19	0.12	18	18	0.2	7	115	36	2.4
Critical level	-	-	2.0	0.80	0.20	-	14	14	0.2	1	10	5	2.0

TABLE 2
Yield and yield attributes of aquatic taro (*Colocasia esculenta* L.) as influenced by different fertilizer levels

Treat.	Plant height (cm)		Number of leaves		Number of stolon		Stolon length (cm)		Stolon yield (t ha⁻¹)	
	2008-09	2010-2011	2008-09	2010-2011	2008-09	2010-2011	2008-09	2010-2011	2008-09	2010-2011
T₁	66.6 cd	72.2 c	3.4 c	3.7 ab	22.8 ef	25.1 abc	73.5 fg	80.8 bc	13.4 fg	14.8 ef
T₂	70.1 bc	77.1 bc	3.6 bc	3.9 ab	26.6 bcd	29.3 ab	84.0 def	92.4 abc	21.6 cd	23.8 b
T₃	79.9 a	87.9 a	4.0 a	4.4 a	29.7 a	32.7 a	100.7 a	110.8 a	25.6 a	28.2 a
T₄	76.3 ab	83.9 ab	3.9 ab	4.1 ab	27.1 bc	27.8 ab	95.7 abc	99.0 ab	22.7 bc	24.9 b
T₅	69.2 bcd	73.4 c	3.5 bc	3.8 ab	21.5 f	23.7 bc	77.5 ef	85.2 abc	14.9 e	16.4 e
T₆	70.0 bc	77.0 bc	3.5 bc	3.8 ab	24.5 cde	26.9 ab	84.0 c-f	92.4 abc	19.7 d	21.7 c
T₇	71.8 abc	83.9 ab	3.7 abc	4.3 ab	27.9 ab	30.8 ab	92.0 a-d	101.2 ab	21.9 cd	24.1 b
T₈	66.7 bcd	79.0 ab	3.4 c	3.9 ab	24.1 def	23.5 bc	81.5 def	79.8 bc	12.6 g	13.9 f
T₉	70.7 bc	77.7 bc	3.5 bc	3.9 ab	26.8 bc	29.5 ab	88.2 b-e	97.0 ab	21.7 cd	23.8 b
T₁₀	73.3 abc	80.6 abc	3.7 abc	4.1 ab	27.9 ab	30.7 ab	95.7 abc	105.2 ab	22.6 bc	24.8 b
T₁₁	69.1 bcd	73.0 bc	3.4 c	4.1 ab	24.1 def	26.5 ab	72.6 fg	89.7 abc	12.4 g	13.6 f
T₁₂	69.5 bcd	76.0 c	3.5 c	3.8 ab	24.0 def	26.4 ab	78.3 def	86.1 abc	16.6 e	18.2 d
T₁₃	71.8 abc	76.1 bc	3.7 abc	4.0 ab	27.9 ab	29.7 ab	90.0 a-d	105.3 ab	21.8 cd	23.9 b
T₁₄	60.6 d	63.1 d	3.0 d	3.3 b	17.4 g	19.2 c	63.7 g	70.1 c	8.80 h	7.68 g
CV (%)	6.90	5.52	5.9	12.8	5.7	13.9	7.5	14.7	7.12	5.64

Figures in a column having common letters do not differ significantly at 5% level of LSD. Notes: T1=$N_0P_{45}K_{100}S_{20}$ kg ha⁻¹, T2=$N_{75}P_{45}K_{100}S_{20}$ kg ha⁻¹, T3=$N_{100}P_{45}K_{100}S_{20}$ kg ha⁻¹, T4=$N_{125}P_{45}K_{100}S_{20}$ kg ha⁻¹, T5=$N_{100}P_0K_{100}S_{20}$ kg ha⁻¹, T6=$N_{100}P_{30}K_{100}S_{20}$ kg ha⁻¹, T7=$N_{100}P_{60}K_{100}S_{20}$ kg ha⁻¹, T8=$N_{100}P_{45}K_0S_{20}$ kg ha⁻¹, T9=$N_{100}P_{45}K_{80}S_{20}$ kg ha⁻¹, T10=$N_{100}P_{45}K_{120}S_{20}$ kg ha⁻¹, T11=$N_{100}P_{45}K_{100}S_0$ kg ha⁻¹, T12=$N_{100}P_{45}K_{100}S1_0$ kg ha⁻¹, T13=$N_{100}P_{45}K_{100}S3_0$ kg ha⁻¹, T14=Native nutrient.

S. Noor, M. R. Talukder, M. K. R. Bhuiyan, M. M. Islam, M. A. Haque and S. Akhter

TABLE 3
Response function of aquatic taro (*Colocasia esculenta* L.) to nitrogen, phosphorous, potassium and sulphur

Nutrient	Regression equation	Optimum dose (kg ha⁻¹)	Economic dose (kg ha⁻¹)	Maximum stolon yield (t ha⁻¹) for optimum dose	Production of stolon (t ha⁻¹) for 1 kg nutrient (use efficiency)	Beyond optimum dose the reduction of stolon yield (t ha⁻¹) for 1 kg nutrient
N 2008-09	$y = -0.0009x^2 + 0.1982x + 13.295$	110.1	109.4	24.21	0.10	0.9
N 2010-11	$y = -0.001x^2 + 0.2179x + 14.623$	108.95	108.30	26.49	0.24	0.1
P 2008-09	$y = -0.0033x^2 + 0.3349x + 14.545$	50.7	47.7	23.03	0.17	3.3
P 2010-11	$y = -0.0036x^2 + 0.3683x + 16.002$	51.15	50.28	25.42	0.51	3.6
K 2008-09	$y = -0.001x^2 + 0.2088x + 12.545$	104.4	103.6	23.44	0.10	1.0
K 2010-11	$y = -0.0011x^2 + 0.2297x + 13.797$	104.4	103.27	25.78	0.25	1.1
S 2008-09	$y = -0.0201x^2 + 0.9745x + 11.49$	24.4	24.3	23.40	0.49	20.0
S 2010-11	$y = -0.0223x^2 + 1.0757x + 12.638$	24.12	24.08	25.59	1.06	22.3

Considering the fact that 1 kg N, P, K, S and stolon is priced at 26, 125, 50, 34 and 20 Tk, respectively.

88

Pertanika J. Trop. Agric. Sci. 38 (1) 83 - 92 (2015)

this was followed by T10 (22.56 and 24.82 t ha⁻¹ in 2008-09 and 2010-11, respectively). The lowest stolon yield (8.80 and 7.86 t ha⁻¹ for 2008-09 and 2010-11, respectively) was in the control (Table 2). Prajapati *et al.* (2003) reported that maximum yield attributes and yield were found with 120 kg N ha⁻¹. Since nitrogen is the constituent of chlorophyll and protein, its application might have increased the photosynthetic activities, increased length of stolon and rhizomes. Similarly, Alam *et al.* (2010) also reported that the highest stolon yield (24.5 t ha⁻¹) was recorded with 125 kg N and 125 kg K ha⁻¹, which is in agreement with the findings of our study. Mandal *et al.* (1982) also observed the highest cormel yield with $N_{120}K_{120}$ kg ha⁻¹.

Response Function

Positive but quadratic relationship was observed between the stolon yield of aquatic taro and added nutrients (N, P, K and S) in both the years (Fig.1 and Fig.2). From the regression equation, the optimum doses of nitrogen appeared as 110.1 and 108.95 kg ha⁻¹ during 2008-09 and 2010-11, respectively. Similarly, the optimum doses of phosphorus were 50.7 and 51.15 kg ha⁻¹ for 2008-09 and 2010-11, respectively. In
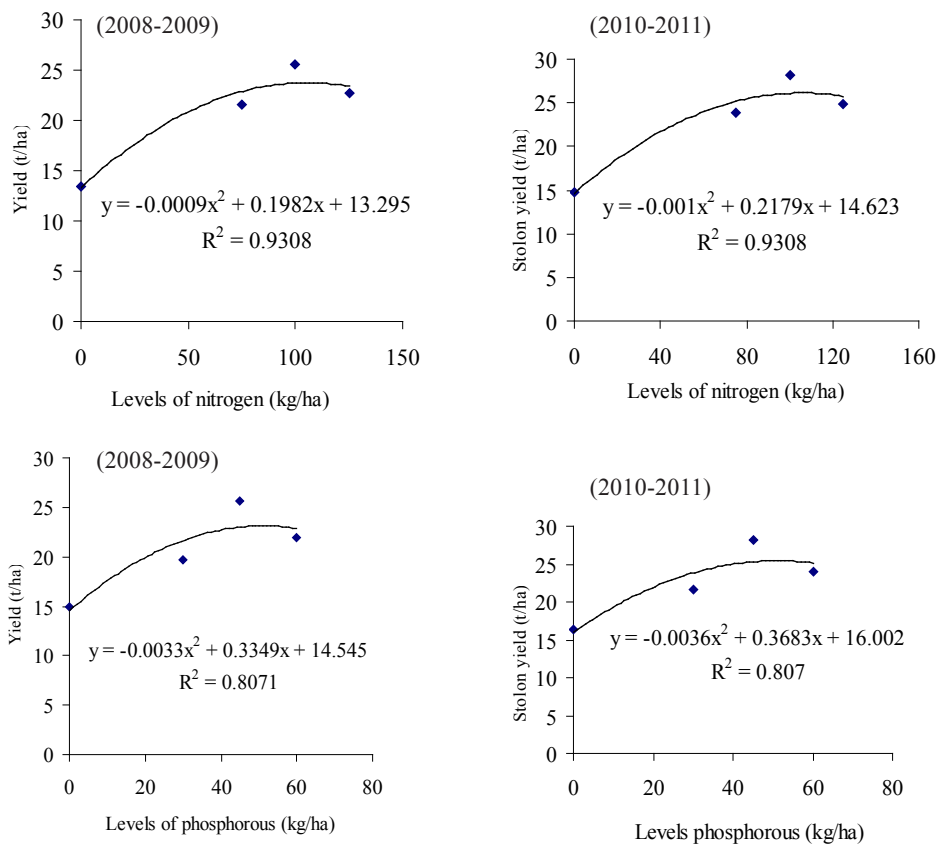
Fig.1: Responses of aquatic taro (*Colocasia esculenta* L.) to the added nitrogen and phosphorous
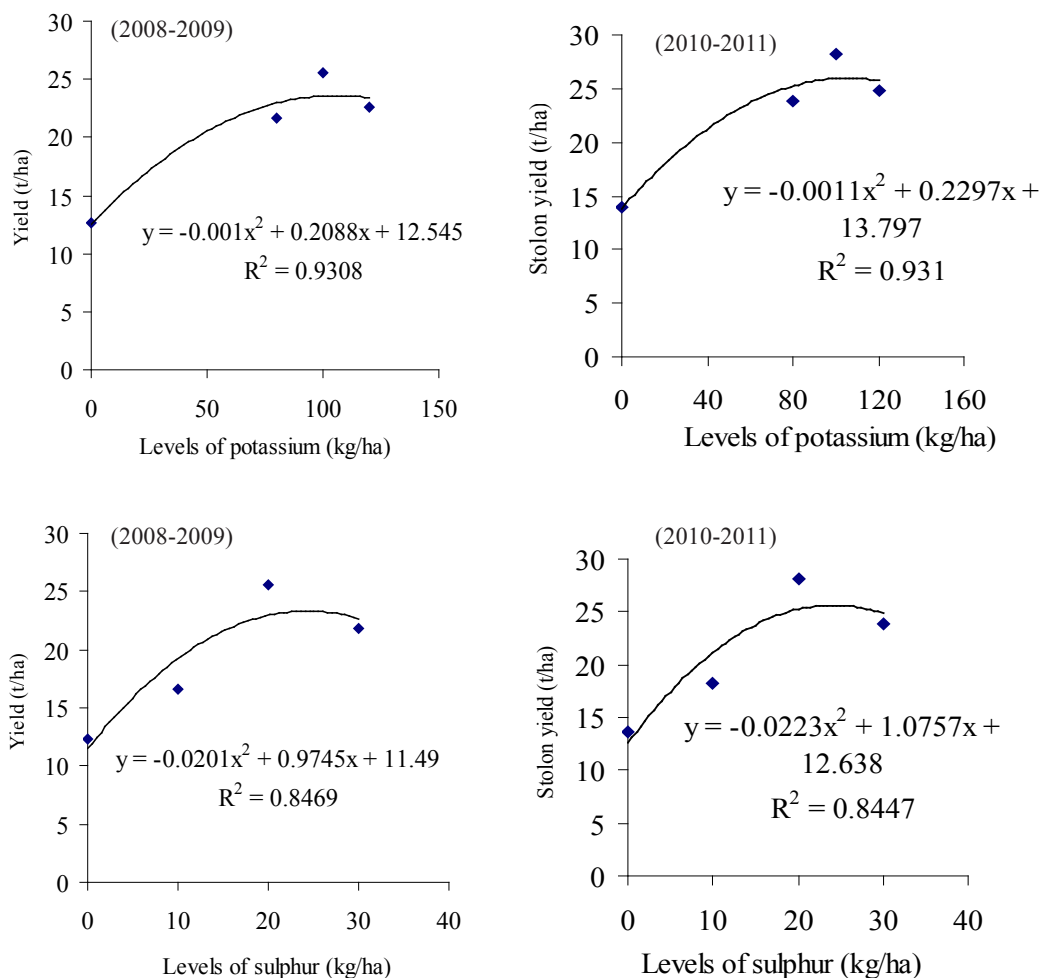
Fig.2: Responses of aquatic taro (*Colocasia esculenta* L.) to the added potassium and sulphur

the case of potassium, the optimum dose was 104.4 kg ha^{-1} in both the years. The optimum doses of sulphur were 24.4 and 24.22 kg ha^{-1} for 2008-09 and 2010-11, respectively (Table 3). Beyond the stated optimum dose, there is a possibility of losing certain amounts of yield if higher levels of nutrients were applied.

CONCLUSION

The different combinations of inorganic fertilizers showed significant effects on the yield parameters and yield of aquatic taro. The highest stolon yields (25.60 and 28.16 t ha^{-1} for 2008-09 and 2010-11, respectively) were found in the $N_{100}P_{45}K_{100}S_{20}$ kg ha^{-1} combination. The optimum doses

of nitrogen, phosphorus, potassium and sulphur were 110, 50, 105 and 24 kg ha^{-1}, respectively.

REFERENCES

Alam, M. S., Reza, M. H., Islam, M. M., Bhuiyan, M. K. R., & Hossain, M. (2010). Effect of split application of NK fertilizers on the yield of panikachu (*Colocasia esculenta* L.)

Annual Report for 2109-10. Tuber Crops Research Centre, Bangladesh Agril. Res. Inst., Gazipur-1701, Bangladesh.

BBS (Bangladesh Bureau of Statistics). (2010). *Statistical Year Book of Bangladesh-2010*. Bangladesh Bureau of Statistics, Stat. Div. Minis. Planning, Govt. Peoples Repub. Bangladesh, Dhaka.

Blake, G. R. (1965). Bulk density. In C.A. Black (Ed.), *Methods of Soil Analysis, Part I* (pp. 374-390). Madison, Wisconsin, USA: American Society of Agronomy Inc.

Bhuiyan, M. K. R., Rahman, M. M., Alam, M. S., Islam, A. T. M. T., Hossain, M., & Begum S. N. (2008). *Production technology of newly released aroids variety* (In Bangla). Tuber Crops Research Centre, Bangladesh Agricul. Res. Inst., Gazipur: TCRC Publication.

Brammer, H. (1996). *The Geography of the Soils of Bangladesh*. Dhaka, Bangladesh: University Press Ltd.

FAO. (1988). *Land resources appraisal of Bangladesh for agricultural development*. Rome, Italy: FAO Publications.

Jackson, M. L. (1973). *Soil Chemical Analysis*. New Delhi, India: Prentice Hall of India Pvt. Ltd.

Mandal, R. C., Singh, K.D., & Maini, S. B. (1982). Effect of nitrogen and potash fertilization on tuber yield and quality of colocasia. *Vegetable Science*, *9*, 82-84.

Matthews, P.J. (2000). An introduction to the history of taro as food. In M. Nakatani & K. Komaki (Eds.), *Potential of root crops for food and industrial research* (pp. 484-497). Proceedings of the 12th Symposium of the International Society of Tropical Root Crops. Tsukuba, Japan: ISTRC Press.

Mehla, C.P., Ram, M., & Singh J. (1997). Effect of spacing and fertilizer levels on growth and yield of colocasia (*Colocasia esculenta* L.). *Agricultural Science Digest, 17*, 205-208.

Nasreen, S. & Islam, M. S. (1989). Response of Chilli to N P K and S fertilization. *Bangladesh Horticulture*, 17, 5-9.

Onwueme, I. (1999). *Taro cultivation in Asia and the Pacific*. Food and Agricultural Organization of the United Nations Regional Office for Asia and the Pacific, Bangkok.

Page, A.L., Miller, R.H., & Keeney, D.R. (1982). *Methods of Soil Analysis* (2nd Edn.). Madison, WI., USA: Amercen Society of Agronomy.

Pardales, J.R. & Villanueva, M.R. (1984). Cultural management for lowland taro under monoculture system in the Philippines. In S. Chandra (Ed.), *Edible Aroids*. UK: Clarendon Press.

Prajapati, M., Singh, P.K., Verma, R.B., & Singh, H.K. (2003). Yield and quality response of Colocasia (*Colocasia esculenta* L.) to FYM, nitrogen and potassium application. *Journal of Root Crops, 29*, 42-45.

Rana, P. & Adhikary, D. (2005). *Developing agricultural markets through business services.* Paper presented at the ADB-DFID MMW4P Workshop held during February 2005 at Manila, Philippines.

Sadhu, M. K. (1993). Root crops. In T. K. Bose, M.G. Som, & J. Kabir (Eds.), *Vegetable Crops* (pp. 470-578). Calcutta, India: Naya Prokash.

Saud, B.K. & Baruah, R.K.S.M. (2000). Pani-Kachu-special taro cultivation in Southern Assam. *Intensive Agriculture, 38*, 26-27.

Sen, H., Das, S., & Dwivedi, A.K. (1998). Stolon production of different organic and inorganic nitrogenous fertilizers. *Journal of Root Crops, 24*, 81-84.

Steel, R. C. B. & Torrie, J. H. (1960). *Principles and procedures of statistics* (pp. 377-398). New York, USA: McGraw Hall Book Publication.

Verma, R. B., Singh, P.K., & Singh, S.B. (1996). Effect of nitrogen and potassium levels on growth, yield and nutrient uptake of *Colocasia esculenta*. *Journal of Root Crops, 22,* 139-143.

Walkley, A.C. & Black, T.A. (1935). Estimation of soil organic carbon by chromic acid titration method. *Soil Science, 47*, 29-38.

Yongping, Y., Jianchu, X., & Eyzaguirre, P. (2003). Taro biodiversity management and conservation in Yunnan, China. In CIP-ESEAP (Ed.), *Conservation and Sustainable Use of Agricultural Biodiversity* (p.89-99). Bogor, Indonesia: CIP-UPWARD Press.

Lithium Levels in Peninsular Malaysian Coastal Areas: An Assessment Based on Mangrove Snail *Nerita lineata* and Surface Sediments

Wan Hee Cheng[1], Chee Kong Yap[1]*, Mohamad Pauzi Zakaria[2], Ahmad Zaharin Aris[2] and Tan Soon Guan[3]

[1]*Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Environmental Forensics Research Centre, Faculty of Environmental Studies, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Sampling for the mangrove snails, *Nerita lineata*, and surface sediments was carried out from nine geographical sites of Peninsular Malaysia in April 2011. The concentrations of Lithium (Li) were determined in the shells, opercula and soft tissues of *N. lineata* and in the surface sediments by using ICP-MS. The ranges of Li concentrations (µg/g dry weight) were found to be 0.107-0.283 for shells, 0.021-0.177 for opercula and 0.011-0.634 for total soft tissues of *N. lineata*. For sediments, Li ranges were found to be between 21.84-146.22 µg/g dry weight). The distribution of Li was found to be: sediment> ST> shell> opercula. The Li sediment data in the present study were comparable with the results of Li contaminated sediments which had been previously reported in the literature and higher than those of continental crust materials and igneous rocks. There was no significant correlation (P>0.05) for the Li levels between the sediments. The snails (shells, opercula and soft tissues) and this indicated that Li is an essential metal for metabolism and thus is being regulated in the body of the snail.

Keywords: Li, biomonitoring, snails

INTRODUCTION

Lithium (Li) is the lightest of the alkali metals belonging to group 1 of the Periodic Table, along with Na, K, Rb and Cs. Li has been extensively utilized in a wide number of industrial, scientific, and clinical

applications (Hu, 2013). In particular, the commonly known usage of Li in our daily life involves Li batteries. Lithium batteries, both rechargeable and nonrechargeable, are now used in a very wide range of devices. This is because of the fact that Li has the greatest electrochemical potential of any metal (~3 V) (Hu 2012). However, its anthropogenic use result in Li waste in our environment.

All trace metals occur naturally in the environment but they may exhibit high levels due to human activities, which could cause harmful effects on the marine coastal ecosystem (Bodin *et al.,* 2013). In this study, surface sediments are focused upon because they are the ultimate sink for trace metals and can reflect the degree of pollution of an environment (Yap *et al.,* 2009). In the study of sedimentary geochemistry, Li is generally considered a conservative element in marine sediments and in metal enrichment studies. Therefore, it is often used to normalize metal concentrations to compensate for the natural textural and mineralogical variability (Aloupi & Angelidis, 2001).

Seafood such as mollusks, collected from mangrove areas, have been of worldwide concern nowadays due to trace element bioaccumulation in the tissues (Guerin *et al.,* 2011; Palpandi & Kesavan 2012; Bodin *et al.,* 2013; Fung *et al.,* 2013; Yap & Cheng, 2013). Most gastropod species have been employed as biomonitors of trace metal pollution in the coastal environment (Pearce & Mann, 2006) due to their abilities to accumulate trace metals in their tissues (Rainbow, 2002) on exposure to various sources of anthropogenic pollution (Wang *et al.,* 2005) and ubiquitous distribution (Oehlmann & Schulte-Oehlmann 2003) in marine coastal ecosystems and easy sampling.

Since there has been no report on Li in snails and sediments from Malaysia, the objective of this study was to provide the baseline levels of Li in the mangrove snail *Nerita lineata* and surface sediments from Peninsular Malaysia.

MATERIALS AND METHODS

The snails, *N. lineata*, and sediments were randomly collected from nine sampling sites in the mangrove areas of Peninsular Malaysia in April 2011 (see Fig.1). Sampling information is given in Table 1. About 20 individuals of the snails of similar size were selected from each sampling site, dissected and pooled into soft tissues, opercula and shells. The snails' gut contents were not depurated in this study, as suggested by Yap *et al.* (2010).

The dissected tissue parts were then dried at 60°C until constant dry weights. Three aliquots of each tissue part were measured, with an approximate amount of 0.5 g each, and placed in the TFM vessels. A mixture of acids (7 ml of HNO_3 65% + 1 ml H_2O_2 30%) was added into the dried samples before inserting them into the microwave cavity. For the sediment samples, they were dried at 60°C until constant dry weights and sifted using a stainless steel sift of 63µm in mesh. Triplicates of 0.5 g each were obtained from the sampling sites and placed in TFM vessels. A mixture of acids (9 ml of HCl + 3 ml of HNO_3 65%) was added into

the dried sediment samples.

For digestion of snail and sediment samples, the microwave digester used was the Milestone ETHOS labstation with easyWAVE or easyCONTROL software HPR1000/10S high pressure segmented rotor. To digest the snail samples, the microwave digester was set to increase the temperature to 200°C for the first 10 minutes and maintained at 200°C for the following 20 minutes, with the application of 1000 W of microwave power. Similar

procedures from the snail tissue preparation were also applied to the sediments but with the temperature raised to 200°C for the first 10 minutes and maintained at 200°C for the following 15 minutes. The samples were left in the microwave digester to cool down to room temperature for 10 minutes after the digestion had been completed. The digested samples were then diluted to 100 ml with double distilled water (DDW) and filtered with Whatman No. 1 filter paper before they were stored for metal analysis. All the
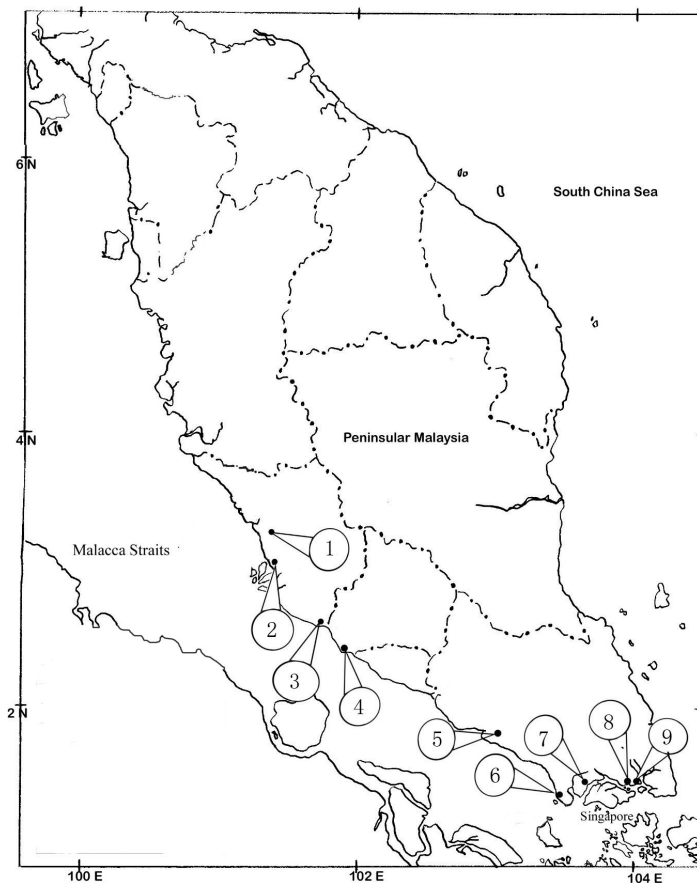
Fig.1: Sampling map. (Sites: 1=SJanggut; 2=JKetam; 3=Sepang; 4=Lukut; 5=SAyam; 6=Kukup; 7=KSMelayu; 8=KPPuteh; 9=TLangsat)

TABLE 1
Descriptions of the sampling sites

No	Sampling Sites	Date	GPS	Shell length (mm)	Site activities
1	Sungai Janggut (SJanggut)	8-Apr-11	N 03° 10.307' E 101° 18.524'	25.19 - 33.82	It is a jetty in mangrove area with fishing village nearby. Water irrigation was also observed here.
2	Pulau Ketam (JKetam)	8-Apr-11	N 03° 00.567' E 101° 21.649'	25.28 - 28.34	A jetty to Pulau Ketam, fishing and shipping activities were observed here. Construction work (building) were being carried out during sampling.
3	Sepang	8-Apr-11	N 02° 36.076' E 101° 42.565'	19.56 - 27.87	Nearby housing and mangrove area, prawn and mussel aquaculture, fishing and shipping activities were observed here.
4	Lukut	8-Apr-11	N 02° 34.511' E 101°47.529"	21.58 - 28.64	Industrial and urban area. It is a fishing village with mooring activities.
5	Sungai Ayam (SAyam)	15-Apr-11	02° 34.423' E 102° 02.346'	23.78 - 30.4	Fishing village with water irrigation. Massive amount of domestic waste were observed in this area. Mussel aquaculture, fishing and shipping are the main activities here.
6	Kukup	15-Apr-11	N 01° 19.471' E 103° 26.521'	23.08 - 30.82	It is a port and tourist attraction site with restaurants and resorts. The main activity here are fishing and shipping.
7	Kampung Sungai Melayu (KSMelayu)	16-Apr-11	N 01° 27.043' E 103° 41.699'	21.05 - 26.44	A jetty where the main activities here are fishing and shipping. Mussel aquaculture was observed here.
8	Kampung Pasir Puteh (KPPuteh)	16-Apr-11	N 01° 26.082' E 103° 56.094'	22.54 - 30.91	It's a jetty with restaurants and a fishing village. Shipping and industrial activities were observed here.
9	Tanjung Langsat (TLangsat)	16-Apr-11	N 01° 28.190' E 104° 0.041'	24.46 - 31.46	It's a jetty with restaurants and a fishing village. Shipping and industrial activities were observed here.

prepared samples were determined for Li by using the Perkin Elmer SCIEX ELAN DRC-e ICP-MS with the limit of detection of < 0.1 ppt.

For quality control, the precision and quality of the method were checked with Certified Reference Material for Marine Sediment (MESS-3) (Li certified value: 73.6 ± 5.2 µg/g dry weight; Li measured value: 66.5 ± 5.0 µg/g dry weight) with an obtained percentage of recovery and standard deviation of 90.3 ± 6.8 %.

For statistical analysis, One-way ANOVA Student-Newman-Keuls test was carried out to determine the differences between the metal levels in the different tissues of the *N. lineata* and the sediments. Pearson's correlation coefficient (IBM SPSS Statistics version 19) and Single Linkage Euclidean distances (STATISTICA version 8.0) were used to determine the relationships of Li between the snails and sediments.

In this study, the distributions of Li levels between the snails (three parts) and in the associated surface sediments, estimated based on the biota-sediment accumulation factors (BSAF), were calculated (Szefer *et al.,* 1999). The formula used is given as follows:

$$\text{BSAF} = \frac{Cx}{Cs}$$

where Cx and Cs are the mean Li concentrations in the different parts of the snails and in the surface sediment, respectively.

RESULTS AND DISCUSSION

The Li concentrations of the snails and sediments are shown in Fig.2. The ranges of the Li concentrations (µg/g dry weight) were 0.107-0.283 (mean: 0.195 ± 0.020) for shells, 0.021-0.177 (mean: 0.099 ± 0.018) for opercula and 0.011-0.634 (mean: 0.310 ± 0.062) for total soft tissues. Interestingly, Sepang recorded the highest Li levels in both the shells and opercula, while KSMelayu recorded the highest levels of Li in soft tissues. The present ranges (0.107-0.283 µg/g dry weight) of Li in the snail shells were below those of the *Mytilus edulis* shells sampled from the Polish coast of the Baltic Sea, which were reported as being 0.275-3.484 µg/g dry weight (Protasowicki *et al.,* 2008). However, comparison of Li in the soft tissues and opercula of mollusks is difficult to be made due to the lack of similar Li data in the literature.

As for sediments, the ranges of Li were between 21.84-146.22 (mean: 50.44) with SAyam recording the highest and Lukut the lowest. The data of SAyam were reported by Yap *et al.* (2009) with elevated levels of Pb due to hidden industrial activities in the nearby area, which could also be the source of the high level of Li in this site. As for KPPuteh, with the second highest level of Li, it is known to be surrounded by heavy anthropogenic activities that could contribute to the high level of Li in this area (Yap *et al.,* 2004). The other sites were basically fishing villages or aquaculture sites that had lower levels of Li pollution (see Table 1).
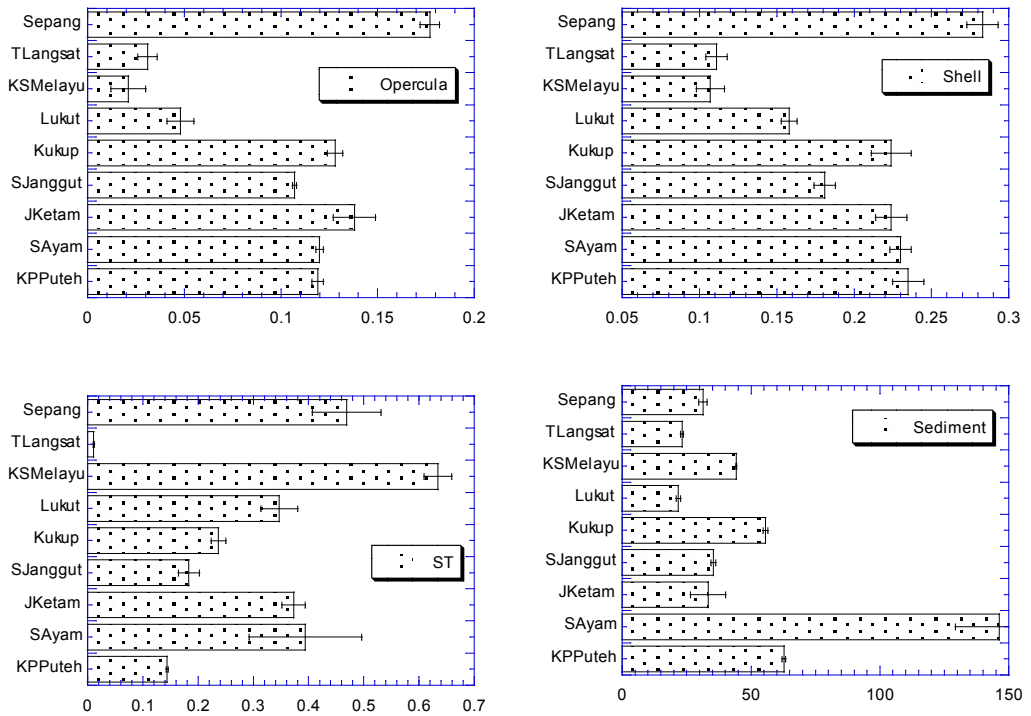
Fig.2: Lithium levels (mean ± SE, µg/g dry weight) in the shells, opercula and soft tissues of *Nerita lineata*, and surface sediments collected from nine sampling sites in Peninsular Malaysian mangrove areas.

Therefore, Li concentrations generally follow sediment> ST> shell> opercula. In order to estimate the status of Li, the present ranges of Li in the sediment were compared to the background Li value, upper and lower continental crust materials (CCM) for Li which were reported as being 22 and 13 µg/g dry weight, respectively (Wedepohl, 1995), and the Li level in the igneous rock (32.0 µg/g dry weight) (Vinogradov, 1962). The ranges of Li in the current work appeared to be higher than CCM and the igneous rocks, and thus, posed an environmental concern whether our Malaysian mangrove area is facing Li pollution.

The present Li ranges were further compared to the Li data in the sediments reported in the literature. Miranda-Avilés *et al.* (2012) reported the Li levels as being 125.10 µg/g dry weight (0-20 cm) and 58.03 µg/g dry weight (0-70 cm) for metal-contaminated overbank-sediment and pristine overbank-sediment, respectively, from Guanajuato City, Mexico. Christiansen *et al.* (2009) reported the Li content as being high (>50 µg/g dry weight) in Skagerrak and northern Kattegat, whereas the sediments in the Belt area and the western part of the Baltic Sea have low concentrations at 20-40 µg/g dry weight. The present ranges (21.84-

98

Pertanika J. Trop. Agric. Sci. 38 (1) 93 - 101 (2015)

146.22 µg/g dry weight) of Li in the surface sediments were higher than those reported for Medway and Swale (6.00 - 39.00 µg/g dry weight) (Spencer *et al.,* 2006), South China Sea (27.6 - 51.6 µg/g dry weight) (Rezaee *et al.,* 2011) and Senegal (3.50 - 8.40 µg/g dry weight) (Bodin *et al.,* 2013). All the above comparisons showed that the ranges of Li in the current work were close to the known Li contaminated range. Knutzen and Skei (1990) defined "high background levels" for heavy metals in sediments and biota to indicate areas affected by heavy metal pollution.

It appeared that the positive relationships are weak for shell-sediment (R= 0.322), opercula-sediment (R= 0.240) and ST-sediment (R= 0.148), but these three pair-wise values were not significantly (P> 0.05) correlated. These insignificant correlation (P>0.05) for the Li levels between the sediments and the snails (shells, opercula and soft tissues) indicated Li regulation in the tissues of *N. lineate,* Therefore, it could poorly reflect the ambient Li level.

Our finding was supported by that reported by Templeman *et al.* (2010) in which Li appeared to be actively regulated within the tissues of the benthic jellyfish, *Cassiopea* sp. collected from the northern and eastern coast of Australia. The available experimental evidence now appears to be sufficient to accept Li as being essentially important for humans and the metal is normally present in all organs and tissues (Schrauzer, 2002). Previous knowledge indicated that essential metals are generally regulated in the tissues of mollusks (Rainbow, 1995) and the present insignificant relationship of Li between snails and sediments is no exception.

The BSAF values are presented in Table 2. It was found that all the BSAF values were below 1.00 (0.05-1.50×10^{-2} for soft tissue/sediment, 0.05-0.56×10^{-2} for opercula/sediment and 0.16-0.90×10^{-2} for shell/sediment). Therefore, according to Dallinger (1993), all the values of BCF are categorized as deconcentrators. Moreover, the differences of the Li levels between the three parts of the snails and the surface

TABLE 2
Biota-sediment accumulation factor (BSAF) values ($\times 10^{-2}$); Li concentrations between the snail parts (soft tissues (ST), opercula (Oper), and shell) and surface sediment (SED)

Sites	ST/SED	Oper/SED	Shell/SED
KPPuteh	0.23	0.19	0.37
SAyam	0.27	0.08	0.16
JKetam	1.12	0.41	0.67
SJanggut	0.52	0.30	0.51
Kukup	0.43	0.23	0.40
Lukut	1.50	0.21	0.68
KSMelayu	1.43	0.05	0.24
TLangsat	0.05	0.14	0.51
Sepang	1.49	0.56	0.90

sediment are significant (P< 0.001, T-test). Therefore, the snails are generally not good bioaccumulators of Li and the present BSAF values showed that Li was not significantly bioaccumulated in the *Nerita* from their environmental habitats.

CONCLUSION

This study serves to provide the baseline data of Li levels in the shells, opercula and total soft tissues of *N. lineata* and the surface sediment from Peninsular Malaysia. The present insignificant relationships of Li between the snails and sediment showed that Li is an essential metal for metabolism and thus it is being regulated in the body of the snails. The fact that the snails are categorized as deconcentrators indicated that further studies are needed to justify them as being reliably employed as good biomonitors of Li pollution.

REFERENCES

Aloupi, M. & Angelidis, M. O. (2001). Normalization to Lithium for the assessment of metal contamination in coastal sediment cores from the Aegean Sea, Greece. *Marine Environmental Research, 52,* 1-12.

Bodin, N., N'Gom-Kâ, R., Kâ, S., Thiaw, O. T., Tito de Morais, L., Le Loc'h, F., Rozuel-Chartier, E., Auger, D., & Chiffoleau, J. F. (2013). Assessment of trace metal contamination in mangrove ecosystems from Senegal, West Africa. *Chemosphere, 90,* 150–157.

Christiansen, C., Leipe, T., Witt, G., Christoffersen, P. L., & Lund-Hansen., L. C. (2009). Selected elements, PCBs, and PAHs in sediments of the North Sea – Baltic Sea transition zone: Sources and transport as derived from the distribution pattern. *Geografisk Tidsskrift-Danish Journal of Geography, 109*(1)*,* 81- 94.

Dallinger, R. (1993). Strategies of metal detoxification in terrestrial invertebrates. In Dallinger, R. & P.S. Rainbow (Eds.), *Ecotoxicology of metals in invertebrates* (pp. 245-289). Boca Raton: Lewis Publishers.

Fung, L. A. H., Antoine, J. M. R., Grant, C. N., & Buddo, D. S. A. (2013). Evaluation of dietary exposure to minerals, trace elements and heavy metals from the muscle tissue of the lionfish *Pterois volitans* (Linnaeus 1758). *Food and Chemical Toxicology, 60,* 205–212.

Guerin, T., Chekri, R., Vastel, C., Sirot, V., Volatier, J., Leblanc, J., & Noël, L. (2011). Determination of 20 trace elements in fish and other seafood from the French market. *Food Chemistry, 127,* 934–942.

Hu, Q. (2012). Lithium: The literature regarding its uses in chemistry, psychiatry, and the engineering of materials and batteries. *Science & Technology Libraries, 31,* 190–199.

Knutzen, J. & Skei, J. (1990). Quality criteria for micropollutants in water, sediment and organisms and preliminary proposals for classification of environmental quality NIVA-report, I.nr. 2540 (139). Oslo, Norway: NIVA.

Miranda-Avilés, R., Puy-Alquiza, M. J., & Arvizu, O.P. (2012). Anthropogenic metal content and natural background of overbank sediments from the mining district of Guanajuato, Mexico. *Soil and Sediment Contamination: An International Journal, 21*(5), 604-624.

Oehlmann, J. & Schulte-Oehlmann, U. (2003). Molluscs as bioindicators. In B.A. Markert, A.M. Breure & H.G. Zechmeister (Eds.), *Bioindicators and biomonitors* (pp. 557-635). Elsevier Science Ltd.

Palpandi, C. & Kesavan, K. (2012). Heavy metal monitoring using *Nerita crepidularia*-mangrove

mollusc from the Vellar estuary, Southeast coast of India. *Asian Pacific Journal of Tropical Biomedicine*, *2*(1), S358-S367.

Pearce, N. J. G. & Mann, V. L. (2006). Trace metal variations in the shells of *Ensis siliqua* record pollution and environmental conditions in the sea to the west of mainland Britain. *Marine Pollution Bulletin, 52,* 739–755.

Protasowicki, M., Dural, M., & Jaremek, J. (2008). Trace metals in the shells of blue mussels (*Mytilus edulis*) from the Poland coast of Baltic Sea. *Environmental Monitoring and Assessment, 141,* 329–337.

Rainbow, P. S. (1995). Biomonitoring of heavy metal availability in the marine environment. *Marine Pollution Bulletin, 31,* 183-192.

Rainbow, P. S. (2002). Trace metal concentrations in aquatic invertebrates: why and so what? *Environmental Pollution, 120,* 497-507.

Rezaee, K., Abdi, M. R., Saion, E. B., Naghavi, K., & Shafaei, M. A. (2011). Distribution of trace elements in the marine sediments along the South China Sea, Malaysia *Journal of Radioanalytical and Nuclear Chemistry, 287,* 733–740.

Schrauzer, G. N. (2002). Lithium: Occurrence, dietary intakes, nutritional essentiality. *Journal of the American College of Nutrition, 21*(1), 14-21.

Spencer, K. L., MacLeod, C. L., Tuckett, A., & Johnson, S.M. (2006). Source and distribution of trace metals in the Medway and Swale estuaries, Kent, UK. *Marine Pollution Bulletin, 52,* 214–238.

Szefer, P., Ali, A. A., Ba-Haroon, A. A., Rajeh, A. A., Geldon, J., & Nabrzyski, M. (1999). Distribution and relationships of selected trace metals in molluscs and associated sediment from the Gulf of Aden, Yemen. *Environmental Pollution, 106,* 299-314.

Templeman, M. A. & Kingsford, M. J. (2010). Trace element accumulation in *Cassiopea* sp.

(Scyphozoa) from urban marine environments in Australia. *Marine Environmental Research, 69,* 63–72.

Vinogradov, A. P. (1962). Mean element contents of the main types of crustal igneous rocks. *Geokhimiva, 1956,* 1-43.

Wang, Y., Liang, L., Shi, J., & Jiang, G. (2005). Study on the contamination of heavy metals and their correlations in mollusks collected from coastal sites along the Chinese Bohai Sea. *Environment International, 31,* 1103-1113.

Wedepohl, K. H. (1995). The composition of the continental crust. *Geochimica et Cosmochimica Acta, 59,* 1217–1239.

Yap, C. K., Ismail, A., Tan, S. G., & Ismail, A. R. (2004). The impact of anthropogenic activities on heavy metal (Cd, Cu, Pb and Zn) pollution: Comparison of the heavy metal levels in the green-lipped mussel *Perna viridis* (Linnaeus) and in the sediment from a high activity site at Kg. Pasir Puteh and a relatively low activity site at Pasir Panjang. *Pertanika Journal of Tropical Agricultural Science, 27*(1), 73-78.

Yap, C. K., Cheng, W. H., Ismail, A., Ismail, A. R., & Tan, S. G. (2009). Biomonitoring of heavy metal (Cd, Cu, Pb, and Zn) concentrations in the west intertidal area of Peninsular Malaysia by using *Nerita lineata. Toxicological & Environmental Chemistry, 91*(1), 29–41.

Yap, C. K. & Cheng, W. H. (2010). Depuration of gut contents in the intertidal snail *Nerita lineata* is not necessary for the study of heavy metal contamination and bioavailability: A laboratory study. *Pertanika. Pertanika Journal of Tropical Agricultural Science, 33*(2), 167-170.

Yap, C. K. & Cheng, W. H. (2013). Distributions of heavy metal concentrations in different tissues of the mangrove snail *Nerita lineata. Sains Malaysiana, 42*(5), 597-603.

PERTANIKA
JOURNALS

Fractionation of Potent Antioxidative Components from Langsat (*Lansium domesticum*) Peel

Kee, M. E., Khoo, H. E., Sia, C. M. and Yim, H. S.*

[1]Department of Food Science and Nutrition, Faculty of Applied Sciences, UCSI University, No. 1, Jalan Menara Gading, UCSI Heights, 56000 Kuala Lumpur, Malaysia
[2]Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Lansium domesticum (langsat) is a popular tropical fruit bearing trees across the Southeast Asia region. It is cultivated mainly for its fruit, while the peel of langsat as wastes of fruit consumption has been traditionally used as medicine. The aim of this study was to evaluate the total phenolic content (TPC) and antioxidant activity of different fractions of ethanolic extract of langsat peel. The extract was fractionated using open column chromatography and the antioxidant components were observed based on high-performance thin layer chromatography (HPTLC). TPC of the extract and extract fractions (FI-FIII) was estimated using Folin-Ciocalteu reagent assay, while their antioxidant activity was evaluated using DPPH radical scavenging assay. Highest TPC was estimated in the ethanolic extract of langsat peel compared to its extract fractions. No significant difference was found for TPC among the extract fractions. The extract also had the highest DPPH radical scavenging activity, followed by FIII and FII, but no radical scavenging activity was observed in FI. The result obtained from HPTLC also showed that no band of antioxidative compounds was observed under UV or white light. Based on the antioxidative components found in the peel extract of langsat, the fruit peel is a potential source of antioxidants for development of nutraceutical. Fractionation of the extract is not an ideal technique for producing nutraceutical.

INTRODUCTION

Fruits are rich in antioxidants such as vitamins, phenolic compounds, carotenoids and other phytochemicals that help in reducing risks of chronic diseases (Feskanich *et al.*, 2000; Hamid *et al.*, 2010). Studies have shown that excessive free radicals in the body cause oxidative damage to all the constituents of the body and increase the risks of developing diseases including hypertension, congestive heart failure and other degenerative diseases (Cornelli, 2009; Scheibmeir *et al.*, 2005). Antioxidants are compounds that delay or prevent the oxidation of cellular constituents of the body by scavenging free radicals such as reactive oxygen species (ROS) (Halliwell, 1994).

For more than fifty years, synthetic antioxidants, particularly butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary-butylhydroquinone (TBHQ) have been widely used in the food industry for the prevention of oxidation and off-flavour development in fats and oils. However, it is known that these chemicals could be carcinogenic (Ajila *et al.*, 2007; Moure *et al.*, 2001). Recent evidence suggests that the crude extracts or purified constituents from various medicinal plants are stronger antioxidants than the synthetic antioxidants (Shiban *et al.*, 2012). Pourmorad *et al.* (2006) also reported that the antioxidant activity of Iranian medicinal plant was four times greater than BHT. Hence, it can be concluded that natural antioxidants could potentially be alternatives for the synthetic ones.

Langsat (*Lansium domesticum*) is a tropical fruit which contains a variety of nutrients especially vitamins and minerals that are health beneficial. Langsat contains several phytochemicals that possess high antioxidant activities. Lim *et al.* (2007) found that among several tropical fruits, langsat exhibited high antioxidant activity as determined based on DPPH assay. Huang *et al.* (2010) reported a higher total antioxidant capacity, total phenolic content, and carotenoids were determined in the peel of langsat as compared to its pulp. Langsat peel also possesses strong antimicrobial activity against *Candida lypolytica* (Mohamed *et al.*, 1994).

In the past, many studies mainly focused on antioxidant activity of extracts from edible portions of fruits, mainly pulps. However, information regarding inedible part of a fruit such as peel of langsat is insufficient. Nonetheless, studies have revealed that the peel extracts of certain fruits possessed higher antioxidant activity than the pulp extracts (Ajila *et al.*, 2007; Contreras-Calderon e*t al.*, 2011). For example, dragon fruit peel showed higher radical scavenging activities than the pulp (Nurliyana *et al.*, 2010). Therefore, this study aimed to determine the total phenolic content and antioxidant activity of langsat peel extract, as well as its extract fractions obtained from open column chromatography. Confirmation of the occurrence of phenolic compounds in the extract and extract fractions was performed based on the HTPLC method.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents used were of analytical grade whereas all solvents used for HPLC were of HPLC grade. Gallic acid, ethanol, ethyl acetate, formic acid, glacial acetic acid, and sodium carbonate anhydrous were purchased from Fisher Scientific (Leicestershire, UK). Folin-Ciocalteu reagent, methanol, chloroform, sulfuric acid, silica gel 60 F_{254} high performance thin-layer chromatography (HPTLC) plates, and silica gel 60 (230–400 meshes) were purchased from Merck (Darmstadt, Germany). BHA and DPPH were purchased from Sigma Chemicals (St. Louis, MO). Water used was Millipore quality (Millipore, Billerica, MA).

Preparation and Extraction of the Sample

Lansium domesticum (langsat) fruit were purchased from a fruit orchard in Selangor, Malaysia. The langsat fruit has been registered with the Department of Agriculture, Malaysia (No. DL2: Langsat) (DOA, 2013). The fruit was washed and the fruit pulp was removed. The thickness of the peel varied from 2mm to 6mm. The fruit peel was air-dried in an oven at 40°C for 24 h. After 24 h, the fully dried langsat peel was collected, while the not fully dried peel further oven-dried for another 5 h. The dried langsat peel was substantially crisp and dry. The air-dried sample was ground into fine powder using a household grinder, and sieved using a laboratory sieve of 0.5 mm mesh size. The sample powder was vacuum-packed and stored at □20°C before further analysis. The sample was extracted by adding 5g of sample powder to 50 ml of 80% ethanol. The mixture was shaken at room temperature (25°C) for 120 min using an orbital shaker as optimized using langsat peel (Chua, 2012). The residue was re-extracted once under similar conditions. The extracts were pooled, followed by filtration and concentration of the supernatant using a rotary evaporator at 45°C to remove the solvent. The concentrated extract was freeze-dried and stored at -20°C. Triplicate extraction was done for the langsat peel.

Extract Fractionation and HPTLC Analysis

Fractionation of the extract was carried out using 23g of silica gel 60 (230–400 meshes) as an adsorbent for the open-column chromatography (25 cm × 2.5 cm). The column was loaded with the extract (equivalent to 0.3 g of sample) and eluted with a linear gradient of chloroform: ethyl acetate: methanol from 100:0:0 to 0:0:100 (*v/v/v*) as described by Prasad *et al.* (2005). It was achieved by increasing solvent polarity by 5% each time. The obtained sample fractions were subjected to HPTLC analysis using HPTLC silica gel plates, where an aliquot of each fraction (20 μl, 5 mg/ml of sample) was spotted with a developing solvent system of ethyl acetate/ water/ formic acid/ acetic acid (100:26:11:11, *v/v/v/v*) (Reich & Schibli, 2007). The sample fractions that have antioxidative compounds of similar retention factor (R_f) values were pooled into three major fractions (extract fractions: FI, FII, and FIII). The solvents

in each extract fraction were evaporated to dryness using a rotary evaporator. Bioautographic reagent of 0.04% DPPH in methanol was sprayed on the developed HPTLC plates for the three extract fractions. Yellowish bands were visualised in the purplish background and this indicated the presence of reducing agents, the most possibly phenolic compounds. On the other hand, 10% sulfuric acid was sprayed on another two developed plates and the colour bands were visualised under white light and UV 366 nm, indicating the present of antioxidative compounds. Each extract fraction was collected as triplicate analyses.

Estimation of Total Phenolic Content

Total phenolic content (TPC) of langsat peel extract and its extract fractions were estimated based on the method described by Lim e*t al*. (2007). The test tubes were first added with 1 ml of extract/ extract fraction (200 µg/ml), followed by addition of 4 ml Folin-Ciocalteu reagent. The mixture was vortexed and incubated at room temperature for 3 min. After 3 min, 5 ml of sodium carbonate solution was added. The reacting mixtures were vortexed and kept in the dark for 30 min at room temperature. Absorbance of the reaction mixtures was measured at 765nm using a UV-visible spectrophotometer (Secomam, France). TPC of the extract and extract fractions was expressed as gallic acid equivalent (GAE). The standard curve of gallic acid was obtained using concentrations of gallic acid at 0.2-50 µg/ml.

$$y = 0.015x + 0.006 \ (R^2 = 0.996) \qquad [1]$$

DPPH Radical Scavenging Activity

DPPH radical scavenging activity of the extract and extract fractions was determined using DPPH radical scavenging assay as described by Ajila *et al*. (2007) with modifications. Aliquots of 1 ml extract or extract fraction with a range of concentrations from 10 to 300 µg/ml were added to 1 ml of 0.2 mM DPPH reagent and vortexed for few seconds. The reacting mixtures were incubated in dark at room temperature for 30 min. Absorbance of the mixture was measured at 517 nm against blank (ethanol). Distilled water was used as a control with an addition of extract or extract fraction. The radical scavenging activity was calculated using the equation as follows:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{sample}{control}\right) \times 100 \qquad [2]$$

where A_{sample} is the absorbance of the extract/ extract fraction, and $A_{control}$ is the absorbance of the control. EC_{50} value, i.e. the amount of antioxidant required to reduce the initial free radicals concentration by 50%, was calculated using the linear regression equation, as follows:

$$y = 0.1299x + 0.1294 \ (R^2 = 0.937) \qquad [3]$$

Statistical Analysis

All data were presented as mean ± standard deviation. The data were statistically

106

Pertanika J. Trop. Agric. Sci. 38 (1) 103 - 112 (2015)

analysed using SPSS software (version 19.0). One-way analysis of variance coupled with Tukey's post-hoc test was used to assess significant differences between the mean values. The significant value was at $p<0.05$.

RESULTS AND DISCUSSION

Sample and Extraction Yield

In the present study, the oven-dried langsat peel was extracted with 80% ethanol for 2 h at 25°C. The oven-dried langsat peel has 70.3% of moisture loss. As mentioned earlier, the extraction condition had been optimised by Chua (2012). The extract yield of the *Lansium domesticum* peel was 66.11%. It was found that binary solvent system offered higher extraction yield compared to the mono-solvent system (Zubair *et al.*, 2012). The finding is also consistent with the result reported by Sultana e*t al*. (2009) that the extraction of medicinal plant using 80% ethanol exhibits higher extracts yields and total phenolic content as compared to 100% ethanol. Besides, ethanol and water were selected as the extraction solvent because ethanol is less toxic than other solvents such as methanol and acetone (Chew e*t al*., 2011).

Extract Fractionation and HPTLC Analysis

The eighty sample fractions collected were pooled as three extract fractions (FI-FIII) based on the R_f values obtained from HPTLC. Fractions 1–28 were obtained as colourless sample fractions, fractions 29–77 as yellow colour sample fractions,

while fractions 78–80 were the light yellow colour fractions. HPTLC analysis of all the 80 sample fractions collected from column chromatography was performed in order to detect the antioxidant compounds in the sample fractions. The developed plates of fractions 1–21 and visualised under UV 366 nm showed no bands on the plates. Therefore, these sample fractions were pooled as extract fraction FI. Fractions 22–29 revealed a significant band at $R_F =$ 0.35 under UV 366 nm and were pooled as FII. As for the subsequent fractions 30–80, seven bands (four major bands and three minor bands) at R_F values of 0.85, 0.73, 0.60, 0.50, 0.39, 0.15 and 0.07, respectively were visualised under UV 366 nm as well. Two dark blue bands and five light blue bands were detected under UV 366 nm after derivatisation. These fractions were pooled as FIII. The total yields of FI-FIII are shown in Table 1.

Bioautographic HPTLC assay was performed as a screening tool that provides quick access for detection of active bands on the plate which could be examined through white light, UV 254 nm or UV 366 nm. The specific phenolic compounds were not determined using HPLTC as this study aimed to screen for the occurrence of phenolic compounds using HPTLC. As shown in Fig.1, the plates for FII and FIII that sprayed with DPPH solution were visualised as light yellow bands in the purple colour background under white light. The plate for the langsat peel had the most obvious light yellow bands compared to the extract fractions, followed by four obviously

seen light yellow bands in the plate of FIII and weakly coloured bands in FII. In contrast, no potential reaction of reducing compounds was seen in FI. In FIII, the four strong, active bands (a, b, c, and d) have R_F values of 0.81, 0.69, 0.57, 0.42, respectively. As the plates were dipped in 10% sulfuric acid, similar R_F values (0.82, 0.71, 0.58 and 0.45, respectively) were obtained for the four band when visualised under white light and UV 366 nm demonstrated that four major antioxidative compounds were detected in langsat.

Total Phenolic Content

TPC of the extract and extract fractions each sample was calculated and expressed as mg GAE per gram sample. The results showed that the colour of the mixture of Folin-Ciocalteu reagent and the extract of langsat peel turned blue, suggesting that phenolic compound is present in the langsat peel extract. To the contrary, the reaction mixtures of FI-FIII was light blue in colour. The lighter blue colour indicates that the extract fractions contain a lower amount of total phenolics as compared to the crude extract.

TPC of the extract and extract fractions of langsat peel are shown in Fig.2. In general, TPC of the langsat peel extract was 3-4 times higher than all the extract fractions. while TPC of the extract fractions was not significantly different. Surprisingly, the TPC of the langsat peel extract (140.5 mg GAE/g extract = 9.2 mg GAE/100 dry weight, DW) obtained was 2.5 times higher than TPC of the langsat peel (3.7 g GAE/100 g DW) reported by Huang *et al.* (2010). A possible explanation for the high

TABLE 1
The yields of extract fractions obtained from a pooled of sample fractions

Extract fraction	Sample fraction	Yield (mg)
Fraction 1	1 – 21	81.4
Fraction 2	22 – 29	70.9
Fraction 3	30 – 80	123.5

TABLE 2
EC_{50} values of the langsat peel extract and its extract fractions

Sample	EC_{50} (μg/ml)
Peel extract	47.94 ± 0.46
FI (Fraction 1)	ND
FII (Fraction 2)	ND
FIII (Fraction 3)	251.44 ± 13.13
BHA	9.66 ± 0.59

Each value is expressed as mean ± standard deviation of triplicate measurements. EC_{50} value is the effective concentration value at which the DPPH radical is scavenged by 50%. ND denotes not determined due to the highest concentration of the extract tested could not give a scavenging activity of ≥50%.
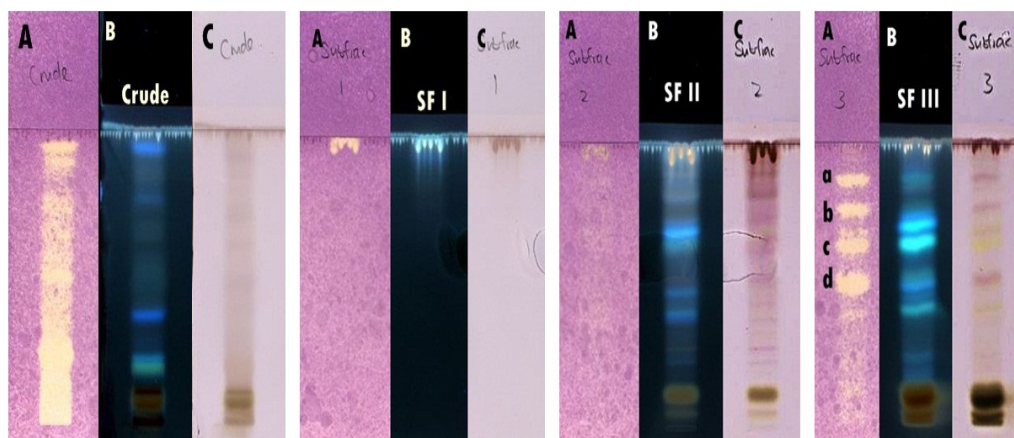
Fig.1: Bioautographic HPTLC analysis for the langsat peel extract and its extract fractions. (A) HPTLC plate sprayed with 0.04% DPPH solution, (B) HPTLC plate dipped in 10% sulfuric acid, under UV 366 nm, (C) HPTLC plate dipped in 10% sulfuric acid, under white light. "Crude", "SF I", "SF II", and "SF III" denote langsat peel extract, extract fraction 1 (FI), extract fraction 2 (FII), and extract fraction 3 (FIII), respectively.
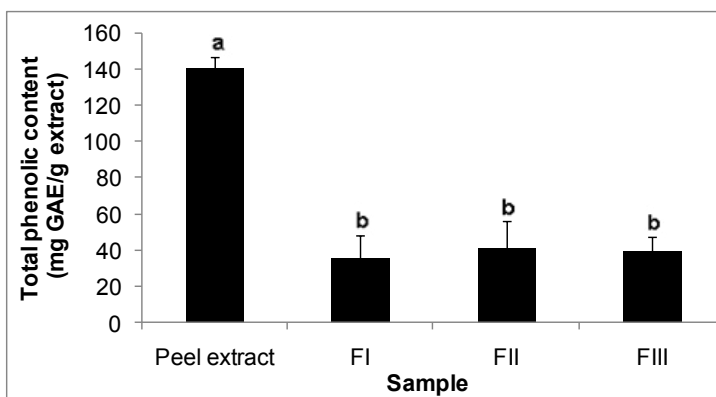
Fig.2: Total phenolic contents of langsat peel extract and its extract fractions estimated using Folin-Ciocalteu reagent assay. Different lowercase letters denote significant difference at $p<0.05$.

TPC in langsat peel is the presence of non-phenolic substances such as vitamin C and other organic acid that might have reacted with Folin-Ciocalteu reagent. Besides, other organic compounds might also interfere with the reaction between phenolic compounds and Folin-Ciocalteu reagent (Yan & Asmah, 2010). Moreover, the lower TPC in langsat peel reported by Huang *et al.* (2010) is due to the fact that the langsat fruit has been bought from the local market in Hong Kong. As langsat is one of the native fruit of Malaysia and Thailand, the fruit sold in the local market in Hong Kong was possibly

imported from Southeast Asia. It could help to explain the low TPC in the langsat peel reported by Huang *et al.* (2010) as the fruit has longer storage duration than the ones purchased in Malaysia. In addition, langsat peel was also reported to have high vitamin C content (22.5 µmol ascorbate/g dry weight) (Huang *et al.*, 2010). Therefore, Folin-Ciocalteu reagent assay seems to overestimate the TPC of the langsat peel extract (Azlan *et al.*, 2011).

DPPH Radical Scavenging Activity

It was observed that, as the concentration of the langsat peel increased, the scavenging activity also increased. At the extract concentration of 300 µg/ml, FIII showed significantly higher scavenging activity (58.34 ± 2.73%) compared to FII (36.27 ± 0.29%). At the extract concentration of 50 µg/ml, the radical scavenging activity of the langsat peel extract was 52.48 ± 0.66% compared to the scavenging activity of the extract fractions (<50%). Thus, crude extract had highest scavenging activity as compared to its extract fractions.

In terms of EC_{50}, the synthetic antioxidant, BHA showed the lowest EC_{50} value (9.66 ± 0.59 µg/ml), followed by the langsat peel extract (47.94 ± 0.46 µg/ml) and FIII (251.44 ± 13.13 µg/ml). EC_{50} for FI and FII could not be determined as the use of 500 µg/ml of the extract fractions could not achieve the DPPH radical scavenging activity of 50% and higher. The results showed that the highest scavenging activity that could be achieved by FI and FII at the concentration of 300 µg/ml was lower than 40%. At a higher concentration of the extract fraction (>500 µg/ml), it could possibly give a scavenging activity of higher than 50%. The EC_{50} values of the langsat peel extract, its extract fractions and BHA are shown in Table 2.

As observed from the experiment, the BHA gave the rapidest colour change from purple to yellow for the DPPH solution. As for the langsat peel extract and FIII, the colour changes of DPPH solution could be obviously seen. However, the experiment carried out for FII showed no obvious colour change. Even worst, no colour change was observed in FI. Therefore, it can be concluded that the antioxidants in FI could not act as reducing agents in scavenge DPPH radical. As reported by Ismail *et al.* (2013), DPPH assay involves in electron-transfer reaction pathway. Based on this mechanism, an antioxidant is known to donate an electron to a DPPH radical molecule as the molecule is unstable under this condition. The presence of antioxidant in the DPPH solution resulted in a decrease of the absorbance that measured at 517 nm (Charhardehi *et al*., 2009).

CONCLUSION

The present study demonstrated that the langsat peel extract contained antioxidant components. The antioxidant components in the extract fractions were also found to vary. Higher TPC and antioxidant activity were determined in FI compared to other extract fractions, but FI had moderately low TPC with undetectable antioxidant activity assessed using DPPH radical scavenging

assay. The highest DPPH radical scavenging activity was determined in the langsat peel extract with the lowest EC_{50} value when compared to its extract fractions. Based on the results obtained, the extract fractions of langsat peel did not have high TPC and antioxidant activity. However, future studies are needed to address some of the factors that caused the discrepancy in TPC and antioxidant activity in Langsat peels in the present study and those reported in the literature. Fractionation of any plant extract is not the ideal way to prepare nutraceutical ingredient unless for purification purpose. Thus, further studies are necessary to identify and elucidate the structure of the phenolic compounds present in langsat peel. The nutraceutical potential of langsat peel extract may also be explored for disease prevention.

ACKNOWLEDGEMENTS

REFERENCES

Ajila, C. M., Naidu, K. A., Bhat, S. G., & Prasada Rao, U. J. S. (2007). Bioactive compounds and antioxidant potential of mango peel extract. *Food Chemistry, 105*, 982-988.

Azlan, A., Ismail, A., Ibrahim, M., Shakirin, F. H., & Khoo, H. E. (2011). Health-promoting properties of selected Malaysian underutilized fruits. II International Symposium on Underutilized Plant Species: Crops for the Future-Beyond Food Security. *ISHS Acta Horticulturae, 979*, 203-210.

Charhardehi, A. M., Ibrahim, D., & Sulaiman, S. F. (2009). Antioxidant activity and total phenolic content of some medicinal plants in Urticaceae family. *Journal of Applied Biological Sciences, 2*, 1-5.

Chew, K. K., Ng, S. Y., Thoo, Y. Y., Khoo, M. Z., Wan Aida, W. M., & Ho, C. W. (2011). Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of *Centella asiatica* extracts. *International Food Research Journal, 18*, 571-578.

Chua, C. H. (2012). *Comparative antioxidant activities and total phenolic content of mangosteen and langsat peels*. In Bachelor Thesis. Malaysia: UCSI University.

Contreras-Calderon, J., Calderon-Jaimes, L., Guerra-Hernandez, E., & Garcia-Villanova, B. (2011). Antioxidant capacity, phenolic content and vitamin C in pulp, peel and seed from 24 exotic fruits from Colombia. *Food Research International, 44*, 2047-2053.

Cornelli, U. (2009). Antioxidant use in nutraceuticals. *Clinics in Dermatology, 27*, 175-194.

Department of Agriculture, Malaysia. (2013). *Plant Variety Protection Malaysia*. Retrieved June 21, 2013, from http://pvpbkkt.doa.gov.my/NationalList/Search.php.

Feskanich, D., Ziegler, R. G., Michaud, D. S., Giovannucci, E. L., Speizer, F. E., Willett, W. C., & Colditz, G. A. (2000). Prospective study of fruit and vegetable consumption and risk of lung cancer among men and women. *Journal of the National Cancer Institute, 92*, 1812-1823.

Halliwell, B. (1994). Free radicals and antioxidants: a personal view. *Nutrition Reviews, 52*, 253-265.

Hamid, A. A., Aiyelaagbe, O. O., Usman, L. A., Ameen, O. M., & Lawal, A. (2010). Antioxidants: Its medicinal and pharmacological applications.

African Journal of Pure and Applied Chemistry, 4, 142-151.

Huang, W. Y., Cai, Y. Z., Corke, H., & Sun, M. (2010). Survey of antioxidant capacity and nutritional quality of selected edible and medicinal fruit plants in Hong Kong. *Journal of Food Composition and Analysis, 23*, 510-517.

Ismail, A., Azlan, A., Khoo, H. E., Prasad, K. N., & Kong, K. W. (2013). *Antioxidant Assays: Principles, Methods and Analyses*. Selangor: Universiti Putra Malaysia Press.

Lim, Y. Y., Lim, T. T., & Tee, J. J. (2007). Antioxidant properties of several tropical fruits: A comparative study. *Food Chemistry, 103*, 1003-1008.

Mohamed, S., Hassan, Z., & Abd Hamid, N. (1994). Antimicrobial activity of some tropical fruit wastes (guava, starfruit, banana, papaya, passionfruit, langsat, duku, rambutan and rambai). *Pertanika Journal of Tropical Agricultural Science, 17*, 219-227.

Moure, A., Cruz, J. M., Franco, D., Dominquez, J. M., Sineiro, J., Dominguez, H., Nunez, M. J., & Parajo, J. C. (2001). Natural antioxidants from residual sources. *Food Chemistry, 72*, 145-171.

Nurliyana, R., Syed Zahir, I., Mustapha Suleiman, K., Aisyah, M. R., & Kamarul Rahim, K. (2010). Antioxidant study of pulps and peels of dragon fruits: a comparative study. *International Food Research Journal, 17*, 367-375.

Pourmorad, F., Hosseinimehr, S. J., & Shahabimajd, N. (2006). Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *African Journal of Biotechnology, 5*, 1142-1145.

Prasad, K. N., Divakar, S., Shivamurthy, G. R., & Aradhya, S. M. (2005). Isolation of a free radical-scavenging antioxidant from water spinach (*Ipomoea aquatic* Forsk). *Journal of the Science of Food and Agriculture, 85*, 1461-1468.

Reich, E., & Schibli, A. (2007). *High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants*. New York: Thieme Medical Publishers.

Scheibmeir, H. D., Christensen, K., Whitaker, S. H., Jegaethesan, J., Clancy, R., & Pierce, J. D. (2005). A review of free radicals and antioxidants for critical care nurses. *Intensive and Critical Care Nursing, 21*, 24-28.

Shiban, M. S., Al-Otaibi, M. M., & Al-Zoreky, N. S. (2012). Antioxidant Activity of Pomegranate (*Punica granatum* L.) Fruit Peels. *Food and Nutrition Sciences, 3*, 991-996.

Sultana, B., Anwar, F., & Ashraf, M. (2009). Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. *Molecules, 14*, 2167-2180.

Yan, S. W. & Asmah, R. (2010). Comparison of total phenolic contents and antioxidant activities of turmeric leaf, pandan leaf and torch ginger flower. *International Food Research Journal, 17*, 417-423.

Zubair, M., Anwar, F., & Shahid, S. A. (2012). Effect of extraction solvents on phenolics and antioxidant activity of selected varieties of Pakistani Rice (*Oryza sativa*). *International Journal of Agriculture & Biology, 14*, 935-940.

Screening for Optimum Concentrations of Boron, Copper and Manganese for the Growth of Three-Month Old Oil Palm Seedlings in Solution Culture

Tengoua, F. F.[1]*, Hanafi, M. M.[1], Idris, A. S.[2] and Syed-Omar, S. R.[3]

[1]*Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*GanoDROP Unit, Biological Research Division, Malaysia Palm Oil Board (MPOB), No. 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia.*
[3]*Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Micronutrient application has been in general overlooked in oil palm fertilisation programmes. Considering the importance of a balanced fertiliser in plant nutrition, it was necessary to determine the requirements of important micronutrients for their further recommendation in oil palm fertilisation programmes to ensure better growth and good yield. An experiment was conducted to identify the optimum concentrations of B, Cu and Mn for the growth of oil palm seedlings. The concentrations tested were 0, 0.25, 0.50, 1 and 2 mg B /L; 0, 0.5, 1, 2 and 4 mg Cu /L; 5, 10, 15 and 20 mg Mn /L. Germinated oil palm seeds were supplied with different concentrations of the selected micronutrients for three months in soilless culture. The assessment of the growth and physiological parameters showed that 2 mg/L for both B and Cu gave better response, while all the tested Mn concentrations were suppressive to the growth of oil palm seedlings. Therefore, 2 mg/L for both B and Cu and a minimal concentration of 2 mg Mn/L are being tested in new experiments in single and different possible combinations on nutritional, biochemical and growth parameters of oil palm seedlings from two to eight months for their future incorporation in oil palm fertilisers

INTRODUCTION

The increasing demand for oil and fats to meet the need of the increasing world population is one of the major concerns of the agricultural sector nowadays and will still be a concern in the future. The world's oil and fat consumption was estimated at 188 million tons in 2012/2013 (Mielke, 2014). Oil palm is the highest oil yielding crop with an average of 5 tons of crude palm oil (CPO) per hectare per year (Barison & Ma, 2000), almost three times the yield of coconut, and more 10 times that of soybean (0.4 ton per hectare) (Rajanaidu & Jalani, 1994).

In 2008, the major vegetable oil production was 111.13 million tons. Palm oil contributed about 40% and ranked first just before soybean oil (33%), amounting to about 67% of world export (Jackson *et al.*, 2009). World palm oil production has multiplied 15-fold since 1948 to reach 38 x 10^6 tons in 2007 (Rival, 2007). Southeast Asia (Malaysia and Indonesia) contributed 86% of global palm oil production. Over the years, oil palm fertilisation has been a macronutrient-based practice involving N, P, K, and Mg, and has been carried out especially on mineral soils, overlooking micronutrients; however, boron application is based on leaf sampling level. Consequently, soils in oil palm plantations seem to have been depleted in micronutrients through continuous removal of these elements by high fresh fruit bunch production.

Currently, the total area planted with oil palm is approximately 15 million ha (FAO, 2009; Turner *et al.*, 2011) with

Malaysia and Indonesia, the two leading palm oil producing countries of the world, accounting for 14 million ha with about 5 and 9 million ha, respectively. Palm oil is the highest oil-yielding crop, producing 10 times more oil than soybean. However, there is still a big gap between its current yield (4.5-5 tons crude palm oil (CPO) ha^{-1}year^{-1}) and its potential yield of about 19 tons CPO ha^{-1}year^{-1} (Corley, 2014), which could be in part due to unbalanced fertilisers, especially oversight in the use of micronutrients. The potential yield of a progeny for a given soil and climate can be seriously influenced by nutrient management. A good nutrient management and other practices can help to minimise or to close the gap between achievable yield and actual yield.

Boron, copper and manganese, besides their other physiological functions, are all involved in phenol synthesis in plants and have major effects on host susceptibility or resistance to disease (Graham, 1983). Boron is reported to be able to form boro-carbohydrate complexes with cis-hydroxyl groups. This affects the substrate flux between glycolysis and the pentose phosphate partway, resulting in the regulation of phenols and lignification, quinones and free radical production (Stangoulis & Graham, 2007). In oil palm, in addition to other functions, B is particularly important for the formation of pollen, its viability, its germination and the growth of pollen tubes (Fairhurst *et al.*, 2005). Copper plays an important role in photosynthesis. As the metal component of plastocyanins, it is involved in electron transport in

photosystem I. In addition to its biocidal effect, which makes it a common component of many copper-based pesticides, copper constitutes the active site of laccase, a multi-copper enzyme involved in the polymerisation of monolignols during the lignin biosynthesis (Fairhurst *et al.,* 2005; Evans & Solberg, 2007; Chmielowska *et al.,* 2008). In oil palm, Cu is required for lipid and nitrogen metabolism and pollen viability (Fairhurst *et al.*, 2005). Thanks to its high redox activities, manganese is among the most abundant elements in most surface environments. Manganese is an activator of several enzymes involved in the synthesis of important secondary metabolites (Thompson & Huber, 2007). Manganese activates the production of phenylalanine ammonia-lyase, the first enzyme committed to the common phenylpropanoid pathway leading to the biosynthesis of lignin precursors in the monolignol specific pathway (Boudet, 2000). Manganese deficiency symptoms are rare in oil palm, but are observed when Mn concentration in frond 17 is less than 25 mg/kg (Goh & Härdter, 2003). Considering the importance of B, Cu and Mn in oil palm, and their involvement in lignification and other plant defence mechanisms, the objective of this research was to gain an insight on how these micronutrients affect the growth of oil palm seedlings as a whole, prior to studying the necessity of their further consideration in oil palm fertilisation.

MATERIALS AND METHODS

The experiment was carried out in experimental field 2, Universiti Putra Malaysia, Serdang, Selangor, Malaysia (2º59'220.56''N, 101º42'44.42''E, about 45 m above sea level) in a shaded green house with 50% light filtration.

Oil palm germinated seeds

The commercial DxP (Yangambi ML 161) oil palm germinated seeds were supplied by FELDA Agricultural Services Sdn Bhd (FASSB), Sungai Tekam, Jerantut, Pahang, Malaysia. Homogeneous well differentiated seeds were selected and used when the radicle was 4 to 5 cm long.

Nutrient solution

The nutrient solution was prepared according to Hoagland and Arnon (1950). Each polypropylene tray containing 10 L of nutrient solution was planted with 3 germinated seeds supported by pieces of sponge and polystyrene. The solution was permanently aerated using an air pump and was renewed every week. The pH was adjusted at 5.5 with 0.1 N HCl or 0.1 N NaOH. The experiment was carried out for three months.

Growth parameters

The parameters measured were plant height (PH), net photosynthetic rate (*P*N), root fresh weight (RFW) and dry weight (RDW), root length (RL), root surface (RS), number of root tips (RT), root volume (RV), shoot fresh weight (SFW) and dry weight (SDW), SPAD chlorophyll value (SPAD Chl), total leaf area (TLA)and biomass dry weight i.e. total dry weight (TDW). Plant height and SPAD Chl were taken at 1.5 months and at

the end of the experiment while the other parameters were measured only at the end (3 months). The SPAD Chl was read on leaf 3 using Chlorophyll Meter SPAD – 502 Plus Konica Minolta Sensing, INC – Japan 20001421. The TLA was measured by the Leaf Area Meter LI – COR Model LI – 3100 Area Meter, USA, the PN by Portable LI – 6400 XT Photosynthesis System (LiCOR, Lincoln, NE, USA), and the RL, RS, RT and RV recorded by the Root Scanner Epson Expression 1680, Model G780B, Singapore, equipped with the software WinRHIZO – Pro.

Nutrient analysis

At the end of the experiment, the oil palm seedlings were washed with distilled water and separated into roots and shoot. The fresh weight was recorded and the samples were oven-dried at 65°C for three days, and the dry weights recorded. The samples were then ground to pass through a 1-mm sieve using Grinder Culleti Typ MEC CZ 13, Polymix Dispersing and Mixing Technology by KINEMATICA, Switzerland. Nutrients were extracted by dry ashing according to Benton Jones (2003). Nutrient analysis was basically focused on our nutrient of interest, namely B, Cu and Mn, to appreciate their levels in oil palm roots and shoots under our experimental conditions. Shoot samples were made up of composite of all leaves, including petiole and bulb. Boron was determined by inductively coupled plasma-mass spectrometry (ICP-MS), Model ELAN – DRC – e Canada, while Cu and Mn were determined by atomic absorption

spectrophotometer (AAS) Perkin Elmer, A Analyst 400, Model SS 103, USA.

Experimental design

The treatments were arranged in a randomised complete block design (RCBD) with three replicates. Each experimental unit contained three oil palm seedlings.

Data analysis

All the data were analysed by analysis of variance (ANOVA) using Statistix 8.0 (USDA and NRCS, 2007). Least significant difference (LSD) test or Tukey's honestly significant difference (HSD) all-pairwise comparison test was used to detect statistical differences among the means at $P = 0.05$ significance level. Where necessary, data were subjected to log or square root transformation to achieve the Shapiro-Wilk normality test. Also, polynomial analysis was applied to identify the best trend when needed, especially for copper concentrations.

RESULTS AND DISCUSSION

Growth parameters

Effect of boron

The tested concentrations of boron (B) did not differ significantly ($P \geq 0.05$) in their effects on morphological and physiological growth parameters studied (Table 1). This may be due to the fact that B is not directly involved in any of those parameters. In plants, the number of growth processes which require B include new cell development in meristematic tissues;

proper pollination and fruit or seed set; translocation of sugars, starches, nitrogen, and phosphorus; synthesis of amino acids and proteins; nodule formation in legumes; regulation of carbohydrate metabolism; root elongation and nucleic acid metabolism, auxin and phenol metabolism (Havlin *et al.,* 1999; Gupta, 2007).

Since there were no significant differences among the B concentrations, the ranking with respect to the parameters studied was used to establish the optimum value (Table 2). As a result, 2 mg B/L was either first or second for the majority of parameters, allowing it to be considered as optimum concentration for subsequent studies. This 2 mg B/L lies within 1 – 2 mg B/kg adequate range for oil palm (Syed Omar, 2007 unpublished data), 0.4 – 5 mg B/kg available range reported by Mills and Jones (1996), and 0.1 – 2.5 mg B/kg available range for oil palm determined by Eschbach (1980).

Effect of copper

Highly significant differences (P ≤ 0.01) were observed among the tested concentrations of copper (Cu) for shoot dry weight with 2 mg Cu/L giving the highest value (Fig.1). This suggests that at 2 mg Cu/L in nutrient solution, oil palm seedlings produce and accumulate more biomass in shoots compared to other concentrations. This may be due to the more efficient photosynthetic activity at 2 mg Cu/L.

Plant height values recorded in the middle of the experiment (1.5 months) were significantly different (P ≤0.05). The graphic

representation (Fig.2) showed 2 mg Cu/L to be the optimum concentration.

The polynomial analysis performed on this parameter and on the shoot dry weight allowed the identification of 2 mg Cu/L as the optimum concentration (Fig.3) after derivation of the equation describing the trend at the end of the experiment (3 months) as well as at mid-term.

This indicates that the maximum height of oil palm seedlings was achieved at 2 mg Cu/L, while the higher levels of Cu led to a decreased height. As observed in the case of boron, 2 mg Cu/L was within the range of 1 – 2 adequate for oil palm (Syed Omar, 2007, unpublished data), close to 0.5 – 1.5 considered as moderate by Munevar (2001). In another study, Sabrina (2011) found 2 mg Cu/L to be optimum for peroxidase activity, laccase activity and lignin content in the roots of oil palm seedlings under sand culture using tap water supplemented with various concentrations of copper.

For most of the parameters studied, the lowest values corresponding to reduced plant height were recorded with the control (0 mg Cu/L) and 4 mg Cu/L, indication of insufficiency and toxicity, respectively.

Effect of manganese

All the tested concentrations of manganese (Mn) showed a suppressive effect on all the parameters evaluated apart from the control (0 mg Mn/L), which gave significantly (P ≤ 0.05) higher performance than the others for 11 parameters out of 14 (i.e. 78.57% of the cases) (Table 3). Even when no significant difference existed among the Mn

TABLE 1
Effects of boron concentrations on morphological and physiological growth parameters of oil palm seedlings

B concentration	Growth parameters													
	PH1	PH2	P_N	RFW	RDW	TRL	RSA	RT	RV	SFW	SDW	TLA	SPAD Chl1	SPAD Chl2
mg/L	—— cm ——		$\mu molICO_2m^{-2}s^{-1}$	—— g ——		cm	cm²		cm³	—— g ——		cm²		
0	22.22 [a]	29.05 [a]	4.15 [a]	1.71 [a]	0.20 [a]	350.87 [a]	68.34 [a]	2723 [a]	1.07 [a]	8.14 [a]	1.42 [a]	155.51 [a]	42.03 [a]	39.77 [a]
0.25	22 [a]	28.77 [a]	4.30 [a]	1.57 [a]	0.17 [a]	453.02 [a]	90.75 [a]	2928 [a]	1.44 [a]	6.99 [a]	1.15 [a]	176.86 [a]	45.07 [a]	37.60 [a]
0.50	20.55 [a]	27.04 [a]	2.94 [a]	1.37 [a]	0.15 [a]	377.98 [a]	79.65 [a]	2590 [a]	1.34 [a]	7.01 [a]	1.17 [a]	170.62 [a]	42.53 [a]	38.93 [a]
1	22.64 [a]	30 [a]	2.38 [a]	1.37 [a]	0.17 [a]	397.15 [a]	78.79 [a]	2855 [a]	1.26 [a]	5.80 [a]	1.12 [a]	162.94 [a]	41.87 [a]	34.23 [a]
2	22.31 [a]	29.33 [a]	4.34 [a]	1.75 [a]	0.19 [a]	538.03 [a]	98.14 [a]	3922 [a]	1.42 [a]	7.41 [a]	1.28 [a]	194.82 [a]	45.30 [a]	39.12 [a]
Coefficient of variation (CV)	8.26	9.87	32.13	19.03	24.60	37.35	37.21	34.21	40.20	22.14	20.56	21.49	15.45	16.11

Means of boron concentration with the same letter within the same column are not significantly different at P = 0.05 (Tukey's HSD test)
PH1 = plant height 1 (first measurement at 1.5 month); PH2 = plant height 2 (second measurement at 3 months);
P_N = net photosynthetic rate; RFW = root fresh weight; RDW = root dry weight; TRL = total root length; RSA = root surface area;
RT = root tip; RV = root volume; SFW = shoot fresh weight; SDW = shoot dry weight; TLA = total leaf area;
SPAD Chl2 = SPAD Chlorophyll value 1 (first reading at 1.5 month); SPAD Chl2 = SPAD Chlorophyll value 2 (second reading at 3 months).

TABLE 2
Ranking of boron concentration with respect to growth parameters

B concentration	Growth parameters													
	PH1	PH2	P_N	RFW	RDW	TRL	RSA	RT	RV	SFW	SDW	TLA	SPAD Chl1	SPAD Chl2
mg/L	—— cm ——		$\mu molICO_2m^{-2}s^{-1}$	—— g ——		cm	cm²		cm³	—— g ——		cm²		
0	22.22 [a]	29.05 [a]	4.15 [a]	1.71 [a]	0.20 [a]	350.87 [a]	68.34 [a]	2723 [a]	1.07 [a]	8.14 [a]	1.42 [a]	155.51 [a]	42.03 [a]	39.77 [a]
0.25	22 [a]	28.77 [a]	4.30 [a]	1.57 [a]	0.17 [a]	453.02 [a]	90.75 [a]	2928 [a]	1.44 [a]	6.99 [a]	1.15 [a]	176.86 [a]	45.07 [a]	37.60 [a]
0.50	20.55 [a]	27.04 [a]	2.94 [a]	1.37 [a]	0.15 [a]	377.98 [a]	79.65 [a]	2590 [a]	1.34 [a]	7.01 [a]	1.17 [a]	170.62 [a]	42.53 [a]	38.93 [a]
1	22.64 [a]	30 [a]	2.38 [a]	1.37 [a]	0.17 [a]	397.15 [a]	78.79 [a]	2855 [a]	1.26 [a]	5.80 [a]	1.12 [a]	162.94 [a]	41.87 [a]	34.23 [a]

TABLE 2 (continue)

	PH1	PH2	P_N	RFW	RDW	TRL	RSA	RT	RV	SFW	SDW	TLA	SPAD Chl1	SPAD Chl2
2	22.31[a]	29.33[a]	4.34[a]	1.75[a]	0.19[a]	538.03[a]	98.14[a]	3922[a]	1.42[a]	7.41[a]	1.28[a]	194.82[a]	45.30[a]	39.12[a]
Coefficient of variation (CV)	8.26	9.87	32.13	19.03	24.60	37.35	37.21	34.21	40.20	22.14	20.56	21.49	15.45	16.11

PH1 = plant height 1 (first measurement at 1.5 month); PH2 = plant height 2 (second measurement at 3 months);

P_N = net photosynthetic rate; RFW = root fresh weight; RDW = root dry weight; TRL = total root length; RSA = root surface area;

RT = root tip; RV = root volume; SFW = shoot fresh weight; SDW = shoot dry weight; TLA = total leaf area;

SPAD Chl2 = SPAD Chlorophyll value 1 (first reading at 1.5 month); SPAD Chl2 = SPAD Chlorophyll value 2 (second reading at 3 months).

Note the position of 2 mg B/L in the ranking (either first or second).

TABLE 3
Effects of manganese concentrations on morphological and physiological growth parameters of oil palm seedlings

Mn concentration							Growth parameters							
	PH1	PH2	P_N	RFW	RDW	TRL	RSA	RT	RV	SFW	SDW	TLA	SPAD Chl1	SPAD Chl2
mg/L	cm	cm	$\mu mol CO_2 m^{-2}s^{-1}$	g	g	cm	cm^2		cm^3	g	g	cm^2		
0	24.50[a]	29.16[a]	5.28[a]	2.01[a]	0.24[a]	577.82[a]	113.13[a]	4260[a]	1.76[a]	8.94[a]	1.61[a]	214.66[a]	54.43[a]	53.83[a]
5	20.83[a]	26.46[ab]	3.11[a]	1.62[ab]	0.18[a]	413.52[ab]	77.08[ab]	3102[a]	1.14[ab]	6.90[ab]	1.10[ab]	171.06[ab]	29.77[b]	39.03[bc]
10	19.66[ab]	26.05[ab]	3.72[a]	1.25[ab]	0.16[a]	316.28[ab]	56.89[b]	2507[a]	0.81[b]	6.31[ab]	1.07[ab]	140.21[bc]	37.07[b]	43.80[b]
15	20.22[ab]	25.55[ab]	5.99[a]	1.46[ab]	0.17[a]	365.03[ab]	76.99[ab]	2541[a]	1.29[ab]	6.58[ab]	1.07[ab]	144.97[bc]	37[b]	38.63[bc]
20	15.94[b]	19.22[b]	1.69[a]	1.07[b]	0.12[a]	254.97[b]	50.21[b]	1984[a]	0.79[b]	4.30[b]	0.65[b]	92.33[c]	29.43[b]	31.47[c]
Coefficient of Variation (CV)	8.93	11.57	52.46	22.25	26.58	26.72	22.57	43.03	20.38	25.05	32.07	15.15	15.64	13.13

Means of boron concentration with the same letter within the same column are not significantly different at P = 0.05 (Tukey's HSD test)

PH1 = plant height 1 (first measurement at 1.5 month); PH2 = plant height 2 (second measurement at 3 months);

P_N = net photosynthetic rate; RFW = root fresh weight; RDW = root dry weight; TRL = total root length; RSA = root surface area;

RT = root tip; RV = root volume; SFW = shoot fresh weight; SDW = shoot dry weight; TLA = total leaf area;

SPAD Chl2 = SPAD Chlorophyll value 1 (first reading at 1.5 month); SPAD Chl2 = SPAD Chlorophyll value 2 (second reading at 3 months).
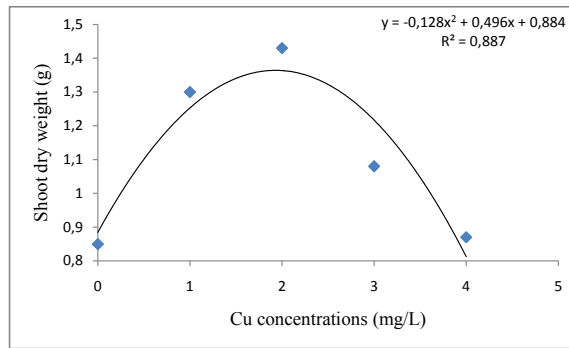
Fig.1: Effect of different concentrations of copper on shoot dry weight of oil palm seedlings at 3 months
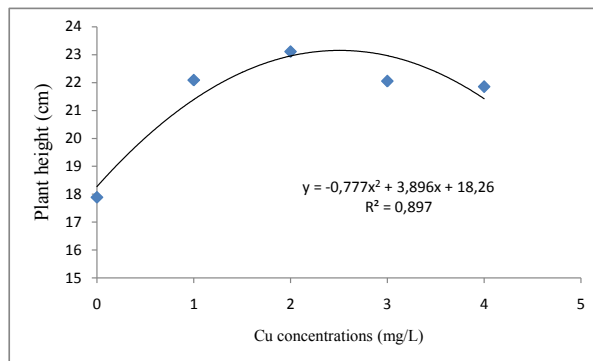
Fig.2: Effect of different concentrations of copper on the height of oil palm seedlings at 1.5 months

concentrations for a given parameter, such as PN, RDW and RT (Table 3), 0 mg Mn/L still gave the highest values.

The improving effect of 0 mg Mn/L and the suppressive effect of other concentrations, especially 20 mg Mn/L were more striking and more discernible on plant height (Fig.4) and shoot fresh weight (Fig.5).

These results suggest that the nutrient reserves in the endosperm of the oil palm kernel might be enough to sustain the adequate growth of the seedlings during the pre-nursery stage and probably for the early nursery stage or until their complete exhaustion. The nutrient analysis of oil palm kernel (Table 4) revealed 82 mg Mn/kg of dry matter indicating that Mn supply might not be necessary in the early stages of oil palm seedlings.

About 4.5 kg Mn is reported to be accumulated in mature oil palms per ha per year (Ng *et al*., 1968), while fresh fruit bunches (FFB) remove only 0.4 – 0.5 kg per ha (Fairhurst & Härdter, 2003). The high level of Mn reserves in kernel, the high accumulation of Mn in mature oil palms compared to low need for FFB production could explain why the requirement of Mn fertiliser application is very rare in oil palm cultivation, except in case of remarkable Mn deficiency.
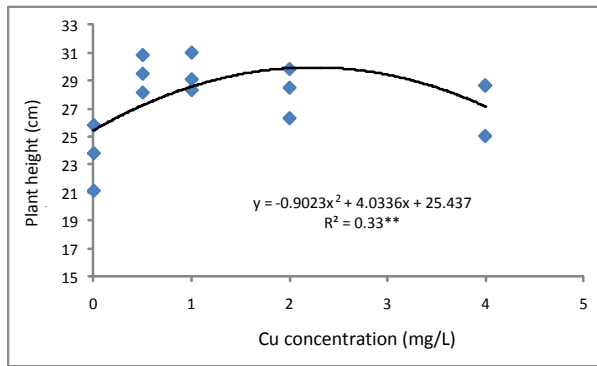
Fig.3: Effect of copper in different concentrations of copper on the height of oil palm seedlings at 3 months
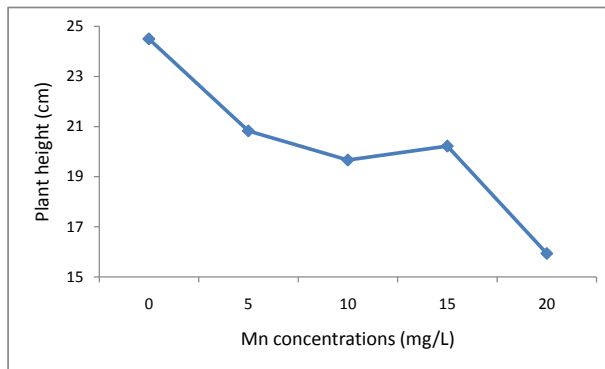
Fig.4: Effect of different concentrations of manganese on the height of oil palm seedlings at 1.5 months
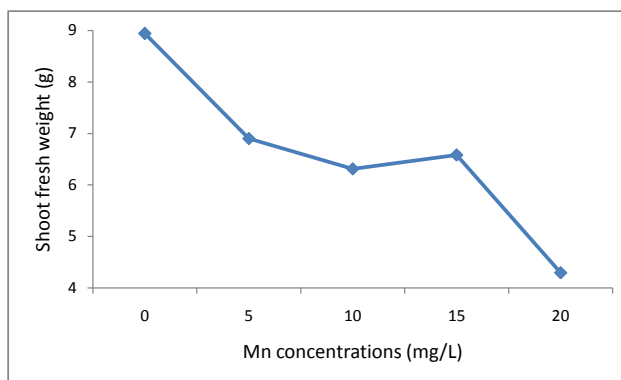
Fig.5: Effect of different concentrations of manganese on shoot fresh weight of oil palm seedlings at 3 months

Effects of Boron, Copper and Manganese on Total Dry Weight

As observed for the previous parameters, no significant differences (P ≥ 0.05) were detected among B concentrations for total dry biomass (Table 5). Unexpectedly, the control gave the highest total dry weight, followed by 2 mg B/L. Once again the consistent conservation of 2 mg B/L at the first or second position supported the choice of this concentration as optimum for B.

Highly significant differences (P ≤ 0.01) were found among Cu concentrations for total dry weight with 0.50 mg Cu/L surprisingly giving the highest value, followed by 2 mg Cu/L (Table 5). Lowest values were recorded at 0 mg Cu/L and 4 mg Cu/L with no statistical difference.

For Mn, as the concentration increased, the total dry weight decreased, indicating a negative correlation between Mn concentration and total dry biomass (Table 5). Once more, 0 mg Mn/L (control) gave the highest total dry weight and 20 mg Mn/L the lowest, confirming the overall suppressive effect of Mn on the growth of oil palm seedlings, with 20 mg Mn/L being more detrimental.

Nutrient Analysis: Effects of Boron, Copper and Manganese on their Concentrations in Roots and Shoots

With the exception of Mn, it could be noticed that a high concentration of each element in nutrient solution led to a high concentration of the said element in roots and shoots (Table 6). As found by Abidemi *et al*. (2006) for phosphorus, a high correlation may exist between B and Cu levels and B and Cu uptake in plants.

TABLE 4
Nutrient composition of oil palm kernel

	Element									
	Macroelements					Microelements				
	N	P	K	Mg	Ca	B	Cu	Fe	**Mn**	Zn
Concentration	——————— g/kg ———————					——————— mg/kg ———————				
	17.45	7.54	6.13	2.18	2.40	89	22	64	**82**	31

TABLE 5
Effects of boron, copper and manganese on biomass dry weight

B concentration	TDW	Cu concentration	TDW	Mn concentration	TDW
mg/L	g	mg/L	g	mg/L	g
0	1.62[a]	0	0.99[c]	0	1.85[a]
0.25	1.31[a]	0.5	1.63[a]	5	1.28[ab]
0.5	1.32[a]	1	1.24[bc]	10	1.23[ab]
1	1.29[a]	2	1.48[ab]	15	1.23[ab]
2	1.48[a]	4	1.01[c]	20	0.77[b]

Means of concentrations with the same letter within the same column are not significantly different at P = 0.05 (Least significant difference (LSD) test). TDW = Total dry weight.

TABLE 6
Effects of boron, copper and manganese on their concentrations in roots and shoots

B concentration	Root B	Shoot B	Cu concentration	Root Cu	Shoot Cu	Mn concentration	Root Mn	Shoot Mn
mg/L	——mg/kg——		mg/L	——mg/kg——		mg/L	——mg/kg——	
0	74.89[a]	44.15[b]	0	72[b]	25.67[a]	0	475[a]	283.30[b]
0.25	74.80[a]	46.50[b]	0.5	70.33[b]	34.53[ab]	5	1073.30[a]	1038.70[a]
0.5	73.14[a]	49.13[b]	1	63.17[b]	28.38[ab]	10	1128.30[a]	975.30[a]
1	76.91[a]	62.42[a]	2	99.17[ab]	37.27[ab]	15	1595[a]	977.50[a]
2	81.24[a]	70.87[a]	4	128.33[a]	46.33[a]	20	875[a]	713.30[a]
Coefficient of Variation (CV)	9.54	9.70		26.17	29.31		65.78	23.81

Means of concentrations with the same letter within the same column are not significantly different at P = 0.05 (Least significant difference (LSD) test)

However, the general pattern is not linear apart from B content in shoots which proportionally increased with the increase of B in the nutrient solution. The non-linearity could be due to multiple interactions that naturally exist between microelements themselves and between micro and macro elements. The different pattern observed particularly for Mn can be explained by the variation of Mn content in the oil palm kernel that might have interfered with Mn in solution. It is worth noting that the nutrient levels in roots and shoots are by far above the plant reference (Benton Jones, 2003) and oil palm reference (Ng *et al*., 1968) (Table 7), but no sign of toxicity was observed. This may suggest that oil palm at the earlier and active growing stage has the ability to accumulate high levels of nutrients. To date, no nutrient reference for oil palm seedlings has been established, probably because of the short period of pre-nursery and nursery, four and eight months, respectively.

CONCLUSION

The concentration of both B and Cu at 2 mg/L nutrient solution has been identified to be optimum for the growth of oil palm seedlings. The superior effect of 0 mg Mn/L (control) over other Mn concentrations is an indication that Mn might not be needed at the pre-nursery stage.

ACKNOWLEDGEMENTS

Pertanika J. Trop. Agric. Sci. 38 (1): 113 - 126 (2015)

123

TABLE 7
Nutrient reference concentrations in plant and oil palm

Elements		Plant reference (a)	Young palms (b)	Mature palms (b)
1- Macro nutrients (g/kg)	N	10 – 60	14	4.4 – 6.5
	P	2 – 5	1.4	0.52
	K	15 – 40	10 – 13	
	Mg	1.5 – 4	2.2	1.6
	Ca	5 – 15	1.4	2.5
	S	1.5 – 5	1.7 – 3.6	
	Cl	50 – 200	0.4 – 6	
2- Micronutrients (mg/kg)	**B**	**20**	**7.0 – 8.5 in above-ground biomass of oil palm. Optimum in frond 17: 15 – 25**
	Cu	**2 – 20**	**7 – 10 in above-ground biomass of oil palm. Optimum in frond 17: 5 - 8**
	Mn	**10 – 200 (10 – 50 acceptable)**	**64 – 166 in above-ground biomass of oil palm. Optimum in frond 17: 50 - 200**
	Fe	150	107 – 221 in above-ground biomass of oil palm. Optimum in frond 17: 50 - 250
	Zn	15 – 50	18 – 31 in above-ground biomass of oil palm
	Mo	0.15 – 0.30	Optimum: 0.5 – 0.8 in leaf Deficiency when < 0.1

a: Benton Jones, 2003; b: Ng *et al*., 1968

REFERENCES

Abidemi, A. A., Akinrinde, E. A., & Obigbesan, G. O. (2006). Oil palm (*Elaeis guineensis*) seedling performance in response to phosphorus fertilization in two benchmark soils of Nigeria. *Asian Journal of Plant Sciences*, *5*(5), 767-775.

Barison, Y. & Ma, A. N. (2000). Malaysian palm oil industry: Moving towards the future. *Palm Oil Development*, *32*, 1-9.

Benton Jones, J. Jr. (2003). *Agronomic Handbook. Management of Crops, Soils, and their Fertility*. CRC Press, New York, USA.

Boudet, A. M. (2000). Lignins and lignification: Selected issues. *Plant Physiology and Biochemistry*, *38*(1/2), 81-96.

Chmielowska, J., Deckert, J., & Diaz, J. (2008). Acivity of peroxidises and phenylalanine ammonia – lyase in luprine and soybean seedlings treated with copper and an ethylene inhibitor. *Biological Letters*, *45*, 59-67.

Corley, R. H. V. (2014). *Potential yield of oil palm – An update*. Retrieved from www.isopb.og/docs/keynoteCorley (Accessed on 25.07.2014)

Eschbach, J. M. (1980). Micronutrients in oil palm. *Oleagineux*, *35*, 291-294.

Evans, I., Solberg, E. & Huber, D. M. (2007). Copper and plant disease. In Datnoff, L. E., Elmer, W. H. & Huber, D. M. (Eds.), *Mineral Nutrition and Plant Disease* (pp. 177-188). APS Press St. Paul, Minnesota, USA.

Fairhurst, T. & Härdter, R. (2003). *Oil Palm: Management for Large and Sustainable Yields*. Potash and Phosphate Institute, Potash and Phosphate Institute of Canada and International Potash Institute.

Fairhurst, T., Caliman, J. P. Härdter, R., & Wilt, C. (2005). *Oil Palm: Nutrient Disorders and Nutrient Management*. Potash and Phosphate Institute, Potash and Phosphate Institute of Canada, and International Potash Institute, Southeast Asia Program, Singapore.

FAO. (2009). *FAOSTAT online statistical service*. Food and Agriculture Organization of the United Nations, Rome. Reteieved from http://faostat.fao.org/ (Accessed August 2011).

Goh, K.-J. & Härdter, R. (2003). General oil palm nutrition. In: Fairhurst, T. and Härdter, R. (Eds). *Oil palm: Management for large and sustainable yield*. Potash and Institute (PPI), Potash and Institute of Canada (PPIC) and International Potash institute (IPI), pp 191-228.

Graham, R. D. (1983). Effects of nutrient stress on susceptibility of plants to disease with particular reference to the trace elements. *Advances in Botanical Research*, *10*, 221-276.

Gupta, U. C. (2007). Boron. In Barker, A.V. & Pilbeam, D.G. (Eds.), *Handbook of Plant Nutrition*, (pp.241-277). Taylor and Francis Group, LLC. CRC Press, New York, USA.

Havlin, J. L., Beaton, J. D., Tisdale, S. L., & Nelson, W. L. (1999). *Soil Fertility and Fertilizers. An Introduction to Nutrient Management.* Prentice Hall, Inc, New Jersey, USA.

Hoagland, D. R. & Arnon, D. I. (1950). *The Water – Culture Method for Growing Plants without Soil*. California Agricultural Experiment Station, Circular 347.

Jackson, D., Fry, J., Fitton, C., Hickingbottom, S., Hogg, M., Fereday, N., & Finley, W. (2009). *Oil-Seeds, Oils and Meals Analysis*, *March 2009*. LMC International Ltd. 16 p.

Mielke, T. (2014). *Global oils and fats outlook-Forecats on demand and consumption*. Paper presented at Palm Oil Trade Fairs and Seminar (POTS) in Tehran (Iran) on the 20th January 2014.

Mills, H. A. & Benton Jones, J. Jr. (1996). *Plant Analysis Handbook II. A Practical Sampling, Preparation, Analysis and Interpretation Guide.* Micro – Macro Publishing, Inc, Georgia, USA.

Munevar, M. F. (2001). Fertilization of oil palm to obtain high yields. *Palmas*, 22, 9-17.

Ng, S. K., Cheah, T. E., & Tamboo, S. (1968). Nutrient contents of oil palm in Malaya. III. Micronutrients in vegetative tissues. *The Malaysian Agricultural Journal*, *46*, 4, 421-434.

Rajanaidu, N. & Jalani, B. S. (1994). Prospects for breeding for kernels in oil palm (*Elaeis guineensis*). *The Planter, 70*(820), 309-318.

Rival, A. (2007). Oil palm. In Pua, E. C. & Davey, M. R. (Eds.), *Transgenic Crops VI. Biotechnology in Agriculture, 61,* 59-80. Springer - Verlag Berlin Heidelberg.

Sabrina, A. A. (2011). *Effects of calcium and copper on lignin biosynthesis and suppression of* Ganoderma boninense *infection in oil palm seedlings*. (MSc Thesis). Universiti Putra Malaysia, Institute of Tropical Agriculture.

Stangoulis, J. C. R. & Graham, R. D. (2007). Boron and plant disease. In Datnoff, L. E., Elmer, W. H. & Huber, D. M. (eds.), *Mineral Nutrition and Plant Disease* (pp. 207-214). APS Press St. Paul, Minnesota, USA.

Thompson, I. A., & Huber, D. M. (2007). Manganese and plant disease. In Datnoff, L. E., Elmer, W. H. & Huber, D. M. (Eds.), *Mineral Nutrition and*

Plant Disease (pp. 139-153). APS Press St. Paul, Minnesota, USA.

Turner, E. C., Snaddon, J. L., Ewers, R. M., Fayle, T. M., & Foster, W. A. (2011). The impact of oil palm expansion on environmental change: Putting environmental research in context. In: Dos Santos Bernardes, M. A. (Ed). *Environmental Impact of Biofuels*. In Tech, Croatia. pp. 1-40.

USDA, NRCS. (2007). *Statistix 8.0 user guide for the plant materials program*. United State Department of Agriculture and National Resources Conservation Service.

Genetic Divergence and Evaluation of Yield Potential of *Jatropha curcas* Accessions Collected from Peninsular Malaysia

Arolu, I. W.[1], Rafii, M. Y.[1,2]*, Hanafi, M. M.[1], Mahmud, T. M. M.[1,2] and Askani, S.[1,3]

[1]*Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[2]*Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[3]*Department of Agronomy and Plant Breeding, Islamic Azad University of Shahr-e-Rey Branch, Tehran, Iran*

ABSTRACT

Widening of the narrow genetic base of *Jatropha curcas* through germplasm collection, diversity study and evaluation is needed to bring about much needed improvement in its seed yield and oil content. This study was carried out to profile the divergence patterns of 45 *Jatropha curcas* from three populations (Kelantan, Selangor and Terengganu) and to evaluate their yield over a period of three years. Eleven (11) morphological traits together with ISSR markers were used in this study. The percentage of polymorphism for the ISSR markers among the three populations was very high, ranging from 90.38-100%. Shannon information index (I) and expected heterozygosity (He) were found to be highest in the Kelantan population, at 0.58 and 0.40 respectively. Genetic differentiation (Analysis of molecular variance) expressed as fixation index (0.46) revealed that variations within the population accounted for about 100% of the total variation. Interestingly, the cluster analysis based on molecular and morphological traits, as presented in the dendrogram, grouped the 45 accessions into seven and five clusters respectively. For morphological traits, variability in terms of coefficient of variation (CV) was very high, as much as 53.19 and 51 % in total number of seeds and oil yield/ha. Small differences were seen between phenotypic and genotypic coefficient of variation ($\leq 10\%$) for the yield trait. Broad sense heritability for virtually all the yield and yield components was very high (≥ 67.8). Accessions B-01-03, D-01-06, T-01-06, B-06-02 have been identified and recommended for further evaluation under field conditions before they are chosen for future breeding programmes for seed yield and oil improvement.

INTRODUCTION

With the increasing rise in global warming, efforts have been intensified to bring down emissions of harmful gases into the environment to an acceptable level (Banković-Ilić *et al*., 2012). Various approaches have been devised to implement this. Such approaches include the use of renewable energy sources (green fuel from seed oil), reduction in burning of fossil fuels, tree planting and other environmentally sustainable practices (Kristensen *et al*., 2011). In an attempt to identify environment-friendly and economically-feasible alternative energy feed stocks, *Jatropha curcas* has been chosen, alongside other oil producing crops. The choice of *Jatropha curcas* was due to its high non-edible seed oil production, fast-growing nature and the low-input requirements for its cultivation (Henning, 2004; Chauhan *et al*., 2012).

Jatropha curcas is a medium-to-tall oil producing tree crop from the euphorbiaceae family. It is able to tolerate drought and grows very well on marginal soil. It also thrives very well with little or no agronomical input. It is widely used as an agroforestry plant for live fences and land reclamation in developing countries. The leaves are used as mulch by farmers while its latex is of great dermatological and ethno-botanical importance. The seed oil content varies, ranging from 30-45 % by weight depending on the genotypes or varieties. The non-toxic *Jatropha curcas* seeds are incorporated into animal feed as a protein source due to its high crude protein content of about 50-60% (Tanya *et al*., 2011).

Despite the great potential of *Jatropha curcas*, the in-depth agronomical requirements necessary for successful and profitable commercial cultivation are yet to be standardised. Similarly, the genetic diversity structures and patterns required for breeding and production of high yielding cultivars are yet to be identified. Morphological alongside ISSR markers have been widely employed in genetic diversity studies for different oil producing tree crops such as coconut, olive, canola plants etc. This is a simple and low-cost method of studying the genetic structure, divergence and variance components in a breeding population (Sunil *et al*., 2011). It gives breeders the opportunity to identify individual plants with high potential in a breeding population.

This research therefore aims to achieve the following objectives: (i) to evaluate the yield performance and genetic divergence existing in the 45 accessions of *Jatropha curcas* collected from three states in Peninsular Malaysia; (ii) to estimate the variance components and broad sense heritability; and iii) to calculate the principal component and carry out cluster analyses with the purpose of identifying high yielding *Jatropha curcas* accessions from the germplasm.

MATERIALS AND METHODS

The seeds and cuttings of 45 accessions of *Jatropha curcas* were collected from three states in Peninsular Malaysia viz. Kelantan, Terengganu and Selangor, and planted in Universiti Putra Malaysia's experimental plot for field evaluation. The experimental plot is in a tropical environment with high humidity and sunshine and receiving about 2500 mm of rainfall annually. The site contains a well-drained sandy-to-loamy soil with moderate pH suitable for crop growth. The cuttings were planted using 2×3 metre spacing in two blocks with six cuttings planted in each block to represent each accession, resulting in 270 plants per block.

DNA Extraction, ISSR Profiling and Analysis.

Samples of fresh young leaves were obtained from each of the trees representing the different accessions. The leaf samples were finely crushed with ceramic mortar and pestle, in the presence of liquid nitrogen. In order to obtain high quality DNA, CTAB extraction protocol of Doyle and Doyle (1990) was employed with some modification in the quantity of the CTAB buffer. The DNA pellets of the samples were completely dissolved in a TE buffer and quantified using the Nanodrop spectrophotometer (Thermoscientific) before being diluted with sterile distilled water to a concentration of 50 ng/μL for PCR analysis and kept in a refrigerator at -20 °C.

Twenty-five ISSR primers, as listed by Gupta *et al*. (2010), were selected and used for profiling this germplasm. The PCR protocol was performed using a Qiagen PCR master mix kit and a total reaction mixture of 25 μl was adopted, while the temperature settings followed standard protocols as described by Murty *et al*. (2013). Following electrophoresis, the gel picture was captured using a Bio-Rad Image Lab. Binary scoring, 1 or 0 representing "presence or absence" of a specific clear and polymorphic band.

Analysis of genetic diversity parameters such as Shannon information index, Nei's genetic diversity and expected heterozygosity were done using GenAIEX 6.5 (Peakall & Smouse, 2006, 2012). Principal component analysis was performed using NTSYS-PC (Rohlf, 1997). Genetic differentiation through analysis of molecular variance (AMOVA) was performed using GenAIEX 6.5.

Data Collection and Statistical Analysis

Data collection on yield and yield components commenced from year one after planting and was carried out for three years, and the mean values were used for analysis. Eleven quantitative traits were measured, which include plant height (cm), number of primary branches, number of secondary branches, stem diameter (mm), seed yield per plant (g), seed length (mm), seed breadth (mm), number of seeds per plant, seed yield per hectare (kg), oil content (%) and oil yield per hectare (kg). The data collection was carried out following the method of Shabanimofrad *et al*. (2013).

Analysis of variance (ANOVA) was carried out using SAS 9.3 to investigate any

significant differences among the accessions based on the traits. Similarly, a SAS code "proc varcomp method=type1" was used for estimating the variance components. Using the results, other heritability components such as genetic advance, broad sense heritability, phenotypic and genotypic coefficients of variation were calculated following the method of Allard (1960) and Kang (1998). Cluster analysis was done using the NTSYS pc software for the construction of the dendrogram and genetic similarity distances among the accessions.

RESULTS

Genetic Diversity Analysis.

The 45 accessions of *Jatropha curcas* collected were profiled using ISSR markers (Table 1 and Fig.1). The accessions were partitioned into three populations (Table 2). Due to their diverse nature, different allelic variations were observed in the three populations, ranging from 1.82 (Terengganu population) to 2.0 (Kelantan population), with a mean value of 1.93. Polymorphism

percentages were very high in the Kelantan, Terenganu and Selangor populations, at 98.08, 90.38 and 100% respectively. The mean percentage of polymorphism for the three populations was found to be 96.3 %. The Shannon information index (I) and observed heterozygosity (Ho) were observed to be the least in the Terengganu population (0.53 and 0.36), while the Kelantan population exhibited the highest at 0.58 and 0.40, respectively.

AMOVA is the genetic differentiation which revealed the allelic variations within and between the Jatropha populations. In this study, about 100% of the variations observed in the germplasm occurred as a result of variations within the populations (Table 3) with fixation index (F-Score) of 0.463.

The cluster analysis was done to construct a dendrogram based on UPGMA analysis using dice similarity index with coefficients ranging from 0.20 to 0.98 (Fig.2). This was done to highlight the overall genetic relationship among the
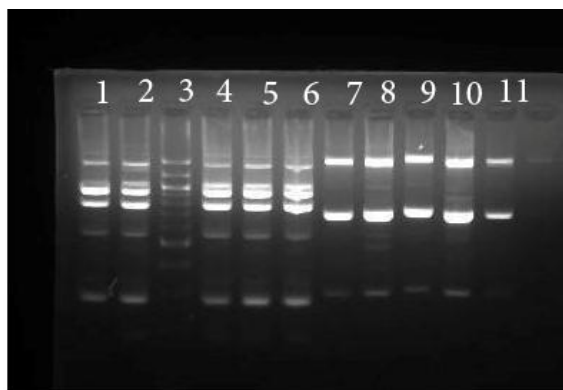
Fig.1: Gel picture showing the polymorphic bands across the eleven accessions of *Jatropha curcas*

TABLE 1
List of 45 accessions from three states with their codes, and locations

Number	Accessions	Population	Origin	Area	Latitude	Longitude
1	B-01-01	Selangor	Seri Serdang	Serdang	3°01.24"	101° 43' 1.1994"
2	B-01-02	Selangor	Seri Serdang	Serdang	3°0'38.88"	101° 42' 35.9994"
3	B-01-03	Selangor	Seri Serdang	Serdang	3°0'38.16"	101° 42' 21.6"
4	B-01-04	Selangor	Taman Serdang Raya	Serdang	3°0'38.52"	101° 42' 25.1994"
5	B-01-05	Selangor	UPM-Cemetery	Serdang	2°59' 52.44"	101° 43' 4.8"
6	B-01-06	Selangor	UPM- Kolej 17	Serdang	2°5845.48"	101° 42' 39.5994"
7	B-01-07	Selangor	UPM- Kolej 17	Serdang	2°5845.479"	101° 42' 39.5994"
8	B-02-01	Selangor	Ladang Raja Musa	Kuala Selangor	2°24'29.519"	101° 16' 55.1994"
9	B-02-02	Selangor	Bukit Belimbing	Kuala Selangor	2°24' 29.88"	101° 16' 51.6"
10	B-02-03	Selangor	Sri Angala Aman	Kuala Selangor	2°23'48.479"	101° 16' 30"
11	B-02-04	Selangor	Kota Hulu Moram	Kuala Selangor	3°23'33.72"	101° 17' 27.5994"
12	B-02-05	Selangor	Taman Sri Blimbing	Kuala Selangor	3°23'23.639"	101° 16' 19.2"
13	B-02-06	Selangor	Lorong Intan A	Kuala Selangor	3°25'10.92"	101° 13' 15.6"
14	B-03-01	Selangor	Sungai Choh, Rawang	Hulu Selangor	3°20' 45.6"	101° 35' 24"
15	B-03-02	Selangor	Batu 16, Kampong Melayu	Hulu Selangor	3°1815.839"	101° 35' 45.6"
16	B-04-01	Selangor	Kampong Sungai Buloh	Kuala Selangor	3°14' 44.16"	101° 28' 22.7994"
17	B-04-02	Selangor	Jalan Rahidin	Kuala Selangor	3°11' 6.3994"	101° 32' 56.4"
18	B-05-01	Selangor	Bangi Lama	Hulu Langat	2°54' 5.04"	101° 46' 40.8"
19	B-05-02	Selangor	Bangi Lama	Hulu Langat	2°54' 2.8794"	101° 46' 37.2"
20	B-05-05	Selangor	Pekan Beromang	Hulu Langat	2°5235.759"	101° 52' 22.8"
21	B-05-06	Selangor	Kampong Sungai Jai	Hulu Langat	2°52' 15.96"	101° 52' 55.2"
22	B-05-11	Selangor	Near Hulu Langat river	Hulu Langat	3°952.9194"	101° 50' 59.9994"
23	B-06-01	Selangor	Batu Laut, Banting	Kuala langat	2° 40' 23.52"	101° 31' 19.2"
24	B-06-02	Selangor	Banting	Kuala langat	2°40'22.439"	101° 31' 19.2"
25	B-06-03	Selangor	Taman Changang	Kuala langat	2°4945.479"	101° 37' 8.3994"

Pertanika J. Trop. Agric. Sci. 38 (1): 127 - 142 (2015)

131

TABLE 1 (*continued*)

Number	Accessions	Population	Origin	Area	Latitude	Longitude
26	D-01-01	Kelantan	PLT. Pasir Puteh	Pasir Puteh	5°49'38.639"	102° 22' 15.5994"
27	D-01-02	Kelantan	PLT. Pasir Puteh	Pasir Puteh	5°49'38.999"	102° 22' 15.5994"
28	D-01-03	Kelantan	PLT. Pasir Puteh	Pasir Puteh	5°49'38.639"	102° 22' 15.5994"
29	D-01-04	Kelantan	PLT. Pasir Puteh	Pasir Puteh	5°49'38.639"	102° 22' 15.5994"
30	D-01-05	Kelantan	PLT. Pasir Puteh	Pasir Puteh	5°49'38.28"	102° 22' 15.5994"
31	D-01-06	Kelantan	PLT. Pasir Puteh	Pasir Puteh	5°49'37.92"	102° 22' 15.5994"
32	D-01-07	Kelantan	Kampong Gong Tinggi	Pasir Puteh	5°48'12.96"	102° 28' 11.9994"
33	D-01-08	Kelantan	Kampong Tebing Tinggi	Pasir Puteh	5°49'33.599"	102° 26' 16.8"
34	D-01-09	Kelantan	Kampong Tok Bali	Pasir Puteh	5°54'28.8"	102° 27' 50.3994"
35	D-02-01	Kelantan	Jabatan Pertanian, Kota Bharu	Kota Bharu	6°6'6.8394"	102° 16' 1.1994"
36	D-02-02	Kelantan	Jabatan Pertanian, Kota Bharu	Kota Bharu	6°6' 6.8394"	102° 16' 1.1994"
37	D-03-01	Kelantan	Jambu Tawar	Machang	5°4'2'48.599"	102° 12' 39.5994"
38	T-01-01	Terengganu	Merang	Setiu	5°30'24.48"	102° 56' 16.8"
39	T-01-03	Terengganu	Merang	Setiu	5°30'24.48"	102° 56' 9.6"
40	T-01-04	Terengganu	Merang	Setiu	5°30'24.48"	102° 56' 6"
41	T-01-05	Terengganu	Merang	Setiu	5°30'25.199"	102° 56' 9.6"
42	T-01-06	Terengganu	Penarik	Setiu	5°28'14.519"	102° 48' 57.6"
43	T-01-08	Terengganu	Merang	Setiu	5°32'13.199"	102° 57' 39.5994"
44	T-01-09	Terengganu	Batu Rakit	Setiu	5°26'53.16"	103° 2' 59.9994"
45	T-01-10	Terengganu	Batu Rakit	Setiu	5°26'35.879"	103° 3' 21.5994"

132

Pertanika J. Trop. Agric. Sci. 38 (1) 127 - 142 (2015)

TABLE 2
Genetic divergence as revealed by ISSR profiling

Pop	N	Na	Ne	I	He	uHe	P (%)
Pop 1	24	2.000	1.687	0.564	0.385	0.394	100.00
Pop 2	13	1.981	1.699	0.572	0.393	0.408	98.08
Pop 3	8	1.827	1.637	0.521	0.357	0.381	90.38
Total mean		1.936	1.674	0.552	0.378	0.394	96.15
SE		0.027	0.024	0.014	0.011	0.012	2.94

Na = No. of Different Alleles; Ne = No. of Effective Alleles; I = Shannon's Information index. He =Expected Heterozygosity P= Percentage of Polymorphic Loci; Pop 1= Selangor, Pop 2= Kelantan and Pop 3= Terengganu.

TABLE 3
Analysis of Molecular variance of three *Jatropha curcas* populations

Source	d.f	SS	MS	Est. Var.	Variation (%)
Among Populations	2	20.611	10.305	0.003	0%
Within Populations	42	430.856	10.258	10.258	100%
Total	44	451.467		10.262	100%

accessions. The dendrogram classified the 45 accessions into seven distinct groups at the mean coefficient of 0.55. Cluster I had the highest number of accessions (35), followed by cluster VI with three members. Cluster III and V had two members each, while cluster II and VII had one member each respectively. Cluster I members were mainly accessions from Kelantan and Selangor, with only seven accessions from the Terengganu population were found to be in Cluster I.

Genetic Diversity and Analysis of Variance Components

From the analysis of variance (Table 4), it was observed that all the yield traits starting with seed yield per plant, seed yield per hectare, total number of seeds, percentage oil content and total oil yield per plant were highly significant. Additionally, Table 5 also shows the mean, coefficient of variation, range and standard deviation values. The highest coefficient of variation was in total number of seeds (53.19%), followed by oil yield per hectare (51%). Total number of seeds ranged from 31.4 to 1183.1, while minimum seed weight per plant was found to be 36.1 g and the maximum was 850.5 g.

Low differences of less than 10% were found between phenotypic and genotypic coefficient of variations (PCV and GCV), except for stem collar diameter, number of secondary and primary branches with differences of 22.04, 34.36 and 15.42 % respectively. Highest PVC was found in total number of seeds (49.39 %), followed by oil yield per hectare (47.94%) while seed length and seed width were found to have the lowest PVC. As for GVC, the highest was for total number of seeds (42.27 %) while the number of secondary branches
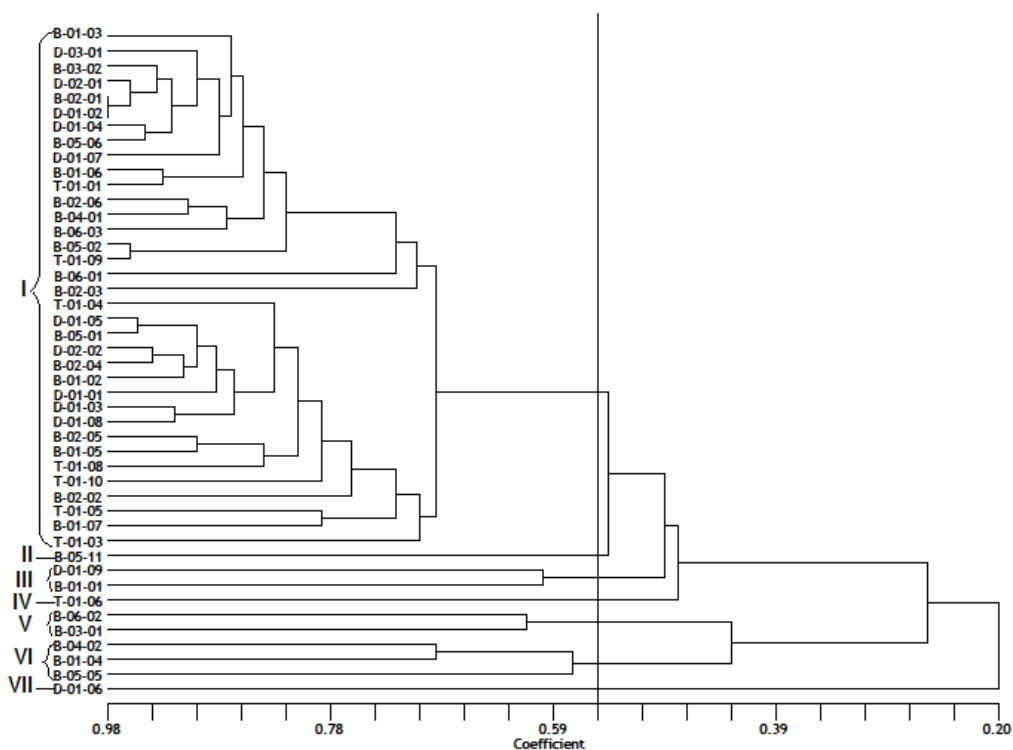
Fig.2: Cluster analysis from ISSR profiling of 45 *Jatropha curcas* accessions based UPGMA and dice similarity index

(0.00), seed length (0.71 %) and seed width (1.61 %) were seen to be the lowest. Broad sense heritability was generally high, with total number of seeds, seed weight per plant, total seed yield per hectare and percentage oil content at 73.25, 70.18, 70.18 and 67.81 % respectively. However, stem collar diameter, number of secondary branches and seed width showed the least amount of broad sense heritability.

From principal components analyses, as shown in Table 6, it was revealed that the first four principal components (PC1-PC4) accounted for 84.3% of the total variations observed in these accessions. PC1 contributed 39%, while PCs 2, 3

and 4 contributed 25.2, 10.9 and 9.3 % respectively. In PC1, the highest positive contribution was made by stem collar diameter followed by number of primary branches, while total number of seeds, seed yield per hectare, oil yield and seed yield per plant contributed negatively. Likewise in Pc 2, all the traits contributed positively except for seed width, seed length and percentage of oil content with 0.011, 0.098 and 0.211 as their positive contributions.

Genetic Distance and Dendrogram Based on Morphological Traits

The genetic distance among the 45 accessions are presented in Table 7. As indicated in the

TABLE 4
Mean squares of all the agro-morphological traits for 45 accessions of *J. curcas*

Source of variation	d.f	collar diameter	no. of Secondary branches	no. of primary branches	Seed width	Seed length	Seed yield/plant	plant height	Oil content	total no. of seed	seed yield/ha	Oil yield/ha
Blocks (b)	1	55373.44**	0.03^{ns}	1.74^{ns}	0.02^{ns}	1.10*	287404.21**	15020.28**	8.68**	569496.86**	782470.08**	69052.78**
Genotypes (g)	44	2165.84^{ns}	12.77^{ns}	1.13^{ns}	0.46^{ns}	0.302^{ns}	33610.95**	561.14^{ns}	8.40**	64823.51**	91505.89**	9038.18**
Error	44	3275.09	13.34	0.73	0.46	0.27	5889.04	536.47	1.61	10009.53	16033.24	1811.47

Note: * = significant at 0.05 level; **= significant at 0.01 level, ns= not significant

TABLE 5
Heritability and variance components of quantitative traits.

Variable	Mean	Std. Error	CV	Mini	Max	PCV	GCV	h$_B$
Stem collar diameter (mm)	150.15	6.07	38.33	49.60	343.73	34.74	12.69	0.00
No. Secondary branches	10.49	0.38	34.17	3.00	20.00	34.36	0.00	0.00
No. Primary branches	3.35	0.10	28.95	1.50	6.50	28.81	13.39	21.60
Seed width (mm)	11.44	0.07	5.91	5.85	12.40	5.94	1.61	0.46
Seed length (mm)	18.73	0.06	2.89	17.50	19.90	2.85	0.71	6.21
Seed weight (g)	295.90	15.90	50.98	36.11	851.50	47.49	39.79	70.18
Plant height (cm)	152.12	2.81	17.53	101.50	228.00	15.40	2.31	2.25
Oil content (%)	31.41	0.24	7.15	27.20	37.10	7.12	5.87	67.81
Total no. of seeds	391.64	21.96	53.19	31.39	1183.09	49.39	42.27	73.25
Seed yield/ha (kg)	488.24	26.24	50.98	59.58	1404.97	47.49	39.79	70.18
Oil yield/ha (kg)	153.63	8.26	51.00	20.61	431.33	47.94	39.13	66.61

Note: CV = Coefficient of variation (%); h$_B$ = Broad sense heritability (%); PCV = Phenotypic coefficient variation (%); GCV = Genotypic coefficient variation (%).

TABLE 6
Principal component analysis and percentage variation contributed by each of the component

Eigenvalue	PC1(%)	PC2(%)	PC3(%)	PC4(%)
Proportion	0.39	0.252	0.109	0.093
Cumulative	0.39	0.641	0.751	0.843
Stem collar diameter (mm)	0.27	-0.364	-0.008	-0.146
No. Secondary branches	0.161	-0.502	-0.027	0.13
No. Primary branches	0.225	-0.48	-0.028	0.072
Seed width (mm)	0.01	0.011	-0.573	-0.659
Seed length (mm)	0.027	0.098	-0.747	0.208
Seed weight (g)	-0.454	-0.201	-0.016	-0.039
Plant height (cm)	0.102	-0.424	-0.215	0.287
Oil content (%)	-0.12	0.211	-0.247	0.621
Total no. of seeds	-0.449	-0.205	0.021	-0.077
Seed yield/ha (kg)	-0.454	-0.201	-0.016	-0.039
Oil yield/ha (kg)	-0.46	-0.17	-0.055	0.045

Fig.3: Cluster analysis of 45 *Jatropha curcas* accessions based on 11 quantitative traits

TABLE 7
Dissimilarity matrix showing the Genetic Distance among the Accessions based on Quantitative traits

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	0.00																													
2	1.11	0.00																												
3	0.79	0.91	0.00																											
4	0.97	1.10	0.72	0.00																										
5	0.87	1.59	0.99	0.91	0.00																									
6	1.21	1.96	1.50	1.40	0.78	0.00																								
7	0.99	0.90	0.94	1.06	1.22	1.11	0.00																							
8	1.16	1.79	1.15	1.16	0.87	1.52	1.1	0.00																						
9	0.42	1.08	0.65	0.90	0.89	1.18	0.87	0.93	0.00																					
10	1.16	1.83	1.18	1.10	0.81	1.05	1.38	0.55	0.92	0.00																				
11	1.09	1.34	0.92	1.13	1.35	1.60	1.18	1.07	0.73	0.97	0.00																			
12	1.38	1.83	1.23	1.26	1.40	1.68	1.74	1.26	1.14	0.94	0.72	0.00																		
13	2.61	3.30	2.64	2.64	1.99	1.69	2.65	1.88	2.53	2.06	2.76	2.74	0.00																	
14	1.29	1.79	1.01	1.39	1.28	1.61	1.48	0.91	1.03	0.97	0.81	0.91	2.32	0.00																
15	0.57	1.27	0.66	0.93	0.64	1.04	0.85	0.77	0.52	0.95	1.06	1.36	2.17	0.99	0.00															
16	0.98	1.92	1.27	1.37	0.86	0.98	1.57	0.95	0.95	0.83	1.18	1.09	2.05	1.00	0.88	0.00														
17	0.75	1.17	0.68	0.73	0.88	1.15	0.96	0.87	0.43	0.75	0.56	0.88	2.47	0.97	0.70	0.96	0.00													
18	1.48	2.20	1.42	1.56	1.12	1.18	1.79	0.99	1.35	1.06	1.42	1.31	1.61	0.91	1.10	0.78	1.26	0.00												
19	1.84	2.62	1.81	1.94	1.34	1.27	2.14	1.22	1.71	1.23	1.81	1.66	1.25	1.26	1.45	1.08	1.64	0.57	0.00											
20	1.15	1.90	1.35	1.22	1.13	1.93	1.78	1.34	1.16	0.72	1.22	0.88	2.52	1.27	1.24	0.73	1.02	1.15	1.55	0.00										
21	0.96	1.58	0.80	1.13	0.85	1.13	1.30	0.93	0.87	1.06	1.18	1.32	1.99	0.84	0.56	0.88	0.91	0.76	1.14	1.21	0.00									
22	1.52	2.12	1.34	1.40	1.24	1.38	1.83	1.04	1.31	0.93	1.16	0.86	2.02	0.78	1.24	0.89	1.10	0.56	0.93	1.06	0.93	0.00								
23	0.83	1.55	0.93	1.13	0.95	1.33	1.51	1.24	0.80	0.99	0.97	0.84	2.58	1.00	0.93	0.68	0.78	1.18	1.54	0.70	0.97	1.06	0.00							
24	1.25	2.18	1.48	1.50	0.84	0.88	1.69	0.78	1.05	1.23	1.43	1.34	1.65	0.69	1.00	0.53	0.53	0.74	0.81	1.55	0.99	0.96	1.09	0.00						
25	1.34	1.57	0.97	1.37	1.51	1.89	1.28	1.04	1.25	0.93	0.87	1.27	2.64	1.00	1.09	1.48	1.08	1.46	1.77	1.14	1.11	1.33	1.39	1.55	0.00					
26	1.42	2.09	1.31	1.41	1.08	1.24	1.61	0.73	1.25	1.44	1.43	1.49	1.64	1.00	1.01	1.15	1.24	0.86	0.95	1.44	0.80	0.99	1.44	0.89	1.14	0.00				
27	2.13	2.77	1.95	2.17	1.73	1.76	2.27	1.34	1.92	1.92	1.83	1.74	1.52	1.17	1.69	1.46	1.83	0.88	0.65	1.89	1.36	1.06	1.82	1.23	1.61	1.05	0.00			
28	1.64	2.29	1.57	1.82	1.49	1.61	1.87	1.07	1.38	1.38	1.12	1.02	2.18	0.73	1.39	0.99	1.27	0.99	1.11	1.38	1.29	0.88	1.21	1.02	1.23	1.24	1.02	0.00		
29	1.41	2.17	1.43	1.57	1.22	1.48	1.73	0.87	1.25	0.82	1.14	0.98	2.11	0.71	1.17	0.72	1.17	0.82	1.06	1.07	1.13	0.77	1.02	0.75	1.27	1.12	1.14	0.56	0.00	
30	1.16	1.89	1.10	1.36	0.98	1.21	1.41	0.60	0.98	0.81	1.10	1.21	1.84	0.58	0.75	0.77	1.00	0.62	0.90	1.26	0.59	0.79	1.08	0.71	0.94	0.57	1.00	0.86	0.70	0.00
31	1.43	2.00	1.46	1.75	1.52	1.64	1.87	1.54	1.20	1.37	1.15	1.01	2.62	1.08	1.39	0.91	1.21	1.18	1.53	1.03	1.31	1.09	0.84	1.34	1.65	1.76	1.70	1.01	1.04	1.28
32	2.16	2.66	2.07	2.10	1.82	1.93	2.55	1.97	1.99	1.47	2.11	1.92	1.51	2.06	2.09	2.00	1.92	2.13	1.97	2.18	1.38	1.98	1.90	1.84	2.20	1.81	1.89	1.95	2.09	1.97
33	1.94	2.70	1.89	2.11	1.67	1.95	2.24	1.30	1.77	1.39	1.73	1.61	1.67	1.07	1.58	1.22	1.74	0.74	0.66	1.65	0.99	0.95	1.64	1.05	1.55	0.99	0.50	0.95	0.96	0.86
34	1.20	2.02	1.37	1.51	0.95	0.99	1.60	0.90	1.03	0.68	1.15	1.10	1.97	1.00	1.02	0.50	0.98	0.90	1.03	1.06	1.05	0.96	0.87	0.57	1.47	1.16	1.32	0.74	0.72	0.81
35	1.33	2.05	1.24	1.18	0.99	1.34	1.69	0.79	1.20	0.73	1.28	1.08	2.01	0.93	1.07	0.96	1.10	0.87	1.10	1.07	0.93	0.74	1.13	0.81	1.21	0.67	1.28	1.16	0.85	0.73
36	1.72	2.35	1.36	1.55	1.14	1.15	1.89	1.35	1.74	1.47	2.17	2.19	1.51	1.95	1.41	1.63	1.73	1.50	1.49	1.87	1.38	1.72	1.93	1.32	2.06	1.12	1.89	2.15	1.89	1.48
37	1.35	2.05	1.72	1.35	1.18	1.29	1.78	1.12	1.24	1.00	1.17	0.88	2.15	0.96	1.19	0.70	1.05	0.67	1.13	1.13	0.99	0.46	0.93	0.92	1.49	1.20	1.39	1.07	0.81	0.92
38	1.71	2.49	1.72	1.78	1.46	1.67	2.19	1.31	1.57	1.03	1.41	0.92	2.29	1.07	1.57	0.92	1.43	1.02	1.15	1.01	1.43	0.79	1.10	0.97	1.64	1.38	1.29	0.78	0.61	1.12
39	1.40	2.10	1.24	1.40	1.13	1.48	1.77	0.96	1.29	0.99	1.31	1.12	1.99	0.69	1.07	0.87	1.22	0.58	0.92	1.10	0.78	0.59	1.06	0.86	1.19	0.81	1.03	0.99	0.68	0.58
40	1.35	2.00	1.24	1.25	0.86	1.04	1.48	0.51	1.15	0.61	1.23	1.21	1.62	0.87	0.92	0.87	1.00	0.61	0.80	1.22	0.80	0.69	1.19	0.64	1.23	0.59	1.01	0.97	0.79	0.53
41	1.27	1.76	1.00	0.99	1.12	1.53	1.44	0.78	1.08	0.87	1.06	1.07	2.26	0.80	0.99	1.19	0.97	1.08	1.41	1.25	0.90	0.89	1.21	1.15	0.84	0.74	1.45	1.28	1.04	0.77
42	2.77	3.15	2.62	2.66	2.23	2.05	2.59	2.18	2.68	2.45	3.57	3.13	1.22	2.60	2.29	2.60	2.66	2.13	1.93	3.02	2.12	2.47	2.95	2.27	2.67	1.84	2.06	2.74	2.70	2.17
43	1.43	2.15	1.44	1.39	0.89	0.94	1.55	0.56	1.28	0.76	1.50	1.53	1.40	1.19	1.01	1.02	1.21	0.83	0.87	1.43	0.97	1.03	1.44	0.64	1.43	0.53	1.15	1.25	1.05	0.71
44	1.08	1.63	0.99	1.03	0.95	1.29	1.09	0.33	0.83	0.62	0.95	1.19	2.13	0.85	0.75	1.08	0.81	1.16	1.42	1.37	0.93	1.11	1.21	0.98	0.80	0.74	1.48	1.17	0.98	0.67
45	1.19	1.98	1.33	1.16	1.04	1.28	1.61	0.81	1.11	0.81	1.30	1.21	2.13	1.16	1.05	0.97	1.09	1.10	1.37	1.04	1.06	1.02	1.21	0.86	1.31	0.84	1.60	1.38	1.03	0.89

Pertanika J. Trop. Agric. Sci. 38 (1): 127 - 142 (2015)

137

TABLE 7 (*continued*)

Genotypes	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
31	0.00														
32	2.34	0.00													
33	1.53	2.20	0.00												
34	0.96	1.75	1.21	0.00											
35	1.55	1.79	1.13	1.08	0.00										
36	2.41	2.10	1.87	1.73	1.31	0.00									
37	0.97	2.19	1.17	0.96	0.86	1.75	0.00								
38	1.11	1.91	1.09	0.92	0.96	2.06	0.84	0.00							
39	1.28	2.03	0.85	1.04	0.54	1.57	0.74	0.86	0.00						
40	1.42	1.86	1.02	0.82	0.62	1.24	0.87	1.09	0.69	0.00					
41	1.63	1.98	1.34	1.30	0.50	1.49	1.03	1.28	0.73	0.79	0.00				
42	3.14	`2.85	2.28	2.54	2.37	1.56	2.66	2.96	2.39	2.01	2.41	0.00			
43	1.73	1.89	1.13	1.00	0.75	0.96	1.14	1.34	0.94	0.41	0.95	1.80	0.00		
44	1.62	1.92	1.42	1.06	0.76	1.44	1.21	1.38	0.98	0.70	0.59	2.32	0.78	0.00	
45	1.68	1.98	1.37	1.17	0.49	1.28	0.96	1.20	0.89	0.85	0.67	2.49	0.84	0.76	0.00

table, the highest dissimilarity (3.57) was found between accession 42 (T-01-06) and four other accessions (11 (B-02-04), 38 (T-01-01), 23 (B-06-01) and 32 (D-01-07)) at 3.57, 2.96, 2.95 and 2.85, respectively. However, the least genetic distance was 0.33, observed between accessions 44 (T-01-09) and 8 (B-02-01), followed by 0.41 between accessions 43 (T-01-08) and 40 (T-01-04), and 0.42 between accessions 9 (b-02-02) and 1 (b-01-03).

Additionally, a dendrogram constructed using 11 quantitative traits grouped the 45 accessions into five clusters at mean coefficient of 1.32 (Fig.3). Cluster III had the largest number of members (29) followed by Cluster I (10), while Clusters II, V and IV had 4, 2 and 1 accessions respectively. Cluster I and II mainly comprised accessions from the Selangor population, except for D-02-02, which is from the Kelantan population.

DISCUSSION

Genetic diversity study of germplasm resources is considered to be an essential activity undertaken before the commencement of any plant breeding programme. Presence of genetic diversity in a genetic resource helps in breeding and crop improvement programmes by helping to ensure the presence of trait variability for selection of individuals containing desirable traits such as high yield and resistance to various biotic and abiotic stresses in the environment. Germplasm exploration and evaluation of the *Jatropha curcas* are essential to widen the narrow genetic base of this crop. The full economic potential of this crop cannot be realised until high yielding materials are identified and obtained through long-term evaluation and selection.

In this study, molecular markers were combined with morphological markers to obtain finger-printing and genetic relationship information among the accessions. The presence of high amounts of

polymorphism (ranging from 90.38-100%) in the three populations studied suggests that the genetic dissimilarity among the accessions is very high. This makes them promising materials for hybridisation. This observation is in agreement with Tanya *et al.* (2011), who made similar observation while studying the genetic variation among 30 Jatropha accessions in Thailand using ISSR markers. It was reported that polymorphism among the individual populations was very high.

Additionally, genetic differentiation through analysis of molecular variance is necessary to reveal the allelic pattern for an in-depth understanding of the population structure. As seen in this study, virtually all the variations (100%) observed in the populations were due to variations within the three populations. This implies that higher differences were present within the accession from the same population. Variation among the population will be more than variation within the population if difference species are involved in the study. High genetic fixation within populations of Jatropha and its related species has been reported in several studies (Kumar *et al.*, 2011a; Barboza *et al.*, 2012; Biabani *et al.*, 2013). Santos *et al.* (2010) reported that genetic variation among 50 Jatropha plants studied was 72.47 % when profiling the Jatropha germplasm with Amplified fragment length polymorphic markers. This occurs because the genotypic composition or genetic make-up of individual plants differ and this will result in higher variations within a population rather than among the populations.

The clustering patterns as presented in the dendrogram, of both morphological and ISSR, showed that the majority of the plants were clustered in the first group. This implies that the accessions are likely to have a similar genetic background. This observation is in agreement with Sudheer *et al.* (2010), who observed that more than 50% of the Jatropha accessions were clustered into one major group. The presence of a large number of accessions of Jatropha from the same population shows that ISSR is capable in finger-printing and identifying plant populations from diverse or similar genetic backgrounds. This observation on ISSR markers' discriminating ability is also seen in the findings reported in many studies (Tanya *et al.*, 2011; Singh *et al.*, 2012; Xu *et al.*, 2012).

The presence of substantial variability as depicted by high coefficients of variation and range indicates that these accessions are genetically diverse in terms of their yield and other trait potentials. This finding complements the results from the molecular finger-printing using ISSR. This variability could also be a reflection of the wild nature of the accessions. These materials were collected from the wild where the plants were growing in their natural populations with a natural or random mating system. This finding is in line with the observations and conclusions on other wild populations of Jatropha studied in countries like China, Brazil, India and Thailand (Grativol *et al.*, 2011; Kumar *et al.*, 2011; Shen *et al.*, 2012). Wani *et al.* (2012) in his study also observed substantial morphological variations in the Jatropha plant's vegetative traits such

as plant height, number of branches and yield traits, such as seed yield per plant, percentage oil content and 100 seeds' weight. The large genetic distance (> 3.5) displayed by some of the accessions suggest that these populations of Jatropha can be successfully introgressed into breeding programmes to enrich and widen their genetic base.

Furthermore, low differences observed between phenotypic and genotypic coefficients of variation for morphological traits suggest that the influence of environmental factors on the expressions of these traits is low. High broad-sense heritability (> 60%) in most of the yield traits further affirms this claim. Broad sense heritability is the proportion of variation which can be inherited by the offspring (Acquaah, 2007). The magnitude of this parameter affects the response to selection in breeding and crop improvement programmes. Higher broad sense heritability in economically important traits is of significant importance to the breeders, as it helps to increase the pace at which progress is made through selection (Bhargava *et al.*, 2007).

Based on all the information obtained from this study, it can be concluded that morphological markers complemented with ISSR markers are suitable for profiling and depicting the genetic diversity of the Jatropha population. The results also show that these populations contain sufficiently divergent materials suitable for introgression into existing breeding programmes. Based on all the results obtained, accessions B-01-03, D-01-06, T-01-06 and B-06-

02 are identified and recommended for further evaluation under field conditions before selection or chosen for use in future breeding programmes for seed yield and oil improvement.

REFERENCES

Acquaah, G. (2007). *Principles of Plant Genetics and Breeding* (p. 569). Blackwell Publishing Ltd.

Allard, R. W. (1960). *Principles of Plant Breeding*. New York: John Wiley and Sons, New York.

Banković-Ilić, I. B., Stamenković, O. S., & Veljković, V. B. (2012). Biodiesel production from non-edible plant oils. *Renewable and Sustainable Energy Reviews*, *16*(6), 3621–3647.

Barboza, N., Albertazzi, F. J., Sibaja-Cordero, J. a., Mora-Umaña, F., Astorga, C., & Ramírez, P. (2012). Analysis of genetic diversity of *Cucurbita moschata* (D.) germplasm accessions from Mesoamerica revealed by PCR SSCP and chloroplast sequence data. *Scientia Horticulturae*, *134*, 60–71.

Bhargava, A., Shukla, S., & Ohri, D. (2007). Genetic variability and interrelationship among various morphological and quality traits in quinoa (*Chenopodium quinoa* Willd.). *Field Crops Research*, *101*(1), 104–116.

Biabani, A., Rafii, M. Y., Saleh, G. B., & Latif, A. (2013). Inter- and intra-population genetic variations in *Jatropha curcas* populations revealed by inter-simple sequence repeat molecular markers. *Maydica, 58*, 111– 118.

Chauhan, B. S., Kumar, N., & Cho, H. M. (2012). A study on the performance and emission of a diesel engine fueled with Jatropha biodiesel oil and its blends. *Energy*, *37*(1), 616–622.

Doyle, J. J. & Doyle, J. L. 1990. A rapid total DNA preparation procedure for fresh plant tissue. *Focus* 12:13-15.

Grativol, C., Da Fonseca Lira-Medeiros, C., Hemerly, A. S., & Ferreira, P. C. G. (2011). High efficiency and reliability of inter-simple sequence repeats (ISSR) markers for evaluation of genetic diversity in Brazilian cultivated Jatropha curcas L. accessions. *Molecular Biology Reports*, *38*(7), 4245–56.

Gupta, S., Srivastava M., Mishra, G., Naik P, Chauhan, R., Tiwari, S., Kumar, M., & Singh, R. (2010) Analogy of ISSR and RAPD markers for comparative analysis of genetic diversity among different Jatropha curcas genotypes. *African Journal of Biotechnology*, *7*(23), 4230-4243

Henning, R. (2004). *Jatropha curcas L. in Africa*. http://www.jatropha de/documents/jci manual. pdf8/3/2004 accessed on 11 July, 2012.

Kang, M. S. (1998). Genotype-by-environment interaction for crop development. *Advances in Agronomy*, *62*, 199– 252.

Kristensen, T., Mogensen, L., Knudsen, M. T., & Hermansen, J. E. (2011). Effect of production system and farming strategy on greenhouse gas emissions from commercial dairy farms in a life cycle approach. *Livestock Science*, *140*(1-3), 136–148.

Kumar, S., Kumaria, S., Sharma, S. K., Rao, S. R., & Tandon, P. (2011a). Genetic diversity assessment of *Jatropha curcas* L. germplasm from Northeast India. *Biomass and Bioenergy*, *35*, 3063–3070.

Kumar, S., Kumaria, S., Sharma, S. K., Rao, S. R., & Tandon, P. (2011b). Genetic diversity assessment of *Jatropha curcas* L. germplasm from Northeast India. *Biomass and Bioenergy*, *35*, 3063–3070.

Murty, S. G., Patel, F., Punwar, B. S., Patel, M., Singh, A. S., & Fougat, R. S. (2013). Comparison of RAPD, ISSR, and DAMD markers for genetic diversity assessment between accessions of *Jatropha curcas* L. and its related species. *Journal of Agricultural Biotechnology and Sustainable Developmentcultural Sceince and Technology*, *15*, 1007–1022.

Peakall, R., & Smouse, P. E. (2006). Genalex 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, *6*(1), 288–295.

Peakall, R., & Smouse, P. E. (2012). GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research--an update. *Bioinformatics (Oxford, England)*, *28*(19), 2537–9.

Rohlf F. J. (1997). NTSYSpc: *Numerical taxonomy and multivariate analysis system, version 2.02*. Setauket (New York): Exeter Publishing.

Santos, C. A. F., Drumond, M. A., Rodrigues, M. A., & Evangelista, M. R. V. (2010). Genetic similarity of *Jatropha curcas* accessions based on AFLP markers. *Crop Breeding and Applied Biotechnology (Online)*, *10*(4), 364–369.

Shabanimofrad, M., Rafii, M. Y., Megat Wahab, P. E., Biabani, A. R., & Latif, M. A. (2013). Phenotypic, genotypic and genetic divergence found in 48 newly collected Malaysian accessions of *Jatropha curcas* L. *Industrial Crops and Products*, *42*, 543–551.

Shen, J., Pinyopusarerk, K., Bush, D., & Chen, X. (2012). AFLP-based molecular characterization of 63 populations of *Jatropha curcas* L. grown in provenance trials in China and Vietnam. *Biomass and Bioenergy*, *37*, 265–274.

Singh, S., Panda, M. K., & Nayak, S. (2012). Evaluation of genetic diversity in turmeric (*Curcuma longa* L.) using RAPD and ISSR markers. *Industrial Crops and Products*, *37*(1), 284–291.

Sudheer, P. D. V. N., Rahman, H., Mastan, S. G., & Reddy, M. P. (2010). Isolation of novel microsatellites using FIASCO by dual probe enrichment from *Jatropha curcas* L. and study on genetic equilibrium and diversity of Indian population revealed by isolated microsatellites. *Molecular Biology Reports*, *37*(8), 3785–93.

Sunil, N., Sujatha, M., Kumar, V., Vanaja, M., Basha, S. D., & Varaprasad, K. S. (2011). Correlating the phenotypic and molecular diversity in *Jatropha curcas* L. *Biomass and Bioenergy*, *35*(3), 1085–1096.

Tanya, P., Dachapak, S., Tar, M. M., & Srinives, P. (2011a). New microsatellite markers classifying nontoxic and toxic *Jatropha curcas. Journal of Genetics, 90*(3), 76–78.

Tanya, P., Taeprayoon, P., Hadkam, Y., & Srinives, P. (2011b). Genetic diversity among Jatropha and Jatropha-related species based on ISSR markers. *Plant Molecular Biology Reporter*, *29*(1), 252–264.

Wani, T. A., Kitchlu, S., & Ram, G. (2012). Genetic variability studies for morphological and qualitative attributes among *Jatropha curcas* L. accessions grown under subtropical conditions of North India. *South African Journal of Botany*, *79*(0), 102–105.

Xu, G. H., Su, W. Y., Shu, Y. J., Cong, W. W., Wu, L., & Guo, C. H. (2012). RAPD and ISSR-assisted identification and development of three new SCAR markers specific for the *Thinopyrum elongatum* E (Poaceae) genome. *Genetics and Molecular Research: GMR*, *11*(2), 1741–51.

Effects of Over-Expressing Ethylene Responsive Transcription Factor on Expression of Selected Fruit Ripening-Related Genes in Oil Palm (*Elaeis guineensis* Jacq.) Mesocarp

Nur Annies Abd Hadi[1], Siti Nor Akmar Abdullah[1,2]*,
Azzreena Mohamad Azzeme[1,3], Ahmed Al-Shanfari[1] and Halimi Mohd Saud[1]

[1]*Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia,*
43400 UPM Serdang, Selangor, Malaysia
[2]*Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia,*
43400 UPM Serdang, Selangor, Malaysia
[3]*Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences,*
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

ABSTRACT

Gene expression is regulated mainly at the transcriptional level through binding of transcription factors to specific promoter regulatory elements. The expression profile of *EgDREB*, a transcription factor belonging to the AP2 family, was determined by reverse transcriptase (RT)-PCR and it was found that it was expressed at different stages of oil palm mesocarp development as well as in vegetative tissues (roots and leaves) but not in the mesocarp at the early ripening stage, which is 12 WAA (weeks after anthesis). Thus, the effects of over-expressing *EgDREB* on the transcriptional regulation of genes from five functional groups related to ripening were investigated in 12 WAA mesocarp of oil palm. Co-bombardment of 12 WAA mesocarp tissues with recombinant vector construct harbouring *EgDREB* and plasmid containing the GFP reporter gene was carried out. Fluorescent detection of GFP and verification via RT-PCR using GFP-specific primers enabled selection of successfully transformed tissues. Using transient expression assay, it was demonstrated that over-expression of *EgDREB* results in up-regulation of translationally controlled tumor protein (*TCTP*) and type 2 metallothionein-like genes (*MET2a* and *MET2b*). These proteins are categorised under biogenesis of the cellular component and proteins with binding functions or cofactor requirements. More specifically, the roles of metallothioneins are in homeostasis of essential metal ions and oxidative stress response. This may suggest that *EgDREB*

is involved in regulating cellular processes related to the roles of these three proteins.

Keywords: Elaeis guineensis Jacq., fruit ripening, microprojectile bombardment, transcription factor, transient expression assay

INTRODUCTION

The oil palm (*Elaeis guineensis* Jacq.) is the most important industrial crop in Malaysia. It is an economically important plant species that provides the world's number one source of edible vegetable oil (Tranbarger *et al.*, 2012). It is a monocotyledonous perennial tree which can produce fruit bunches after two and a half years with good agronomic practices while 20-22 weeks are required from anthesis to fruit maturity. Palm oil and the palm kernel oil are extracted from the fruit mesocarp and nut, respectively (Hartley, 1988).

Generally, fruit development, maturation and ripening are important biological processes unique to plants. The molecular basis of oil palm fruit development, maturation and ripening has, however, received less attention. A series of physical and chemical changes occurs in ripening fruits of oil palm (Prada *et al.*, 2011) where colour development is one of the most significant criteria related to fruit maturity (Alfatni *et al.*, 2008). Differential expression of various genes that are tissue-specific and developmentally regulated genes lead to the occurrence of several physiological and biochemical changes during the fruit ripening stage. Various changes take place in the oil palm mesocarp during the late

period of fruit ripening, including cell wall expansion and oil accumulation as well as carotenoids, vitamin E and ethylene production (Bapat *et al.*, 2010).

Understanding the biological and physiological mechanisms at the molecular levels during the ripening stage of the oil palm fruit will help enhance specific characteristics to improve production and quality of the oil. Al-Shanfari *et al.,* (2012) took an important step in unravelling these mechanisms by characterising the structure and function of genes expressed during the late ripening period of the oil palm fruits. Suppression subtractive hybridisation technique successfully identified twenty unigenes encoding abundant transcripts expressed in the mesocarp at the late fruit ripening stage. Based on gene ontology classifications, the genes were grouped under cellular component biogenesis; cell rescue, defence and virulence; protein with binding function or cofactor requirement; metabolism; cell cycle and DNA processing.

Various approaches have been carried out to unveil the complexity of gene expression and interaction in plants (Low *et al.,* 2008). Understanding the mechanisms of transcriptional regulation underlying gene expression in its entirety is essential. One of the most important mechanisms of gene expression regulation occurs at the transcriptional level through binding of transcription factors to specific promoter motif of genes whose expression they regulate (Laurila & Lahdesmaki, 2009). Transcription appears to be controlled by various transcription factors that mediate

the effects of intracellular and extracellular signals. The transcription factors are responsible for enhancing or suppressing the transcription of specific genes through a complex regulatory network (Phillips & Hoopes, 2008).

The AP2/ERF transcription factors are widely found in both monocot and dicot plant species and are reported to be important in regulating plant development and responses to biotic and abiotic stresses (Nakano *et al.*, 2006). They can be divided into subfamilies with diverse roles (Saleh & Montserrat, 2003). The AP2 family members of transcription factors are distinguished by the presence of the AP2-DNA binding domain specific to plants (Licausi *et al.*, 2010). The AP2 domain sequence comprises approximately 70 amino acids that have a high level of amino acid conservation among its members. DREB is a sub-family of the AP2/ERF superfamily of transcription factors. These genes are involved in regulation of abiotic stress responses, including response to low temperature, dehydration and salt stress through induction of expression of stress response genes (Wang *et al.*, 2011).

From a recent study, a transcription factor belonging to the AP2 family designated as *EgDREB* (Genbank accession no. ABF59742), which is highly expressed in ripening oil palm fruit mesocarp, was isolated and sequenced. In the present study, further characterisation of this transcription factor was conducted in order to identify genes whose expression is regulated by this transcription factor in the mesocarp tissue.

This was achieved through co-bombardment of the mesocarp tissue at an early stage of the ripening period with the transcription factor and GFP reporter gene followed by analysis of the effects on the expression of selected groups of the oil palm ripening-related genes (Al-Shanfari *et al.*, 2012) in GFP positive-bombarded tissues.

MATERIALS AND METHODS

Plant Materials

Oil palm (*Elaeis guineensis* Jacq.) variety Tenera (*Dura × Pisifera*) fruits at different stages of development (7, 10, 12, 15, 17 and 19 WAA), roots and young leaves from 5-month-old polybag-grown seedlings were used in the present study. The oil palm fruits were obtained from the Malaysian Palm Oil Board Research Station, Bangi, Selangor, while the 5-month-old seedlings grown in polybags were obtained from Sime Darby, Banting, Selangor. The samples were sterilised in 20% (v/v) Clorox and followed by thorough rinsing with distilled water. The exocarp was removed and the mesocarp section of the fruits was excised and immediately frozen in liquid nitrogen and stored at −80°C.

Plasmid

pMDC32 plasmid carrying the 35S promoter of Cauliflower Mosaic Virus (CaMV35S) (ABRC) and pMDC32-EgDREB plasmid carrying oil palm ethylene responsive transcription factor gene (*EgDREB*) fused to CaMV35S promoter, constructed by Azzreena Mohamad Azzeme (Institute

of Tropical Agriculture, Universiti Putra Malaysia) were used. [Fig.1 (a)] and 35SpEGFP plasmid carrying GFP reporter gene, fused to CaMV35S promoter (Clontech) [Fig.1 (b)]. The 35SpEGFP plasmid was constructed by inserting an 800 bp *Hin*d III – *Sma*I fragment containing the CaMV35S promoter into the multiple cloning site of pEGFP-1 (Clontech). The plasmids were purified using the QIAprep® Spin Miniprep Kit (Qiagen).

Preparation of Target Tissue for Bombardment

Preparation of the target tissue for bombardment was carried out based on the procedure described by Agius *et al.* (2005). The oil palm fruit bunches at 12 WAA were obtained from University Agriculture Park, Universiti Putra Malaysia. The fruits were surface sterilised in Clorox (20%), then rinsed in sterile distilled water. The mesocarp was excised to produce 1 cm × 1 cm slices. The mesocarp tissue slices were subsequently placed in the middle within a diameter of about 3 cm in petri dishes filled with solid media (Murashige and Skoog 1962). They were kept at 28° 1 day before being used for bombardment.

Preparation of DNA-microcarrier

Gold microcarriers were prepared according to the manufacturer's (Bio-Rad) instructions. Gold microcarriers (60 mg of 1 µm diameter) were re-suspended in 100% ethanol (1 ml) by vigorous vortexing for 2 minutes followed by centrifugation at 10,000 rpm for 1 minute. The supernatant was removed and the pellet was washed with sterile distilled water and centrifuged at 10,000 rpm for 1 minute and the supernatant discarded. The recovered pellet was washed again with sterile distilled water and centrifuged at 10,000 rpm for 1 minute and the supernatant discarded. The microcarriers were re-suspended in 1 ml of sterile distilled water and kept at 4°C.

The oil palm mesocarp tissue slices were co-bombarded with pMDC32-EgDREB and 35SpEGFP. Into an aliquot of 50 µl of microcarriers, 2.5 µl of each plasmid DNA (1 µg/µl) was added followed by 50 µl of 2.5 M $CaCl_2$ and then 20 µl of 0.1 M spermidine, with continuous vortexing. An additional 3 minutes of vortexing was performed followed by recovery of the DNA microcarriers by centrifugation at 10,000 rpm for 10 seconds. After discarding
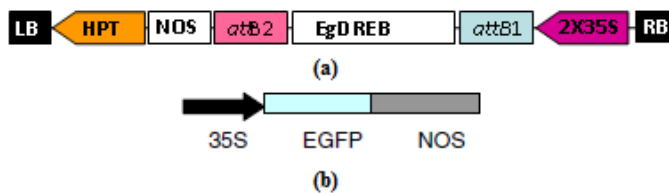
Fig.1: The schematic representation of pMDC32-EgDREB (a), and 35SpEGFP (b) used in this study

146

Pertanika J. Trop. Agric. Sci. 38 (1) 143 - 159 (2015)

the supernatant, the pellet was washed 2X with 250 µl of 100% ethanol and finally re-suspended in 60 µl of 100% ethanol.

Biolistic Bombardment

Biolistic transformation was carried out using PDS-1000/He™ System (Bio-Rad) by co-bombardment of pMDC32-EgDREB and 35SpEGFP plasmids into the mesocarp tissue. For particle bombardment experiments, samples were arranged in a completely randomised design with three independent bombardments (replicates) which were 5 samples per replicate. The controls included unbombarded tissues and tissues bombarded with empty vector (pMDC32), which was plasmid without the *EgDREB* gene.

Each co-bombardment involved 10 µl of DNA-coated microcarriers loaded onto the centre of macrocarrier. The optimised parameters for transient transformation of oil palm tissues as described by Ramli and Abdullah (2003) were followed. The distances between the rupture disk and macrocarrier, macrocarrier and stopping screen, and stopping screen to target were 6 mm, 11 mm and 6 cm, respectively. The rupture disks of 1,550 psi and vacuum pressure of 27 mmHg pressure were used for the bombardment. The rupture disk, macrocarrier and stopping screen were soaked in 70% ethanol for 30 minutes prior to bombardment. The bombarded tissues were kept at 28°C for 48 hours prior to reporter gene assay.

Green Fluorescent Protein Assay

GFP analysis of the mesocarp tissue slices was carried out at 12 hours and 2 days after co-bombardment. The GFP spots were detected 2 days after co-bombardment, using Nikon SMZ1000 microscope equipped with UV source and GFP filter (excitation at 360 to 480 nm and emission at 480 to 500 nm). The transiently transformed tissues expressing GFP were used for RT-PCR analysis. The total RNA from the unbombarded and bombarded mesocarp tissue slices was extracted according to the modified method by Prescott and Martin (1987).

Semi-quantitative RT-PCR

A semi-quantitative RT-PCR was performed to measure the effects of over-expressing *EgDREB* in 12 WAA mesocarp tissues on the expression of fruit ripening-related cDNAs, which had been divided into 5 groups based on gene ontology classification (Table 1). For greater reliability of the results, the RT-PCR was performed in triplicate. The representative gel electrophoresis results of the RT-PCR products were used for product quantification.

The gene-specific primers used were from Al-Shanfari *et al.* (2012). As an internal standard, the GAPDH housekeeping gene was used. Initial standardisation of the PCR using different cycles ranging from 23 to 38 revealed that the 28 cycles provided the optimal result within the exponential range for product quantification for all expressed cDNAs. Hence, 28 cycles were

subsequently used in all of the quantification assays. RT-PCR analysis of *EgDREB* and GFP reporter genes was also carried out in order to provide internal and positive controls, respectively.

QIAGEN®OneStep RT-PCR Kit (Qiagen) was used for RT-PCR. The reactions were set up separately for each gene in a 50 µl reaction mixture, containing 10 µl of 5X QIAGEN OneStep RT-PCR Buffer, 2 µl of 25 mM dNTP mix, 3 µl each 0.6 µM gene-specific forward and reverse primers, 2 µl of QIAGEN OneStep RT-PCR Enzyme Mix, 1 µl of total RNA (1 µg/µl) and 29 µl of RNase-free water. The RT-PCR cycling was as follows: 30 minutes at 50°C for reverse transcription, initial PCR activation step for 15 minutes at 95°C, then 28 cycles of denaturation, annealing and extension steps for 1 minute at 94°C, 1 minute at 51 to 64.2°C (based on T_m values of the primers) and 1 minute at 72°C, respectively. This was followed by a final extension step for 10 minutes at 72°C. Electrophoresis analysis was performed using 1.2% agarose gel at 80 V for 1 hour and 20 minutes. RT-PCR analysis was also carried out for the *EgDREB* gene in fruit (mesocarp) at different stages of development and young leaves and roots from 5-month-old polybag-grown oil palm seedlings in order to check for the expression profile of the gene.

Quantification of Gene Expression

The AlphaEaseFCTM software (version 4.0.0; Alpha Inotech Corporation, San Leandro, CA, USA) was used to quantify the RT-PCR bands. The relative expression level of each target cDNA was represented by the band intensity ratio of the target cDNA/GAPDH. The fold expression was represented by cDNA relative expression level ratio of bombarded mesocarp tissue with pMDC32-EgDREB/unbombarded mesocarp tissue and bombarded mesocarp tissue with pMDC32/unbombarded mesocarp tissue. Only the band intensity that was ≥1.5-fold was considered significant.

Sequence Analysis

Sequence analysis was carried out in order to confirm the identity of the RT-PCR products. The RT-PCR bands were purified using QIAquick Gel Extraction Kit (Qiagen) and sent for sequencing. The obtained sequences were analysed using the workbench software (http://workbench.sdsc.edu).

RESULTS

RT-PCR Analysis of EgDREB Gene in Various Oil Palm Tissues

As shown in Fig.2 (a), RT-PCR analysis of the *EgDREB* gene at different stages of fruit (mesocarp) development (7, 10, 12, 15, 17 and 19 WAA) showed the expression of the *EgDREB* gene at all stages of fruit (mesocarp) development except for the 12 WAA fruit mesocarp tissues. The expected size of the *EgDREB* cDNA based on the primers was 700 bp. However, no expression of *EgDREB* gene was observed in the mesocarp of fruits at 12 WAA, suggesting that there is no endogenous DNA binding activity of the *EgDREB* gene

in unbombarded 12 WAA mesocarp tissues. As shown in Fig.2 (b), RT-PCR analysis of the *EgDREB* gene in the leaf and root tissues showed expression of the *EgDREB* gene in the vegetative tissues.

Green Fluorescent Protein Assay

Fig.3 (a) illustrates the transient expression of the GFP reporter gene in the mesocarp tissue slices 2 days after co-bombardment with pMDC32 and 35SpEGFP plasmids, whereas Fig.3 (b) illustrates the transient expression of the GFP reporter gene in the mesocarp tissue slices 2 days after co-bombardment with pMDC32-EgDREB and 35SpEGFP plasmids. However, no transient expression of the GFP reporter gene was detected in the unbombarded mesocarp tissue slices [Fig.3 (c)].
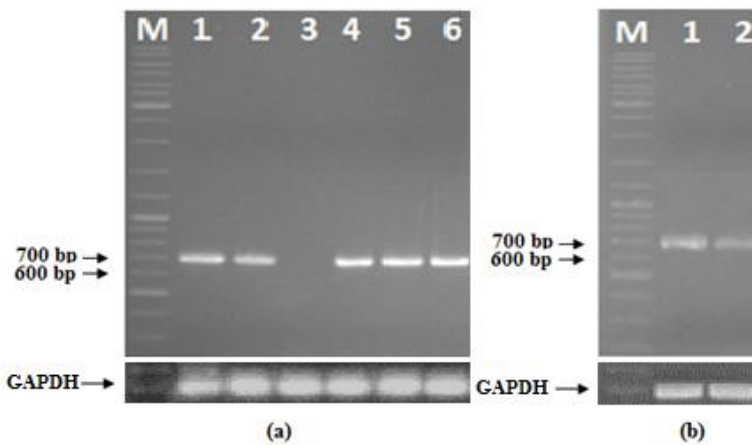
Fig.2: RT-PCR analysis of *EgDREB* gene in different stages of fruit (mesocarp) development. M: DNA Ladder Mix. Lanes 1, 2, 3, 4, 5 and 6 representing mesocarp at 7 WAA, 10 WAA, 12 WAA, 15 WAA, 17 WAA and 19 WAA, respectively(a), and vegetative tissues. M: DNA Ladder Mix. Lane 1 and 2 represents leaves and roots, respectively (b). GAPDH was used as an internal control
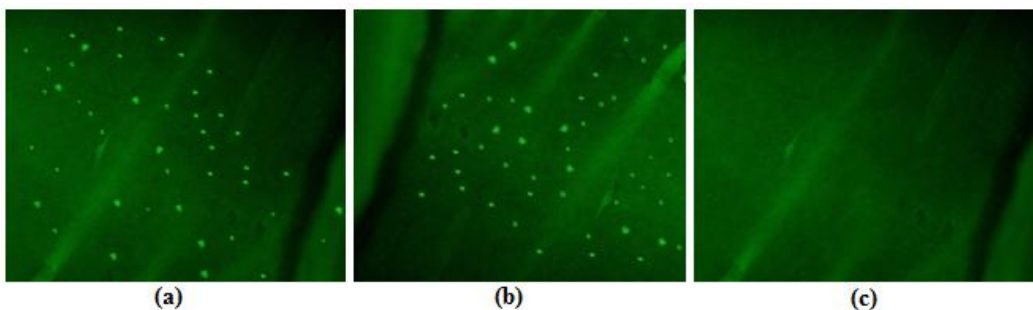
Fig.3: GFP-expressing mesocarp tissue slices co-bombarded with pMDC32 and 35SpEGFP (a), GFP-expressing mesocarp tissue slices co-bombarded with pMDC32-EgDREB and 35SpEGFP(b), and GFP expression not detected in unbombarded mesocarp tissue slices (c)

Co-bombardment of pMDC32 and pMDC32-EgDREB with GFP reporter gene provides a very useful indicator to measure the efficiency of DNA delivery and allows quick and easy selection of transiently transformed tissues.

RT-PCR Analysis of Controls

As shown in Fig.4 (a), RT-PCR analysis of the *EgDREB* gene showed that the expected size band of 700 bp was observed in the 17 WAA mesocarp tissue and the 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB plasmid. No expression of *EgDREB* gene was detected in the unbombarded 12 WAA mesocarp tissue and 12 WAA mesocarp tissue bombarded with pMDC32 plasmid. It was shown that *EgDREB* gene that was highly expressed in ripening oil palm fruit mesocarp (17 WAA) was only present in the 17 WAA mesocarp tissue and the 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB. As shown in Fig.4 (b), RT-PCR analysis of the GFP reporter gene showed that the expected size band of 200 bp was observed in the 12 WAA mesocarp tissue co-bombarded with pMDC32 and 35SpEGFP. It was also detected in the 12 WAA mesocarp tissue co-bombarded with pMDC32-EgDREB and 35SpEGFP. No expression level of GFP reporter gene was detected in the unbombarded 12 WAA mesocarp tissues. Thus the GFP reporter gene was only present in the bombarded mesocarp tissues.

Successful transcription of the *EgDREB* and GFP reporter gene in the bombarded mesocarp tissues showed the efficiency of transformation by biolistics as well as the accuracy of the RT-PCR approach. The RT-PCR was performed in triplicate for more reliable results and interestingly, the same results were observed for all the replicates which showed that the expression of introduced genes was found to be stable and most probably, did not interact with other metabolic pathways that result in suppression of gene expression.

RT-PCR Analysis of Fruit Ripening Related Genes

For Group 1, as shown in Fig.5(a), RT-PCR analysis of the *TCTP* gene showed expression of the *TCTP* gene based on the presence of the expected size of PCR product of 197 bp. The expected size bands were observed in the unbombarded 12 WAA mesocarp tissue, 12 WAA mesocarp tissue bombarded with pMDC32 plasmid, 17 WAA mesocarp tissue and 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB plasmid, with differences in intensity. The RT-PCR analysis showed that over-expression of the *EgDREB* gene under the regulation of the 35S promoter in the bombarded mesocarp tissues had resulted in up-regulation of the *TCTP* gene, based on the presence of bigger and enhanced intensity of the expected size band. From the representative gel electrophoresis result, it can be suggested that the *EgDREB* transcription factor gene regulates the expression of the*TCTP* gene.

For Group 2, RT-PCR analysis of the *Pp* [Fig.6 (a)], *ERP* [Fig.6 (b)], *USP-L* [Fig.6 (c)] and *USP* [Fig.6 (d)] genes showed

that no expression level was detected in the unbombarded mesocarp tissue, 12 WAA mesocarp tissue bombarded with pMDC32 plasmid and 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB plasmid. RT-PCR analysis showed that over-expression of the *EgDREB* gene under the regulation of the 35S promoter in the bombarded 12 WAA mesocarp tissue

had not induced expression of *Pp*, *ERP*, *USP-L* and *USP* genes. This may suggest that the *EgDREB* transcription factor is not involved in regulating the expression of genes involved in cell rescue, defence and virulence.

For Group 3, RT-PCR analysis of *Met2a* [Fig.5 (b)] and *Met2b* [Fig.5 (c)] genes showed expression of these genes

(a)   (b)
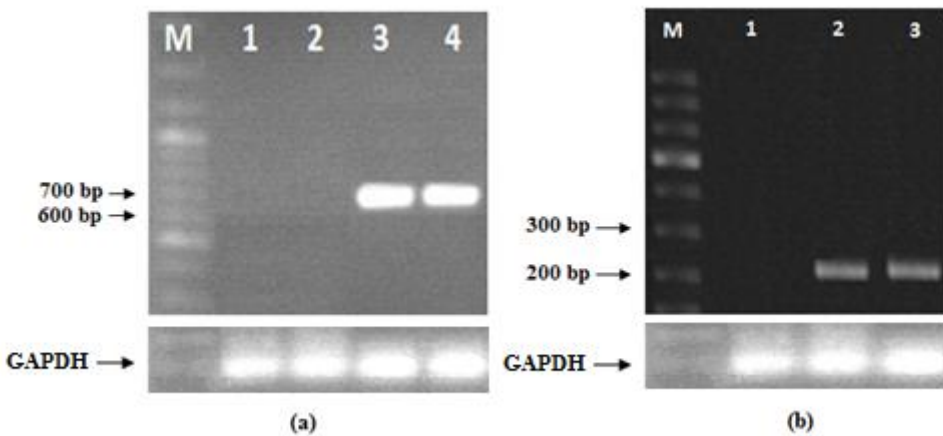
Fig.4: RT-PCR analysis for *EgDREB* gene in oil palm mesocarp tissues. M: DNA Ladder Mix. Lane 1: Unbombarded 12 WAA mesocarp tissue. Lane 2: Bombarded 12 WAA mesocarp tissue with pMDC32. Lane 3: 17 WAA mesocarp tissue. Lane 4: Bombarded 12 WAA mesocarp tissue with pMDC32-EgDREB, and RT-PCR analysis for GFP reporter gene in oil palm mesocarp tissues. M: DNA Ladder Mix. Lane 1: Unbombarded 12 WAA mesocarp tissue. Lane 2: Bombarded 12 WAA mesocarp tissue with pMDC32. Lane 3: Bombarded 12 WAA mesocarp tissue with pMDC32-EgDREB (b). GAPDH was used as an internal control

(a)   (b)   (c)
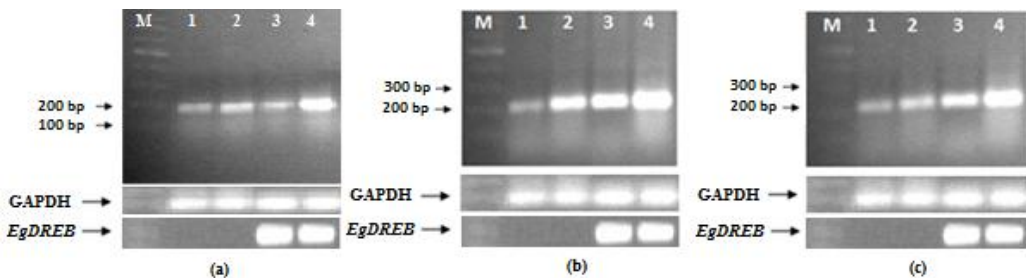
Fig.5: RT-PCR analysis for*TCTP* (a), *Met2a*(b), and*Met2b*(c) genes in oil palm mesocarp tissues. M: DNA Ladder Mix. Lane 1: Unbombarded 12 WAA mesocarp tissue. Lane 2: 12 WAA mesocarp tissue bombarded with pMDC32. Lane 3: 17 WAA mesocarp tissue. Lane 4: 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB. GAPDH and *EgDREB* were used as an internal control

based on the presence of the expected band sizes. The expected sizes of the *Met2a* and *Met2b* genes, based on the primers used, were 209 and 239 bp, respectively. The expected band sizes were observed in the unbombarded mesocarp tissue, bombarded mesocarp tissue with pMDC32 plasmid, 17 WAA mesocarp tissue and bombarded mesocarp tissue with pMDC32-EgDREB plasmid, with differences in intensity. RT-PCR analysis showed that overexpression of the *EgDREB* gene under the regulation of the 35S promoter in the bombarded 12 WAA mesocarp tissue had resulted in up-regulation of the *Met2a* and *Met2b* genes based on the presence of the bigger expected sized bands in the bombarded as compared to the unbombarded 12 WAA mesocarp tissues. From the representative gel electrophoresis results, it can be suggested that the *EgDREB* transcription factor gene regulates the expression of *Met2a* and *Met2b* genes where its expression will lead to an increase in the expression level of both the *Met2a* and *Met2b* genes.

The results of Group 4 and 5 were identical to those of Group 2. For Group 4, as shown in Fig.6 (e), RT-PCR analysis of the *His3p* gene showed no detectable expression in the unbombarded mesocarp tissue, bombarded mesocarp tissue with pMDC32 plasmid and bombarded mesocarp tissue with pMDC32-EgDREB plasmid. As for Group 5, as shown in Fig.6 (f), RT-PCR analysis of the *FtsL* gene showed no detectable expression in the unbombarded mesocarp tissue, bombarded mesocarp tissue with pMDC32 plasmid and bombarded

mesocarp tissue with pMDC32-EgDREB plasmid. Thus, it can be suggested that the *EgDREB* transcription factor gene does not regulate the expression of the *His3p* and the *FtsL* gene.

RT-PCR analysis of the 9 fruit ripening related genes (*TCTP*, *Pp*, *ERP*, *USP-L*, *USP*, *MET2a*, *MET2b, His3p* and *FtsL*) was conducted in triplicate for more reliable results. Interestingly, the same results were observed in all the replicates, which showed that the expression of the introduced *EgDREB* gene was found to be stable and most probably does not interact with other metabolic pathways. The representative gel electrophoresis results of the RT-PCR analysis showed that over-expression of the *EgDREB* gene under the regulation of the 35S promoter in the bombarded 12 WAA mesocarp tissues had resulted in increased expression of *TCTP*, *MET2a* and *MET2b* genes. No detectable expression of *Pp*, *ERP*, *USP-L*, *USP*, *His3p* and *FtsL* genes was observed in the bombarded mesocarp tissues as well as before bombardment of the 12 WAA mesocarp tissues.

Quantification of Gene Expression

The relative expression level of each target cDNA was represented by the band intensity ratio of the target cDNA/GAPDH. According to Barber *et al.* (2005), usually, quantitative expression levels of genes are normalised to the expression levels of the control, which is the housekeeping gene. RT-PCR analysis of the GAPDH gene showed expression in all of the tissues analysed. The expected size of the GAPDH

gene, based on the primers, was 100 bp. The expected size band was observed in the 12 WAA unbombarded mesocarp tissues, 12 WAA mesocarp tissue bombarded with pMDC32 plasmid, 17 WAA mesocarp tissue

and 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB plasmid. RT-PCR analysis of the GAPDH housekeeping gene showed that the gene was expressed in all the mesocarp tissues being analysed.
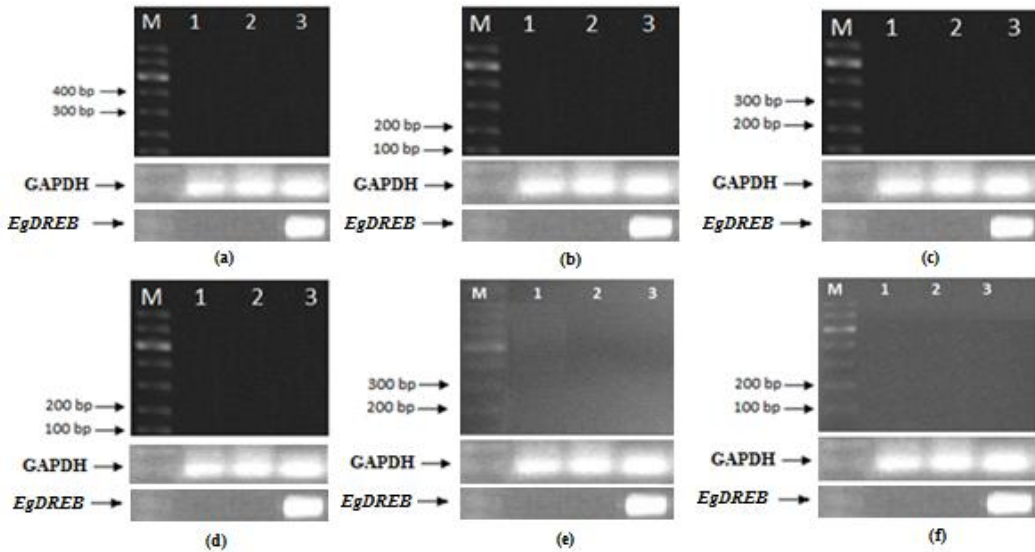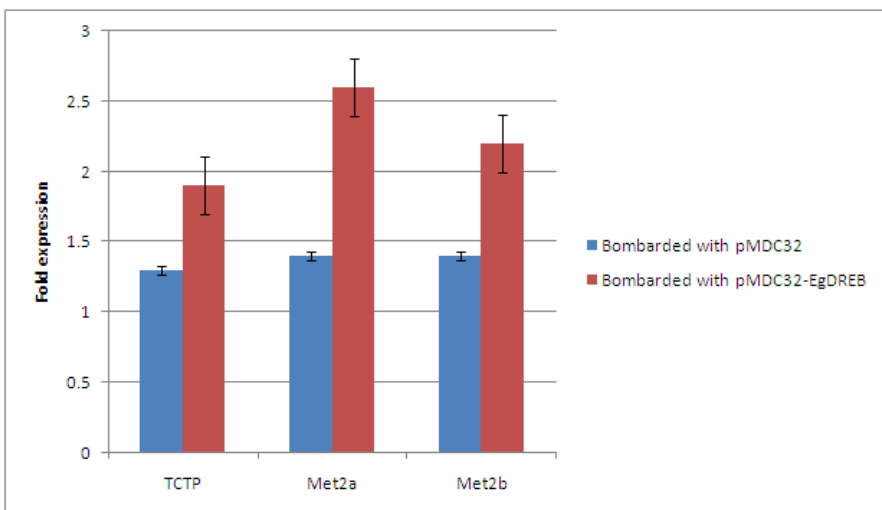

Fig.6: RT-PCR analysis for *Pp* (a), *ERP* (b), *USP-L* (c), *USP* (d) *His3p* (e), and *FtsL* (f) genes in oil palm mesocarp tissues. M: DNA Ladder Mix. Lane 1: Unbombarded 12 WAA mesocarp tissue. Lane 2: 12 WAA mesocarp tissue bombarded with pMDC32. Lane 3: 12 WAA mesocarp tissue bombarded with pMDC32-EgDREB. GAPDH and *EgDREB* were used as an internal control

Fig.7: The fold expression of each target cDNA relative expression level in oil palm mesocarp tissues

The fold expression was represented by each target cDNA relative expression level ratio of bombarded mesocarp tissue with pMDC32-EgDREB/unbombarded mesocarp tissue and bombarded mesocarp tissue with pMDC32/unbombarded mesocarp tissue (Fig. 7). The band intensity that was ≥1.5-fold was considered significant (up-regulated). All 3 genes (*TCTP*, *MET2a* and *MET2b*) in the bombarded 12 WAA mesocarp tissue with pMDC32-EgDREB were found to be up-regulated with various expression values ranging between 1.9- to 2.6-fold. The *TCTP* gene showed 1.9-fold up-regulated expression, the *Met2a* gene showed 2.6-fold-up-regulated expression and the *Met2b* gene showed 2.2-fold up-regulated expression in the oil palm mesocarp tissue at 12 WAA. These genes are involved in biogenesis of the cellular component and protein with binding function or cofactor requirement, and were classified in Groups 1 and 3, respectively.

Bombarded mesocarp tissue with pMDC32 (empty vector without the gene of interest, *EgDREB* gene) showed no effects in the expression levels of *TCTP*, *MET2a* and *MET2b* genes, which indicates that the introduction of the pMDC32 plasmid in the bombarded mesocarp tissues did not affect the expression of these 3 genes and in fact the expression of the *TCTP*, *MET2a* and *MET2b* genes after bombardment with pMDC32-EgDREB plasmid is the absolute result of the over-expression of the *EgDREB* introduced via the pMDC32-EgDREB expression vector construct.

Sequence Analysis

Sequence analysis of *TCTP*, *Met2a* and *Met2b* RT-PCR products was conducted to confirm their identity. Significant sequence identities with 100% similarity with the oil palm genes reported by Al-Shanfari *et al.*, 2012 were observed for all 3 genes (*TCTP*, *Met2a* and *Met2b*).

DISCUSSION

This is the first report on applying transient expression assay as a model system to investigate the effects of a transcription factor in oil palm tissues in order to identify target genes whose expression is regulated. Successful transient gene expression assay was demonstrated by Omidvar *et al.* (2008) using a microprojectile bombardment-based approach in oil palm tissues. In both studies, the GFP gene was selected as a reporter for non-destructive monitoring and verification of successful transformed tissue in a manner similar to that reported by Kanchanapoom *et al.* (2008).GFP has gained widespread attention as a reporter gene system for plants as it need no external substrate for detection (El-Shemy *et al.*, 2008), and there have been no reports of detectable detrimental effects on the fitness of plants that express it (Sheahan *et al.*, 2004). The green fluorescence can be directly, easily and inexpensively assessed with fluorescence microscope as reported by Wurster *et al.* (2012). Even though GFP can provide non-destructive monitoring and be used to confirm the success of the transformation process (Hraska *et al.*, 2006), the transient expression of the GFP reporter

gene in the present study does not promise the successful expression of the *EgDREB* gene.

The *EgDREB* was shown to be constitutively expressed in different oil palm tissues including the roots, leaves and the mesocarp at different developmental stages except for the mesocarp at 12 WAA. Oil palm fruit development can be divided into different phases, including the cell division and expansion starting at 4 WAA, differentiation phase from 9 WAA followed by the ripening or maturation phase, with an active period of oil synthesis at 15-16 WAA until fruit maturity at about 20 WAA (Tranbarger *et al.*, 2011). This may suggest that the *EgDREB* may play a role in cell growth and development in young mesocarp tissues and also during the ripening period when oil accumulation occurs. Similar findings were observed for the fatty acid biosynthetic gene, stearoyl-ACP desaturase, which was found to be highly expressed in actively dividing young mesocarp tissues and its expression picked up again during the active oil synthesis period. It was suggested that it played a role in provision of fatty acids for membrane lipids in young tissues and for production of storage oil during the ripening stage (Siti Nor Akmar *et al.*, 1999). For *EgDREB*, the negligible expression provided an opportunity to look at genes whose expression is induced by this transcription factor by its introduction into mesocarp slices at 12 WAA.

The translationally controlled tumour protein (*TCTP*; also called p21, p23, histamine releasing factor and fortilin) is ubiquitously expressed and widely spread in eukaryotes (Berkowitz *et al.*, 2008; Susini *et al.*, 2008; Bommer & Thiele 2004). *TCTP* is an evolutionarily highly conserved protein (Nagano-Ito & Ichikawa, 2012), and its expression is regulated at the levels of transcription and translation and also by a wide range of extracellular signals. *TCTP* plays a role in important cellular processes such as cell growth, cell cycle progression and malignant transformation and in the defence of cells against several stress conditions and apoptosis (Chen *et al.*, 2007; Bommer & Thiele, 2004). A study conducted by Al-Shanfari *et al.* (2012) reported that *TCTP* was found to be up-regulated at the late fruit ripening stage (17 WAA) of oil palm but is expressed at much lower levels at the earlier stage, at 12 WAA, the stage where the mesocarp tissues were used in the current study. In the present study, *TCTP* was found to be up-regulated with 1.9-fold expression value in response to over-expression of the *EgDREB* gene at the early ripening stage of fruit mesocarp development. The result suggests that the *EgDREB* gene may regulate the expression of genes related to growth and development based on the most widely reported role of *TCTP*. However, in recent years, microarray and proteomic analysis suggest the involvement of plant *TCTP* in abiotic stress signaling such as aluminum, salt and water deficit. Furthermore, over-expression of *Arabidopsis thaliana TCTP* was found to enhance drought tolerance in transgenic plants (Kim *et al.*, 2012). Hence, in oil palm the *TCTP* gene may also play a

role in abiotic stress signaling and the oil palm *EgDREB* is involved in regulating its expression at the transcriptional level.

Metallothioneins (MT) is defined as low molecular weight, cysteine rich metal binding proteins (Kumar *et al.*, 2010; Alizadeh *et al.*, 2011), whose expressions are induced by various factors (Yin *et al.*, 2005). These intracellular proteins are characterised by their unique high cysteine content (30%) and the absence of aromatic amino acids. MTs bind to a few trace metals like cadmium, mercury, platinum and silver, due to the high thiol content, (Sigel *et al.*, 2009) and also defend cells and tissues against toxicity of heavy metals (Thirumoorthy *et al.*, 2007). Research by Al-Shanfari and Abdullah (2014) reported that two different unigenes encoding type 2 MT-like proteins, namely *Met2a* and *Met2b*, were up-regulated in 17 WAA oil palm fruit mesocarp. This result suggests its role as a metal ion binding gene in the stress response to enhance the process of fruit ripening or to defend against oxidative damage. In the present study, *Met2a* and *Met2b* were found to be up-regulated with 2.6 and 2.2-fold expression value, respectively, in response to over-expression of the *EgDREB* gene. Their expression at the early stages of ripening is significantly lower. The result suggests that the *EgDREB* gene may regulate expression of genes that have transition metal ion binding functions or genes involved in oxidative stress response, consistent with the reported roles of metallothioneins. Metallothioneins have been reported to be involved in scavenging reactive oxygen

species during oxidative stress (Ning *et al.*, 2010). Hence if the *EgDREB* is involved in regulating expression of *Met2a* and *Met2b*, its ultimate effect would be directly related to controlling the oxidative stress response. A recent study conducted by Hwang *et al.* (2012) which showed that the oxidative stress tolerance of DREB2C-overexpressing transgenic plants was significantly greater than that of wild-type plants supports our findings.

A study conducted by Al-Shanfari *et al.* (2012) on transcripts which encode the predicted protein (Pp), ethylene-responsive protein (ERP), USP-like protein (USP-L) and universal stress protein (USP) that were related to cellular stress, under cell rescue, defence and virulence, indicates that the oil palm fruit possesses a notably elevated level of stress genes in response to its environment. Genes encoding proteins that consist of the conserved 140-160 residues USP domain have the capability to respond to environmental stresses in bacteria, archaea, fungi, protozoa and plants. Genes containing the USP domain are induced by nutrient starvation, drought, high salinity, extreme temperatures and exposure to toxic chemicals (Isokpehi *et al.*, 2011). ERP proteins are essential in plant responses to stress (Zhang *et al.*, 2009; Wu *et al.*, 2008). In the present study, no detectable expression for Pp, ERP, USP-L and USP was found in response to over-expression of the *EgDREB* gene at the early ripening stage of fruit mesocarp development. The results suggest that the *EgDREB* gene is not involved in inducing expression of these

genes even though these genes have been reported to be involved in abiotic and biotic stress response. Hence, it can be suggested that while not all stress responsive genes are regulated by EgDREB there are certain genes whose expression is influenced by EgDREB.

CONCLUSION

This represents a basic study on screening the effects of *EgDREB* on different genes that have been grouped based on gene ontology classification. The results thus far seem to indicate that *EgDREB* may be involved in enhancing the expression of genes involved in growth, abiotic and oxidative stress response based on its ability to increase the expression of the *TCTP*, *Met2a* and *Met2b* genes. However, it appears that not all abiotic stress responsive genes are regulated by EgDREB as there may be certain signalling pathways or molecular mechanisms of stress response that are affected by EgDREB.

ACKNOWLEDGEMENTS

REFERENCES

Al-Shanfari, A., Abdullah, S. N. A., Saud, H. M., Omidvar, V., & Napis, S. (2012). Differential gene expression identified by suppression subtractive hybridization during late ripening of fruit in oil palm (*Elaeis guineensis* Jacq.). *Plant Molecular Biology Reports, 30*, 768 – 779.

Al-Shanfari, A., & Abdullah, S. N. A. (2014). Isolation and expression analysis of transcripts encoding metallothioneins in oil palm. *Biologia Plantarum*, *58*(1), 18-28.

Alfatni, M. S., Shariff, A. R. M., Shafri, H. Z. M., Saaed, O. M. B., & Eshanta, M. O. (2008). Oil palm fruit bunch grading system using red, green and blue digital number. *Journal of Applied Science, 8*, 1444 – 1452.

Alizadeh, F., Abdullah, S. N. A., Khodavandi, A., Abdullah, F., Yusuf, U. K., & Chong, P. P. (2011). Differential expression of oil palm pathology genes during interactions with *Ganoderma boninense* and *Trichoderma harzianum. Journal of Plant Physiology, 168*, 1106–1113.

Bapat, V. A., Trivedi, P. K., Ghosh, A., Sane, V. A., Ganapathi, T. R., & Nath, P. (2010). Ripening of fleshy fruit: Molecular insight and the role of ethylene. *Biotechnology Advance*, *28*, 94–107.

Barber, R.D., Harmer, D. W., Coleman, R. A., & Clark, B. J. (2005). GAPDH as a housekeeping gene: Analysis of GAPDH mRNA expression in a panel of 72 human tissues. *The Journal of General Physiology*, *3*, 389 – 95.

Berkowitz, O., Jost, R., Pollmann, S., & Masle, J. (2008). Characterization of TCTP, the translationally controlled tumor protein, from *Arabidopsis thaliana. The Plant Cell, 20*, 3430 – 3447.

Bommer, U., & Thiele, B. (2004).The translationally controlled tumour protein (TCTP). *The International Journal of Biochemistry and Cell Biology*, *36*, 379–385.

Chen, S. H., Wu, P., Chou, C., Yan, Y., Liu, H., Weng, S., & Yang-yen, H. (2007). A knockout mouse approach reveals that TCTP functions as an essential factor for cell proliferation and survival in a tissue- or cell type–specific manner. *Biology Cell*, *18*, 2525 – 2532.

El-Shemy, H. A., Khalafalla, M. M., & Ishimoto, M. (2008). The role of green fluorescent protein (GFP) in transgenic plant to reduce gene silencing phenomena. *Current Issue Molecular Biology*, *11*, 21 – 28.

Hartley, C. W. S. (1988). The oil palm (*Elaeisguineensis*Jacq.). 3rd edition. Longman group UK limited, pp. 47-94

Hraska, M., Rakousky, S., & Curn, V. (2006). Green fluorescent protein as a vital marker for non-destructive detection of transformation events in transgenic plants. *Plant Cell Tissue Organ Culture*, *860*, 303–318.

Hwang, J. E., Lim, C. J., Je, J., Song, C., & Lim, C. O. (2012). Overexpression of arabidopsis dehydration-responsive element-binding protein 2c confers tolerance to oxidative stress. *Molecules and Cells*, *33*, 135 – 140.

Isokpehi, R. D., Simmons, S. S., Cohly, H. H. P., Ekunwe, S. I. N., Begonia, G. B., & Ayensu, W. K. (2011). Identification of drought-responsive universal stress proteins in viridiplantae. *Bioinformatics and BiologyInsights*, *5*, 41–58.

Kanchanapoom, K., Nakkaew, A., Kanchanapoom, K., & Amornrat, P. S. (2008). Determination of the most efficient target tissue and helium pressure for biolistic transformation of oil palm (*Elaeis guineensis* Jacq.). *Journal of Science Technology*, *30*, 153 – 157.

Kim, Y. M., Han, Y. J., Hwang, O. J., Lee, S. S., Shin, A. Y., Kim, S. Y., & Kim, J. I. (2012). Overexpression of arabidopsistranslationally controlled tumor protein gene atttctp enhances drought tolerance with rapid aba-induced stomatal closure. *Molecular Cells*, *33*, 617 – 26.

Kumar, A., Ahmad, I., Shukla, S., Singh, D. K., Patel, D. K., Pandey, H. P., & Singh, C. (2010). Effect of zinc and paraquat co-exposure on neurodegeneration: Modulation of oxidative stress and expression of metallothioneins, toxicant responsive and transporter genes in rats. *Free RadicalResearch*, *44*, 950 – 965.

Laurila, K., & Lahdesmaki, H. (2009). Systematic analysis of disease-related regulatory mutation classes reveals distinct effects on transcription factor binding. In Silico *Biology*, *9*, 18.

Licausi, F., Giorgi, F. M., Zenoni, S., Osti, F., Pezzotti, M., & Perata, P. (2010). Genomic and transcriptomic analysis of the AP2/ERF superfamily in *Vitisvinifera*. *BMC Genomics, 11*, 719.

Low, E. L., Alias, H., Boon, S., Shariff, E.M., Tan, C. A., Ooi, L. C. L., Cheah, S., Raha, A. R., Wan, K. L., & Singh, R. (2008). Oil palm (*Elaeis guineensis* Jacq.) tissue culture ESTs: identifying genes associated with callogenesis and embryogenesis. *BMC Plant Biology*, *8*, 62.

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

Nagano-Ito, M., & Ichikawa, S. (2012). Biological effects of mammalian translationally controlled tumor protein (TCTP) on cell death, proliferation, and tumorigenesis. *Biochemistry Research International, 12*, 7.

Nakano, T., Suzuki, K.., Fujimura,T., & Shinshi, H. (2006). Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiology, 140*, 411 – 432.

Ning, J., Li, X., Hicks, L. M., & Xiong, L. (2010). A raf-like MAPKKK gene DSM1 mediates drought resistance through reactive oxygen species scavenging in rice1. *Plant Physiology*, *152*, 876 – 890.

Omidvar, V., Abdullah, S. N. A., Marziah, M., & Maheran, A. A. (2008). A transient assay to evaluate the expression of polyhydroxybutyrate genes regulated by oil palm mesocarp-specific promoter. *Plant Cell Reports, 27*, 1451 – 1459.

Phillips, T., & Hoopes, L. (2008). Transcription factors and transcriptional control in eukaryotic cells. *National Education, 1*, 1.

Prada, F., Ayala-Diaz, I. M., Delgado, W., Ruiz-Romero, R., & Romero, H. M. (2011). Effect of fruit ripening on content and chemical composition of oil from three oil palm cultivars (*Elaeisguineensis* Jacq.) grown in Colombia. *Journal of Agricultural and Food Chemistry, 18*, 10136 – 10142.

Ramli, Z., & Abdullah, S. N. A. (2003). Development of a transient promoter assay system for oil palm. *Journal of Oil Palm Research, 15*, 62–69.

Saleh, A., & Montserrat, P. (2003). Plant AP2/ERF transcription factors. *Genetika, 35*, 37 – 50.

Sigel, A., Sigel, H., & Roland, K. O. (2009). Metallothioneins and related chelators. *RSC Publishing.*

Siti Nor Akmar, A., Cheah, S. C., Aminah, S., Leslie, C. L. O., Sambanthamuthi, R., & Murphy, D. J. (1999). Characterization and regulation of the oil palm (*Elaeis guineensis*) Stearoyl-ACP Desaturase Genes. *Journal of Oil Palm Research* (Special Issue), 1-17.

Sheahan, M. B., Staiger, C. J., Rose, R. J., & McCurdy, D. W. (2004). A green fluorescent protein fusion to actin-binding domain 2 of *Arabidopsis* fimbrin highlights new features of a dynamic actin cytoskeleton in live plant cells. *Plant Physiology, 136*, 3968 – 3978.

Susini,L., Besse, S., Duflaut, S., Lespagnol, A., Beekman, C., Fiucci, G. *et al.* (2008). TCTP protects from apoptotic cell death by antagonizing bax function. *Cell Death and Differentiation, 15*, 1211–1220.

Thirumoorthy, N., Manisenthil, K.T., Shyam, A., & Panayappan, L. (2007). Metallothionein: an overview. *World Journal of Gastroenterology, 7*, 993 – 996.

Tranbarger, T. J., Kluabmongkol, W., Sangsrakru, D., Morcillo, F., Tregear, J. W., Tragoonrung, S., & Billotte, N. (2012). SSR markers in transcripts of genes linked to post-transcriptional and transcriptional regulatory functions during vegetative and reproductive development of *Elaeis guineensis*. *BMC Plant Biology, 12*, 1.

Tranbarger, T. J., Dussert, S., Joët, T., Argout, X., Summo, M., Champion, A., Cros, D., Omore, A., Nouy, B., & Morcillo, F. (2011). Regulatory mechanisms underlying oil palm fruit mesocarp maturation, ripening, and functional specialization in lipid and carotenoid metabolism. *Plant Physiology, 156*, 564 – 584.

Wang, X., Chen, X., Liu, Y., Gao, H., Wang, Z., & Sun, G. (2011). *CkDREB* gene in *Caragana korshinskii* is involved in the regulation of stress response to multiple abiotic stresses as an AP2/EREBP transcription factor. *Molecular Biology Reports, 38*, 2801 – 2811.

Wu, L., Zhang, Z., Zhang, H., Wang, X., & Huang, R. (2008). Transcriptional modulation of ethylene response factor protein JERF3 in the oxidative stress response enhances tolerance of tobacco seedlings to salt, drought and freezing. *Plant Physiology, 148*, 1953 – 1963.

Wurster, T., Pölzelbauer, C., Schönberger, T., Paul, A., & Seizer, P. (2012). Green fluorescent protein (GFP) color reporter gene visualizes parvovirus B19 non-structural segment 1 (NS1) transfected endothelial modification. *PLoS ONE,* doi:10.1371/J.pone.0033602.

Yin, X., Knecht, D. A., & Lynes, M. A. (2005). Metallothionein mediates leukocyte chemotaxis. *BMC Immunology, 6*, 21.

Zhang, M., Yuan, B., & Leng, P. (2009). The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *Journal of Experimental Botany, 6*, 1579 – 1588.

REFEREES FOR THE PERTANIKA
JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

VOL. 38(1) FEB. 2015

The Editorial Board of the Journal of Tropical Agricultural Science wishes to thank the following:

While every effort has been made to include a complete list of referees for the period stated above, however if any name(s) have been omitted unintentionally or spelt incorrectly, please notify the Chief Executive Editor, UPM Journals at nayan@upm.my.

Any inclusion or exclusion of name(s) on this page does not commit the *Pertanika* Editorial Office, nor the UPM Press or the University to provide any liability for whatsoever reason.

Pertanika

Our goal is to bring high quality research to the widest possible audience

Journal of Tropical Agricultural Science

INSTRUCTIONS TO AUTHORS
(Manuscript Preparation & Submission Guidelines)
Revised: June 2014

We aim for excellence, sustained by a responsible and professional approach to journal publishing.
We value and support our authors in the research community.

> Please read the guidelines and follow these instructions carefully; doing so will ensure that the publication of your manuscript is as rapid and efficient as possible. The Editorial Board reserves the right to return manuscripts that are not prepared in accordance with these guidelines.

About the Journal

Pertanika is an international peer-reviewed journal devoted to the publication of original papers, and it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields. *Pertanika* began publication in 1978 as a Journal of Tropical Agricultural Science and became a leading agricultural journal in Malaysia. After 29 years as a multidisciplinary journal, the revamped Journal of Tropical Agricultural Science (JTAS) is now focusing on tropical agricultural research. Other *Pertanika* series include Journal of Science and Technology (JST) and Journal of Social Sciences and Humanities (JSSH).

JTAS is published in **English** and it is open to authors around the world regardless of the nationality. It is currently published four times a year, i.e. in **February**, **May**, **August** and **November**.

Goal of *Pertanika*
Our goal is to bring the highest quality research to the widest possible audience.

Quality
We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 12 weeks. The elapsed time from submission to publication for the articles averages 5-6 months.

Indexing of *Pertanika*
Pertanika is now over 33 years old; this accumulated knowledge has resulted in Pertanika JTAS being indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge [BIOSIS & CAB Abstracts], EBSCO, DOAJ, Google Scholar, AGRICOLA, ISC, MyAIS and Rubriq.

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.

We also have views on the future of our journals. The emergence of the online medium as the predominant vehicle for the 'consumption' and distribution of much academic research will be the ultimate instrument in the dissemination of research news to our scientists and readers.

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.*

Editorial Statement

Pertanika is the official journal of Universiti Putra Malaysia. The abbreviation for *Pertanika* Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

Guidelines for Authors

Publication policies

Pertanika policy prohibits an author from submitting the same manuscript for concurrent consideration by two or more publications. It prohibits as well publication of any manuscript that has already been published either in whole or substantial part elsewhere. It also does not permit publication of manuscript that has been published in full in Proceedings. Please refer to Pertanika's **Code of Ethics** for full details.

Editorial process

Authors are notified on receipt of a manuscript and upon the editorial decision regarding publication.

Manuscript review: Manuscripts deemed suitable for publication are sent to the Editorial Board members and/or other reviewers. We encourage authors to suggest the names of possible reviewers. Notification of the editorial decision is usually provided within to eight to ten 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.

Author approval: Authors are responsible for all statements in articles, including changes made by editors. The liaison author must be available for consultation with an editor of *The Journal* to answer questions during the editorial process and to approve the edited copy. Authors receive edited typescript (not galley proofs) for final approval. Changes **cannot** be made to the copy after the edited version has been approved.

Manuscript preparation

Pertanika accepts submission of mainly four types of manuscripts. Each manuscript is classified as **regular** or **original** articles, **short communications**, **reviews**, and proposals for **special issues**. Articles must be in **English** and they must be competently written and argued in clear and concise grammatical English. Acceptable English usage and syntax are expected. Do not use slang, jargon, or obscure abbreviations or phrasing. Metric measurement is preferred; equivalent English measurement may be included in parentheses. Always provide the complete form of an acronym/abbreviation the first time it is presented in the text. Contributors are strongly recommended to have the manuscript checked by a colleague with ample experience in writing English manuscripts or an English language editor.

Linguistically hopeless manuscripts will be rejected straightaway (e.g., when the language is so poor that one cannot be sure of what the authors really mean). This process, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

The instructions for authors must be followed. Manuscripts not adhering to the instructions will be returned for revision without review. Authors should prepare manuscripts according to the guidelines of *Pertanika*.

1. Regular article

Definition: Full-length original empirical investigations, consisting of introduction, materials and methods, results and discussion, conclusions. Original work must provide references and an explanation on research findings that contain new and significant findings.

Size: Should not exceed 5000 words or 8-10 printed pages (excluding the abstract, references, tables and/or figures). One printed page is roughly equivalent to 3 type-written pages.

2. Short communications

Definition: Significant new information to readers of the Journal in a short but complete form. It is suitable for the publication of technical advance, bioinformatics or insightful findings of plant and animal development and function.

Size: Should not exceed 2000 words or 4 printed pages, is intended for rapid publication. They are not intended for publishing preliminary results or to be a reduced version of Regular Papers or Rapid Papers.

3. Review article

Definition: Critical evaluation of materials about current research that had already been published by organizing, integrating, and evaluating previously published materials. Re-analyses as meta-analysis and systemic reviews are encouraged. Review articles should aim to provide systemic overviews, evaluations and interpretations of research in a given field.

Size: Should not exceed 4000 words or 7-8 printed pages.

4. Special issues

Definition: Usually papers from research presented at a conference, seminar, congress or a symposium.

Size: Should not exceed 5000 words or 8-10 printed pages.

5. Others

Definition: Brief reports, case studies, comments, Letters to the Editor, and replies on previously published articles may be considered.

Size: Should not exceed 2000 words or up to 4 printed pages.

With few exceptions, original manuscripts should not exceed the recommended length of 6 printed pages (about 18 typed pages, double-spaced and in 12-point font, tables and figures included). Printing is expensive, and, for the Journal, postage doubles when an issue exceeds 80 pages. You can understand then that there is little room for flexibility.

Long articles reduce the Journal's possibility to accept other high-quality contributions because of its 80-page restriction. We would like to publish as many good studies as possible, not only a few lengthy ones. (And, who reads overly long articles anyway?) Therefore, in our competition, short and concise manuscripts have a definite advantage.

Format

The paper should be formatted in one column format with at least 4cm margins and 1.5 line spacing throughout. Authors are advised to use Times New Roman 12-point font. Be especially careful when you are inserting special characters, as those inserted in different fonts may be replaced by different characters when converted to PDF files. It is well known that 'μ' will be replaced by other characters when fonts such as 'Symbol' or 'Mincho' are used.

A maximum of eight keywords should be indicated below the abstract to describe the contents of the manuscript. Leave a blank line between each paragraph and between each entry in the list of bibliographic references. Tables should preferably be placed in the same electronic file as the text. Authors should consult a recent issue of the Journal for table layout.

Every page of the manuscript, including the title page, references, tables, etc. should be numbered. However, no reference should be made to page numbers in the text; if necessary, one may refer to sections. Underline words that should be in italics, and do not underline any other words.

We recommend that authors prepare the text as a **Microsoft Word** file.

1. Manuscripts in general should be organised in the following order:

   o **Page 1: Running title.** (Not to exceed 60 characters, counting letters and spaces). This page should **only** contain the running title of your paper. The running title is an abbreviated title used as the running head on every page of the manuscript.

      In addition, the **Subject areas m**ost relevant to the study must be indicated on this page. Select the appropriate subject areas from the Scope of the Journals provided in the Manuscript Submission Guide

   o A **list** of n**umber of black and white / colour figures and tables** should also be indicated on this page. Figures submitted in color will be printed in colour. See "*5. Figures & Photographs"* for details.

   o **Page 2: Author(s) and Corresponding author information**. This page should contain the **full title** of your paper with name(s) of all the authors, institutions and corresponding author's name, institution and full address (Street address, telephone number (including extension), hand phone number, fax number and e-mail address) for editorial correspondence. The names of the authors **must** be abbreviated following the international naming convention. e.g. Salleh, A.B., Tan, S.G., or Sapuan, S.M.

      Authors' addresses. Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers:

      George Swan[1] and Nayan Kanwal[2]
      [1]Department of Biology, Faculty of Science, Duke University, Durham, North Carolina, USA.
      [2]Office of the Deputy Vice Chancellor (R&I), Universiti Putra Malaysia, Serdang, Malaysia.

   o **Page 3**: This page should **repeat** the **full title** of your paper with only the **Abstract** (the abstract should be less than 250 words for a Regular Paper and up to 100 words for a Short Communication). **Keywords** must also be provided on this page (Not more than eight keywords in alphabetical order).

   o **Page 4 and subsequent pages**: This page should begin with the **Introduction** of your article and the rest of your paper should follow from page 5 onwards.

      Abbreviations. Define alphabetically, other than abbreviations that can be used without definition. Words or phrases that are abbreviated in the introduction and following text should be written out in full the first time that they appear in the text, with each abbreviated form in parenthesis. Include the common name or scientific name, or both, of animal and plant materials.

      Footnotes. Current addresses of authors if different from heading.

2. **Text.** Regular Papers should be prepared with the headings **Introduction**, **Materials and Methods**, **Results and Discussion, Conclusions** in this order. Short Communications should be prepared according to "*8. Short Communications."* below.

3. **Tables.** All tables should be prepared in a form consistent with recent issues of *Pertanika* and should be numbered consecutively with Arabic numerals. Explanatory material should be given in the table legends and footnotes. Each table should be prepared on a separate page. (Note that when a manuscript is accepted for publication, tables must be submitted as data - .doc, .rtf, Excel or PowerPoint file- because tables submitted as image data cannot be edited for publication.)

4. **Equations and Formulae.** These must be set up clearly and should be typed triple spaced. Numbers identifying equations should be in square brackets and placed on the right margin of the text.

5. **Figures & Photographs.** Submit an original figure or photograph. Line drawings must be clear, with high black and white contrast. Each figure or photograph should be prepared on a separate sheet and numbered consecutively with Arabic numerals. Appropriate sized numbers, letters and symbols should be used, no smaller than 2 mm in size after reduction to single column width (85 mm), 1.5-column width (120 mm) or full 2-column width (175 mm). Failure to comply with these specifications will require new figures and delay in publication. For electronic figures, create your figures using applications that are capable of preparing **high resolution** TIFF files acceptable for publication. In general, we require **300 dpi or higher resolution for coloured and half-tone artwork** and **1200 dpi or higher for line drawings** to be submitted in separate electronic files.

   For review, you may attach low-resolution figures, which are still clear enough for reviewing, to keep the file of the manuscript under 5 MB. Illustrations may be produced at no extra cost in colour at the discretion of the Publisher; the author could be charged Malaysian Ringgit 50 for each colour page.

6. **References.** Literature citations in the text should be made by name(s) of author(s) and year. For references with more than two authors, the name of the first author followed by '*et al*.' should be used.

   Swan and Kanwal (2007) reported that …
   The results have been interpreted (Kanwal *et al.,* 2009).

   o   References should be listed in alphabetical order, by the authors' last names. For the same author, or for the same set of authors, references should be arranged chronologically. If there is more than one publication in the same year for the same author(s), the letters 'a', 'b', etc., should be added to the year.

   o   When the authors are more than 11, list 5 authors and then *et al*.

   o   Do not use indentations in typing References. Use one line of space to separate each reference. The name of the journal should be written in full. For example:

      ▪ Jalaludin, S. (1997a). Metabolizable energy of some local feeding stuff. *Tumbuh, 1,* 21-24.
      ▪ Jalaludin, S. (1997b). The use of different vegetable oil in chicken ration. *Malayan Agriculturist, 11,* 29-31.
      ▪ Tan, S. G., Omar, M. Y., Mahani, K. W., Rahani, M., & Selvaraj, O. S. (1994). Biochemical genetic studies on wild populations of three species of green leafhoppers *Nephotettix* from Peninsular Malaysia. *Biochemical Genetics*, *32,* 415 - 422.

   o   In case of citing an author(s) who has published more than one paper in the same year, the papers should be distinguished by addition of a small letter as shown above, e.g. Jalaludin (1997a); Jalaludin (1997b).

   o   Unpublished data and personal communications should not be cited as literature citations, but given in the text in parentheses. 'In press' articles that have been accepted for publication may be cited in References. Include in the citation the journal in which the 'in press' article will appear and the publication date, if a date is available.

7. **Examples of other reference citations**:

   Monographs: Turner, H. N., & Yong, S. S. Y. (2006). *Quantitative Genetics in Sheep Breeding*. Ithaca: Cornell University Press.

   Chapter in Book: Kanwal, N .D. S. (1992). Role of plantation crops in Papua New Guinea economy. In Angela R. McLean (Ed.), *Introduction of livestock in the Enga province PNG* (p. 221-250). United Kingdom: Oxford Press.

8. *Proceedings*: Kanwal, N. D. S. (2001). Assessing the visual impact of degraded land management with landscape design software. In Kanwal, N. D. S., & Lecoustre, P. (Eds.), *International forum for Urban Landscape Technologies* (p. 117-127). Lullier, Geneva, Switzerland: CIRAD Press.

9. **Short Communications** should include **Introduction, Materials and Methods, Results and Discussion, Conclusions** in this order. Headings should only be inserted for Materials and Methods. The abstract should be up to 100 words, as stated above. Short Communications must be 5 printed pages or less, including all references, figures and tables. References should be less than 30. A 5 page paper is usually approximately 3000 words plus four figures or tables (if each figure or table is less than 1/4 page).

*Authors should state the total number of words (including the Abstract) in the cover letter. Manuscripts that do not fulfill these criteria will be rejected as Short Communications without review.

STYLE OF THE MANUSCRIPT

Manuscripts should follow the style of the latest version of the Publication Manual of the American Psychological Association (APA). The journal uses American or British spelling and authors may follow the latest edition of the Oxford Advanced Learner's Dictionary for British spellings.

SUBMISSION OF MANUSCRIPTS

All articles should be submitted electronically using the ScholarOne web-based system. ScholarOne, a Thomson Reuters product provides comprehensive workflow management systems for scholarly journals. For more information, go to our web page and click "**Online Submission**".

Alternatively, you may submit the electronic files (cover letter, manuscript, and the **Manuscript Submission Kit** comprising *Declaration* and *Referral* forms) via email directly to the Chief Executive Editor. If the files are too large to email, mail a CD containing the files. The **Manuscript Submission Guide** and **Submission Kit** are available from the *Pertanika*'s home page at http://www.pertanika.upm.edu.my/home.php or from the Executive Editor's office upon request.

All articles submitted to the journal **must comply** with these instructions. Failure to do so will result in return of the manuscript and possible delay in publication.

Please do **not** submit manuscripts to the editor-in-chief or to any other office directly. All manuscripts must be **submitted through the chief executive editor's office** to be properly acknowledged and rapidly processed at the address below:

Dr. Nayan KANWAL
Chief Executive Editor
Pertanika Journals, UPM Press
Office of the Deputy Vice Chancellor (R&I)
IDEA Tower II, UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor
Malaysia

E-mail: nayan@upm.my; journal.officer@gmail.com tel: + 603-8947 1622.
or visit our website at http://www.pertanika.upm.edu.my/home.php for further information.

Authors should retain copies of submitted manuscripts and correspondence, as materials cannot be returned. Authors are required to inform the Chief Executive Editor of any change of address which occurs whilst their papers are in the process of publication.

Cover letter

All submissions must be accompanied by a cover letter detailing what you are submitting. Papers are accepted for publication in the journal on the understanding that the article is original and the content has not been published or submitted for publication elsewhere. This must be stated in the cover letter.

The cover letter must also contain an acknowledgement that all authors have contributed significantly, and that all authors are in agreement with the content of the manuscript.

The cover letter of the paper should contain (i) the title; (ii) the full names of the authors; (iii) the addresses of the institutions at which the work was carried out together with (iv) the full postal and email address, plus facsimile and telephone numbers of the author to whom correspondence about the manuscript should be sent. The present address of any author, if different from that where the work was carried out, should be supplied in a footnote.

As articles are double-blind reviewed, material that might identify authorship of the paper should be placed on a cover sheet.

Peer review

Pertanika follows a **double-blind peer-review** process. 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.

In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts. Authors are encouraged to indicate in the **Referral form** using the **Manuscript Submission Kit** the names of three potential reviewers, but the editors will make the final choice. The editors are not, however, bound by these suggestions.

Manuscripts should be written so that they are intelligible to the professional reader who is not a specialist in the particular field. They should be written in a clear, concise, direct style. Where contributions are judged as acceptable for publication on the basis of content, the Editor reserves the right to modify the typescripts to eliminate ambiguity and repetition and improve communication between author and reader. If extensive alterations are required, the manuscript will be returned to the author for revision.

The Journal's 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 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 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 executive editor asks them to complete the review in three weeks and encloses two forms: (a) referral form B and (b) reviewer's comment form along with reviewer's guidelines. 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 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 Editorial Board, 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 submit a revised version of the paper to the executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor.

5. The executive editor sends the revised paper out for 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 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, the paper is sent to that Press and 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 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.

English language editing

Pertanika **emphasizes** on the linguistic accuracy of every manuscript published. Thus all authors are required to get their manuscripts edited by **professional English language editors**. Author(s) **must provide a certificate** confirming that their manuscripts have been adequately edited. A proof from a recognised editing service should be submitted together with the cover letter at the time of submitting a manuscript to Pertanika**. All costs will be borne by the author(s)**.

This step, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

Author material archive policy

Authors who require the return of any submitted material that is rejected for publication in the journal should indicate on the cover letter. If no indication is given, that author's material should be returned, the Editorial Office will dispose of all hardcopy and electronic material.

Copyright

Authors publishing the Journal will be asked to sign a declaration form. In signing the form, it is assumed that authors have obtained permission to use any copyrighted or previously published material. All authors must read and agree to the conditions outlined in the form, and must sign the form or agree that the corresponding author can sign on their behalf. Articles cannot be published until a signed form has been received.

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.

Hardcopies of the Journals and off prints

Under the Journal's open access initiative, authors can choose to download free material (via PDF link) from any of the journal issues from Pertanika's website. Under "Browse Journals" you will see a link entitled "Current Issues" or "Archives". Here you will get access to all back-issues from 1978 onwards.

The **corresponding author** for all articles will receive one complimentary hardcopy of the journal in which his/her articles is published. In addition, 20 off prints of the full text of their article will also be provided. Additional copies of the journals may be purchased by writing to the executive editor.

Why should you publish in *Pertanika*?

BENEFITS TO AUTHORS

PROFILE: Our journals are circulated in large numbers all over Malaysia, and beyond in Southeast Asia. Our circulation covers other overseas countries as well. We ensure that your work reaches the widest possible audience in print and online, through our wide publicity campaigns held frequently, and through our constantly developing electronic initiatives such as Web of Science Author Connect backed by Thomson Reuters.

QUALITY: Our journals' reputation for quality is unsurpassed ensuring that the originality, authority and accuracy of your work is fully recognised. Each manuscript submitted to Pertanika undergoes a rigid originality check. Our double-blind peer refereeing procedures are fair and open, and we aim to help authors develop and improve their scientific work. Pertanika is now over 35 years old; this accumulated knowledge has resulted in our journals being indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge [BIOSIS & CAB Abstracts], EBSCO, DOAJ, Google Scholar, AGRICOLA, ERA, ISC, Citefactor, Rubriq and MyAIS.

AUTHOR SERVICES: We provide a rapid response service to all our authors, with dedicated support staff for each journal, and a point of contact throughout the refereeing and production processes. Our aim is to ensure that the production process is as smooth as possible, is borne out by the high number of authors who prefer to publish with us.

CODE OF ETHICS: Our Journal has adopted a Code of Ethics to ensure that its commitment to integrity is recognized and adhered to by contributors, editors and reviewers. It warns against plagiarism and self-plagiarism, and provides guidelines on authorship, copyright and submission, among others.

PRESS RELEASES: Landmark academic papers that are published in Pertanika journals are converted into press releases as a unique strategy for increasing visibility of the journal as well as to make major findings accessible to non-specialist readers. These press releases are then featured in the university's UK-based research portal, ResearchSEA, for the perusal of journalists all over the world.

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

An
Award-Winning
International-Malaysian
Journal
—MAY 2014

About the Journal

Pertanika is an international multidisciplinary peer-reviewed leading journal in Malaysia which began publication in 1978. The journal publishes in three different areas — Journal of Tropical Agricultural Science (JTAS); Journal of Science and Technology (JST); and Journal of Social Sciences and Humanities (JSSH).

JTAS is devoted to the publication of original papers that serves as a forum for practical approaches to improving quality in issues pertaining to **tropical agricultural research-** or related fields of study. It is published four times a year in **February**, **May**, **August** and **November**.

JST caters for **science and engineering research-** or related fields of study. It is published twice a year in **January** and **July**.

JSSH deals in **research or theories in social sciences and humanities research**. It aims to develop as a flagship journal with a focus on emerging issues pertaining to the social and behavioural sciences as well as the humanities, particularly in the Asia Pacific region. It is published four times a year in **March**, **June**, **September** and **December**.

Call for Papers 2015-16
now accepting submmissions...

Pertanika invites you to explore frontiers from all key areas of **agriculture**, **science and technology** to **social sciences and humanities**.

Original research and review articles are invited from scholars, scientists, professors, post-docs, and university students who are seeking publishing opportunities for their research papers through the Journal's three titles; JTAS, JST & JSSH. Preference is given to the work on leading and innovative research approaches.

Pertanika is a fast track peer-reviewed and open-access academic journal published by **Universiti Putra Malaysia**. To date, Pertanika Journals have been indexed by many important databases. Authors may contribute their scientific work by publishing in UPM's hallmark SCOPUS & ISI indexed journals.

Our journals are open access - international journals. Researchers worldwide will have full access to all the articles published online and be able to download them with **zero subscription fee**.

Pertanika uses online article submission, review and tracking system for quality and quick review processing backed by Thomson Reuter's ScholarOne™. Journals provide rapid publication of research articles through this system.

For details on the Guide to Online Submissions, visit http://www.pertanika.upm.edu.my/guide_online_submission.php

Questions regarding submissions should only be directed to the **Chief Executive Editor**, Pertanika Journals.

Remember, *Pertanika* is the resource to support you in strengthening research and research management capacity.

Address your submissions to:
The Chief Executive Editor
Tel: +603 8947 1622
nayan@upm.my

Journal's profile: www.pertanika.upm.edu.my

Selected Articles from International Conference on Crop Improvement (ICCI) 2013

Guest Editor: Siti Nor Akmar Abdullah

Contents