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About the Journal

Overview

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

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

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

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

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

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

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

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

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

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

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

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

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

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ABSTRACTING/INDEXING

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Foreword

Welcome to the **First Issue 2018** 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 **35 articles**, out of which **four** are review papers, **three** are short communication papers and **28** are regular papers. The authors of these articles come from different countries, namely, **Malaysia**, **Thailand**, **Iraq**, **Nigeria**, **Bangladesh**, **China**, **Vietnam**, **India**, **Iran**, **Ghana**, **Saudi Arabia** and **Indonesia**.

The first review paper aims to briefly cover the necessity of a herd health management programme for dairy goat farms in Malaysia (*Shahudin, M. S., Ghani, A. A. A., Zamri-Saad, M., Zuki, A. B., Abdullah, F. F. J., Wahid, H.* and *Hassim, H. A.*). The second review paper focusses on the biological pre-treatment of agricultural biomass (*Obeng Abraham Kusi, Duangporn Premjet* and *Siripong Premjet*), while the third examines the Kedah water resources enactment 2008 for sustainable agriculture development (*Siti Zuhaili Hasan* and *Sarah Aziz*). The last review article in this issue reports on sperm DNA impairment in bulls, looking at the causes, influences on reproduction and related evaluation (*Baiee, F. H., Wahid, H., Rosnina, Y., Ariff, O.* and *Yimer, N.*).

One of the short communication papers discusses the seroprevalence of neospora caninum in sheep and goats of the Gua Musang district in Kelantan, Malaysia (*Than Kyaw, Athirah Mohd Mokhtar, Bee Lee Ong, Chee Hoek Hoe, Abd Rahman Aziz, Erkihun Aklilu* and *Suratan Kamarudin*). The subsequent two short communication papers look at 24-epibrassinolide mediated changes on germination and early seedling parameters of vigna mungo (L). hepper var. shekhar-2 under salinity stress (*Sombir Singh* and *Somveer Jakhar*) and light-harvesting complex and how it affects the growth of Arabidopsis thaliana plants (*Nozulaidi, M., Khairi, M., Alamri, S.* and *Jahan, M. S.*).

The 28 regular papers cover a wide range of topics. In the first research paper, simple net rainfall partitioning equations for nearly full to full-canopy stands is studied (*Chong, S. Y., Teh, C. B. S., Ainuddin, A. N.* and *Philip, E.*). The next paper discusses the effect of

mevalonic acid (MVA) and linalool as a precursor in enhancement of limonene in citrus grandis Osbeck albedo tissue culture (*NikNorulaini, N. A. R, Thamare, K. M., Zarina, Z* and *Tengku Norsalwani, T. L*). The other papers are studies on: characterisation of fungi from palm kernel cake (PKC) and the effect of storage temperature on fungi growth (*Razali, S. M., Lee, H. Y., Jinap, S.* and *Mahyudin, N. A.*); biochemical and nutritional composition of the giant African land snail (Archachatina marginata) from Southwest Nigeria (*Bamidele, Julius A., Ademolu, Kehinde O., Idowu, Adewumi B., Aladesida, Adeyinka A. and Oladele, Adewumi O.*); optimisation of soaking conditions to improve the quality of frozen fillets of Bocourti's catfish (pangasius bocourti sauvage) using response surface methodology (RSM) (*Chaluntorn Vichasilp* and *Sutee Wangtueai*); effects of short- and long-term temperature on seed germination, oxidative stress and membrane stability of three rice cultivars (dular, KDML105 and riceberry) (*Borriboon, W., Lontom, W., Pongdontri, P., Theerakulpisut, P.* and *Dongsansuk, A.*); kenaf-based composite posts as alternative supports for black pepper (piper nigrum L.) (*Khew Choy Yuen, Kevin Muyang, Chen Yi Shang, Wong Chin Mee, Zehnder Jarroop* and *Siti Nur Aniza*); genetic diversity and relationship of Sabah traditional rice varieties as revealed by RAPD markers (*Eric Tzyy Jiann Chong, Lucky Poh Wah Goh, Jovita Jun Wong, Zaleha Abdul Aziz, Noumie @ Loumie Surugau, Mariam Abd. Latip* and *Ping-Chin Lee*); effect of naphthalene acetic acid (NAA) on oil content and quality of the mustard plant (brassica campestries L.) (*Ferdousi Begum, Feroza Hossain, Md. Monirul Islam* and *Md. Rafiqul Islam Mondal*); the effects of application of exogenous IAA and GA3 on the physiological activities and quality of abelmoschus esculentus (okra) var. singa 979 (*Khandaker, M. M., H. M. Azam, J. Rosnah, D. Tahir* and *M. Nashriyah*); the anatomy and histochemistry of structures producing aroma in the leaves of sygyzium aromaticum (L.) merr. and clausena excavata burm. f. (*Faridah Qamaruz Zaman, Anisa S. Al-Hakimi, Shamsul Khamis, Fatin F. Ruhaizin* and *Syuhada. M. Zaidi.*); GC-MS analysis of phytochemical compounds in the aqueous leaf extract of abrus precatorius (*Wan Suriyani Wan-Ibrahim, Tuan Nadrah Naim Tuan Ismail, Siti Farhanah Mohd-Salleh* and *Norzila Ismail*); plant growth, nutrient content and water use of rubber (hevea brasiliensis) seedlings grown using root trainers and different irrigation systems (*Nabayi, A., C. B. S. Teh, M. H. A. Husni* and *Z. Sulaiman*); potential mangrove species in the Porong River estuary as an inhibiting agent of heavy metal (Pb, Cu and Zn) pollution (*Sari, S. H. J., Harlyan, L. I.* and *Yona, D.*); patterns of biomass allocation in upland rice cultivars grown on soils along a toposequence (*Olagunju, S. O., Nassir, A. L., Adewusi, K. M., Oguntade, O. A., Odusanya, O. A.* and *Azeez, A. A.*); enhancement of the contents of anticancer bioactive compounds in mutant clones of

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

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

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

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Review Article

The Necessity of a Herd Health Management Programme for Dairy Goat Farms in Malaysia

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ABSTRACT

In Malaysia, an increasing number of new dairy goat farms are being opened by smallholders due to increasing demand for dairy goat products. However, most of the dairy goat farms are not managed well due to poor knowledge and information about the standard management of dairy goat. Indeed, low performance of dairy goats with respect to growth performance, feed utilisation, disease resistance and milk production has been associated with improper rearing protocol, specifically, herd health protocol. For this reason, implementation of a herd health management programme is important as a standard rearing management and disease control programme for dairy goat farms. A herd health management programme is a preventive programme intended to keep the herd healthy and free of disease through comprehensive husbandry management that includes nutrition management, breeding, parasite control, vaccination, biosecurity and environmental management with the goal of improving the herd's performance and productivity. However, the level of acceptance from farmers for implementing herd health management programmes varies, especially among smallholder farmers. Thus, veterinarians play an important role as advisor in transferring knowledge of the importance of herd health management to the farmers.

Keywords: Breeder, farm management, goat, herd health

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INTRODUCTION

Rising human population has resulted in a corresponding increase in demand for food, especially food made from animal products (Sarma & Yeung, 1985). This has triggered an increase in goat farms to meet the demand for goat products such as meat and milk (Derks, 2013). In the last two decades, expanding market demand for goat milk has resulted in the establishment of commercial dairy goat farms in several Southeast Asian countries (Liang, 2014). In Malaysia, dairy goat farms opened by smallholders contribute to the increasing dairy production for the local market. However, these smallholder farmers lack knowledge and information about standard management of dairy goats, resulting in poor farm production practice and methods (Young, 2010).

To cope with the problem, a herd health management programme was developed for use as the standard in rearing management and disease control. The programme, for implementation in dairy goat farms, intended to monitor, treat and prevent health problems as well as ensure the welfare of animals while being cost effective (Sibley, 2000, 2006). The main purpose of the programme is to prevent disease and improve animal health and production by introducing long-term strategies focussing on the whole herd (Hall & Wapenaar, 2012).

Regardless of the fact that research activities have developed comprehensive herd health management programmes, there are still problems in implementation at the farm level, especially among smallholders. Major concerns regarding animal nutrition, disease control, breeding and husbandry that directly affect production and profitability do not seem to have been properly understood by farmers and entrepreneurs (Aziz, 2007). Therefore, there is a need for a multidisciplinary approach involving veterinarians, animal nutritionists and theriogenologists in the work of transferring this knowledge actively to farmers. As the dairy industry has evolved tremendously, the scope of veterinary work also has grown; where in the past veterinarians were called by farmers to diagnose and treat sick animals, they now need to take the lead in approaching farmers to share suitable and relevant advice (Derks, 2013).

The objectives of this review were:

1. To provide a brief profile of the dairy goat industry in Malaysia,
2. To highlight the common practice of goat rearing in Malaysia and aspects of a proper herd health management programme that can be practised in dairy goat farms, and
3. To outline the challenges and problems in implementing a herd health management programme among smallholder goat farmers.

DAIRY GOAT INDUSTRY IN MALAYSIA

The population of goats in Malaysia is small compared to that in other developing countries such as India, China, Thailand and Indonesia. In 2005, the population of goats in Malaysia was 428,263 heads,

with approximately 200,000 heads reared mainly by smallholders (Aziz, 2007). In 2015, the population of goats in Malaysia had increased to 439,667 heads. Although there was an increment of 2.7% in the population of goats, the increment was small and it could not meet local demand. Therefore, the government had encouraged the import of live goats from other countries, and 102,445 heads of imported live goats were brought into Malaysia in 2007 (Department of Veterinary Statistics, DVS, 2012). Nevertheless, in 2013, the number of imported live goats had decreased to 82,821 heads (Table 1). This was due to the government policy of increasing participation of smallholders in goat farming in order to lower dependence on imports to meet the shortfall in domestic production (Jamaluddin, 2012). Compared with the commercialised pig and poultry sector, the ruminant industry, particularly the goat industry, was stagnant and lagging. While the former had achieved domestic self-sufficiency, mainly with the active participation of the private sector, the goat industry is kept going mainly by smallholders, who generally start out on low capital. Although there has been enthusiastic participation by government land development agencies aiming to increase production in goat and sheep rearing integrated with plantation crops, the goat industry continues to lag in meeting local demand (Devendra, 2007).

Table 1
Information of goats in Malaysia from 2011-2015

Item	2011	2012	2013	2014	2015
Goat population (Number)	479,444	462,510	434,202	429,398	439,667
Recorded slaughter of goats (Number)	37,121	37,653	64,368	67,858	66,466
Imports of live goats (Number)	102,445	110,117	82,821	80,065	50,634
Imports of mutton (Tonne)	17,805	18,007	18,400	22,116	18,143
Local production of mutton (Tonne)	3,091.5	4,806.2	4,688.8	4,546.1	4,367.3

The dairy goat industry is part of the world dairy industry and has to confront competition with cow, sheep and even buffalo milk products (Dubeuf, Morand-Fehr, & Rubino, 2004). This is because the organisation of the goat milk sector appears to be reliant on existing competition from the cow milk sector, as goat milk is generally still not widely sold although consumed locally. However, goat milk production could meet its potential role as a significant milk alternative especially in developing tropical countries. This is because in 2000, FAO projected that demand for milk in the developing tropical world would reach an estimated 242 million tonnes, whereas the projected supply was estimated to be only 177.6 million tonnes. This increase in demand for milk production could be met by goat milk as 90% of the total world goat population comes from developing countries in the tropics (Knights & Garcia,

1997). In addition, according to Lai (2012), goats are generally more efficient than other domesticated ruminants in digesting low-quality roughage in both tropical and arid environments and are more efficient than sheep in temperate zones. This shows that goats are well adapted and their abundance of population in the tropics can be used as an important source in milk production.

Dairy goat farming in Malaysia began in 1950 when imported breeds were used such as the Saanen, Anglo Nubian, British Alpine and Jamnapari. While the most common breed used was the Saanen, dual purpose breeds such as Anglo Nubian, Boer and Jamnapari as well as Shami goats have been used in Malaysia since 2009. In 2009, a total of 115 dairy goats were imported to Sarawak by the Agriculture Department; they consisted of the Saanen, Anglo Nubian, Toggenburg, Australian Brown and British Alpine breeds and were meant to meet demand from local dairy goat producers. Table 1 shows information on goats in Malaysia from 2011-2015 (DVS, 2017). Up until now, there is no official recorded data of goat milk production and its consumption in Malaysia. This is because the majority of milk production in Malaysia is still dominated by cow's milk and statistical data often categorised goat milk together with cow and buffalo milk (Table 2). Nevertheless, in 2013, there were 8,195 heads of dairy goats in Peninsular Malaysia, with about 50% of the dairy population located in Johor (Shanmugavelu & Nizamuddin, 2013). The biggest share of dairy goat products in the local market is fresh goat's milk. Also available are fermented goat's milk and pharmaceutical products made from goat's milk such as soap and shampoo. Although there is a lack of official data on goat's milk in Malaysia, it does seem clear that there is a significant increase in demand for goat's milk since claims of its benefit to health rose among the public. Indeed, various studies have been done to compare the nutritional advantage of goat's milk compared to milk from other animals. According to Chadan et al. (1992),

Table 2
Production quantity, ex-farm value and vonsumption of major livestock products in Malaysia for 2012 and 2015

Product	Production (000MT)		Ex-Farm Value (RM million)		Consumption (000MT)	
	2012	2015	2012	2015	2012	2015
Beef	51.28	50.50	1,031.78	1,411.72	181.48	214.87
Chevon/Mutton	4.81	4.37	146.12	143.18	24.38	38.10
Poultry (Live)	1,374.50	1,613.92	6,867.81	9,421.27	1,301.48	1,544.78
Pigs	218.47	215.76	1,988.88	2,459.41	225.82	228.03
Eggs (Mil. units)	643.00	775.05	3,274.03	4,641.24	545.00	683.00
Milk (Mil. L)	72.41	76.04	144.82	152.08	1,046.28	1,087.83

Adapted from DVS (2017)

the average of fat globule size in goat milk is significantly smaller compared to that of cow's milk. It also has a greater amount of selenium, which is an important nutrient requirement for infant milk formulae, and thus, it has been used widely as replacement milk for infants (Haenlein, 1992). In the case of infants who have cow's milk protein intolerance, goat's milk also can act as a viable dairy option in meeting nutritional requirements.

One of the issues and challenges facing dairy goat smallholders in Malaysia is the dependency on imported breeding stock to meet local demand (Jamaluddin, 2012). To overcome the problem, in the 1980s, the development of a breeding programme was started to produce a synthetic goat breed called Jermasia, which is a combination of the German Fawn breed and the Katjang breed. This is a dual-purpose breed developed by University of Malaya and the Department of Veterinary Services (DVS). Nevertheless, the number of goat produced under this programme is small and still not able to meet local demand. Thus, to further encourage development of dairy goat production and its sustainability, Malaysia developed the Malaysian Livestock Breeding Policy in 2013. The objective was to enable breeding of quality livestock through sound genetic principles and practices that satisfy the need for an economical and sustainable livestock industry and to fulfil market requirements (DVS, 2013). Since then, many programmes have been initiated by the DVS to boost the development of dairy goat production. In 2013, the DVS set up a National Boer Breeding Centre (NBBC) in Pondok Tanjung, Perak to harness the superior qualities of the Boer breed, which has undergone systemic genetic selection in South Africa. Besides developing a systematic breeding programme through application of breeding technology, this centre also provides training and incubator programmes to ensure that the technology and knowledge can be transferred to commercial goat farmers and thus, production quality will be consistent.

COMMON PRACTICE OF GOAT REARING IN MALAYSIA AND ASPECTS OF A PROPER HERD HEALTH MANAGEMENT PROGRAMME IN DAIRY GOAT FARMS

Malaysia has a long history of goat rearing, with most of the production being carried out by farmers and lower-income farming families (Devendra, 2013). In the past, the traditional farming system was observed; farms were usually a family-orientated business and the goats were inherited from previous generations of the family. Most of the goat products were used only for family consumption, providing the family with food security as its principal means of survival. The productivity of the goats during this time was low due to the fact that the goats were reared using an extensive system, which allowed them to graze in small plots of land or wander freely while scavenging natural vegetation that had low nutrient content (Gatenby, 1986). However, commercial goat farms in Malaysia began to increase in the last 20 years. There has been some improvement in rearing practices

in this time, as these smallholders have started to use the intensive farming system to increase the production of goat products. However, information on standard rearing management for goats is limited and most farmers still rely on traditional knowledge to rear their goats.

The herd health programme is a preventive programme intended to keep the herd healthy and free from disease (Salisi et al., 2012). The programme is a comprehensive husbandry management programme, which includes nutrition management, breeding, parasite control, vaccination, biosecurity and environmental management aimed at improving herd performance and productivity. Developing an effective herd health programme is very crucial as it can determine the success of a dairy goat farm. A farm management programme that integrated herd health, animal welfare, public health and farm production was developed in the 1990s (Noordhuizen & Wentink, 2001) and it has resulted in improvement of animal on farms (Sol & Renkema, 1984; Hogeveen & Dykhuizen, 1992). Relying solely on feeding and breeding will not result in maximum production if goats are exposed to disease and not kept in good health (Salisi, 2012). Diseases have a major effect on reproduction, growth, survival rate and performance of the goats. When disease occurs and treatment is required, in the event of ineffective treatment, production losses may occur.

Feeding Programme

Common feeding practice by smallholders in Malaysia relies on locally available forage and commercial concentrated feed. Common forage includes Guinea grass, Napier grass and *Bracharia* spp., while goats are usually fed legume species like *Lucaena* spp., *Gliricidia* spp. or *Mulberry* spp. (Shanmugavelu & Nizamuddin, 2013). One of the major constraints in developing dairy goat production is the difficulty of maintaining a balanced diet throughout the year for the animals (Islam, 2000). This is due to shortage of available grazing pasture, the hot and humid climate, which limits the ability to grow quality grass for ruminants (Rahman, 2015). The farmers have been using concentrates (goat pellets) and low-quality forage extensively to overcome the problem. In recent years, various agro-industrial by-products such as oil palm fronds, rice straw and soy bean waste have been utilised as animal feed. Malaysia produces 2 mil tons of agro-industrial by-products every year. Indeed, the use of agricultural by-products as source of feed for animals has been accepted and is widely practised by smallholder farmers in Malaysia.

Most of the farmers have limited knowledge of a systematic feeding management that takes into account all nutritional requirements needed based on the goat's stage (Devendra, 2013). According to a study by Abdullah (2015), only 59% of smallholder goat farmers in

Malaysia understand the importance of good feeding management for herds. They do not comprehend the usage of proper rations and minimal amount of nutrients such as protein, carbohydrate and fat required in the different stages of goat growth. Balanced nutrition is essential for the health and productivity of all animals and is the basis of successful production systems. A well-planned preventive health programme without a proper feeding programme cannot overcome problems that are created by poor nutrition (Hart, 2008). Besides that, nutrition is a production factor that reflects the total production and profit of a farm as it is the easiest aspect of management for farmers (Morand-Fehr, 2005). Therefore, goat nutrition is of paramount importance for successful goat production (Abubakr, 2015).

Like other ruminants, goats have four compartments in their stomach, three of which contain microbes that will break down feed. To promote proper fermentation in the stomach, the microbes need to be provided with an optimum amount of energy, protein, fibre, minerals and vitamins. Each nutrient should be given in correct proportion to meet the minimum daily requirements. The nutritional requirements are expressed on the basis of either percentage or grams per kilogram of the total ration, which comprises the minimum daily requirement for each nutrient group. Indeed, energy and protein are the most crucial nutrients needed by goats to build new tissue for growth or tissue replacement (Mowlem, 1992).

With respect to nutritional management, adequate nutrients should be given to animals according to their sex, age and body size as well as the production system, climate and physiological stages (Rashid, 2008). Planning nutrient requirements for each stage of animal growth should address the requirements for maintenance, growth, pregnancy and lactation, during which energy is needed to maintain a steady live weight as well as special needs related to the stage.

Health Monitoring and Vaccination Programme

Poor animal health due to disease infection may result in low milk production and indirectly lead to low profit margin. Goats in herds, especially in an intensive farming system, are more prone to infection with a higher mortality rate. This is because diseases in a high stocking density environment are more easily transmittable between animals through the air, faeces and urine. Since past times, smallholders have been using traditional medication to treat their goats; for example, goats with respiratory problems are given mulberry leaf, while goats infested with helminth parasites are given areca nut palm in their drink as a means of deworming and goats suffering from bloating have coconut oil rubbed on the affected area and are fed water mixed with tamarind fruit and ginger. However, the growing number of goats imported from other countries has led to the emergence of zoonotic and infectious diseases such as foot

and mouth disease (FMD) and brucellosis; these together with the increasing number of common diseases such as mannheimiosis, caprine arthritis encephalitis (CAE) and enterotoxaemia (pulpy kidney) highlight the importance of proper health monitoring and a vaccination programme to ensure that the production and health of the goats are not affected (Salisi, 2012). A study by Abdullah (2015) showed that only 35% of goat smallholders in Malaysia have complied with good health management. This includes participation in vaccination programmes, deworming programmes, disease monitoring and data recording for disease incidence and mortality. This shows that herd health management is very important as it determines the productivity of the farm and thus, smallholders need to implement herd health programmes on their farms.

A vaccination programme is one of the important components in herd health programmes. It can ensure that goats are protected from various types of disease. Vaccination helps the animal to develop its immune system at an earlier stage of life (Smith & Sherman, 2009). There are two types of vaccine that are commonly given to goats: killed vaccine and modified live vaccine. In Malaysia, the vaccination programme is aimed at treating various infectious diseases such as pneumonia, foot and mouth disease and caseous lymphadenitis (CLA), among others. Pneumonia is a respiratory problem that commonly occurs in goats, especially young goats. The main causative agent of respiratory problems in Malaysia is *Mannheimia haemolytica* serotypes A2, A7 and A9 (Salisi, 2011). Under stressful conditions such as transportation, overcrowding, malnutrition and weaning following concurrent viral infection and other diseases, disease can compromise the animal's immune system, exposing them to infection and disease (Davies et al., 1982; Zamri-Saad et al., 1989). During the rainy season, the number of pneumonia cases increases, especially among young goats (Jasni et al., 1991). Vaccination against *Mannheimia haemolytica* is normally given at six-month intervals, in March and September, prior to the rainy season. Vaccination can also be given to newly introduced goats on a farm to ensure the goats are healthy and protected from disease.

Besides pneumonial infection, vaccination in Malaysia currently also combats foot and mouth disease (FMD) (Abdullah, 2015), which is caused by *Apthovirus* under genus *Picornaviridae* and is highly contagious among cloven-footed animals and can affect livestock production (Abdul-Hamid, 2011). It is characterised by fever, loss of appetite, hypersalivation and vesicular eruptions on the feet, mouth, udder and teats (Wongsathapornchai, 2008). According to Edwards (2004), the disease is endemic in Peninsular Malaysia with mainly serotype O and occasionally A and Asia 1. Nevertheless, Sabah and Sarawak have been declared as FMD-free zones, which means there is no need of an FMD vaccination programme for animals in those states. Besides strict management of

animal movement, vaccination is one of the important measures to control the disease. Vaccination against FMD is normally given twice a year to all goats, especially those in high-risk zones and bordering Thailand (Gleeson, 2002).

Under Malaysian Veterinary Protocol, Mannheimiosis and foot and mouth disease have been categorised as a notifiable disease which should be reported to the Department of Veterinary Diseases (DVS, 2011). Under the protocol, there is a standard operating procedure (SOP) to control and eradicate the disease; the SOP includes treatment to infected goats and a vaccination programme carried out by the authorities. The vaccine for the disease is controlled by the Control and Eradication Section under the DVS and is distributed to each state DVS for farm vaccination programmes.

Parasitism Management and Deworming Programme

Parasitism is one of the biggest problems occurring on farms and needs to be tackled effectively. In Malaysia, infectious diseases such as parasitism, together with mismanagement and nutritional deficiencies, are the main reason for high losses on goat farms (Dorny, 1994, 1995; Fatimah, 1992; Syed, 1976; Symoens, 1992; Zamri-Saad, 1987). Parasites can vary from ectoprasites, endoparasite and blood protozoa. Parasitism results in weakness, low body condition, lowered resistance to disease, mortality and, ultimately, loss of productivity and income from the affected stock. One of the common parasitic gastroenteritis diseases among goats in Malaysia is caused by *Haemoncus contortus* (Symoens, 1992). It is considered to be the most prevalent and pathogenic nematode species to infect small ruminants in Malaysia (Dorny, 1995). Favourable conditions such as a hot and humid climate throughout the year and free grazing usually practised by smallholders encourage the development of these nematodes in the goats' gastrointestinal system, causing haemonchosis ((Dorny, 1994; Ikeme, 1987). Besides haemonchosis, coccidiosis is also typically occurring among goats in Malaysia (Dorny et al., 1995). Coccidiosis, which is caused by *Eimeria* sp., commonly occurs among young goats. It is known as one of the main factors of mortality among kids; the mortality rate among kids in their first year of age can reach up to 63% (Symoens et al., 1993).

A good herd health programme must include a plan to manage the common parasites that are a threat to goats. A good parasite control programme should be focused on preventive rather than therapeutic action. Indeed, treating goats after heavy parasite loads has less impact on reducing future contamination of the environment. Besides that, goats tend to shed worm eggs back into the environment even after treatment.

Whether or not goats are in an intensive system of free grazing, it is best to practise deworming every two or three months for adult goats. Kids should be dewormed at weaning and this should be repeated after 21 days. Antihelminthic drugs such as albendazole or fenbendazole should

also be given orally. These drugs are effective against gastro-intestinal nematodes such as *Haemonchus contortus*, *Oestergia circumcinta*, *Trichostrongylus axel*, lung nematodes such as *Dictyocaulus viviparous*, cestodes such as *Moniezia expansa* and hepatic nematodes such as *Fasciola hepatica*. A good parasite control programme also should include a scheduled screening programme; faecal samples from goats in the herd should be collected once every three months and the most common parasite diagnostic tests, faecal floatation, should be carried out.

Breeding Management Programme

Under the smallholder goat farming system in Malaysia, goats are often kept under a wide range of minimal husbandry conditions (Holst, 1999). Whether they practise intensive or extensive farming, farmers lack understanding and proper implementation of breeding programmes for their farm. Indeed, there is no recorded data on goat breeding by smallholder farmers in Malaysia. Only 48% of smallholders comply with proper breeding management (Abdullah, 2015). Most of them use native local breeds such as the Katjang for milk and meat production. Even though the breed is known to be adaptive to the local environment and is able to breed all year round, it is not an efficient milk producer due to the female's small udder size and low effective reproductive or maternal ability. Furthermore, most smallholders do not keep proper breeding records. Record keeping in breeding management is important as it can help the farmer to select high quality traits from their goats to be used for future breeding. It also can help them to reduce production cost by culling unproductive goats (Doye, 2004).

A good breeding programme should start with a clear objective of farm production and what traits are important for the particular environment of the farm (Carles, 1983; Sölkner et al., 1998). Indeed, different farms have different aims of production i.e. either for meat, milk or both. Thus, breeds suitable for a particular production can be selected to improve production and profit in the long run. Selection and replacement of goats to be used for breeding should be based on the important traits for the purpose of breeding. For instance, the selection of the buck should be based on structural and breeding soundness. The buck should be healthy and free from any reproductive disease and should have good male characteristics such as masculinity, adequate muscling, conformation of head and neck and standard buck vocalisation. The doe or female goat should have a proper oestrous cycle, be free from any reproductive disease, have good conformation and a healthy udder for milk production. Selection of suitable traits for breeding is important because these traits will contribute to the genetic makeup of every kid born and will determine the performance of the herd on the farm.

Biosecurity Programme

Another important aspect of a herd health programme is biosecurity control. Biosecurity can be defined as a prevention

plan to control disease on the farm or spreading around the farm by implementing certain practices or procedures on the farm (Delabbio, 2006). Biosecurity control includes daily cleaning of the animal pens, buying animals and other products from approved sources, control of traffic on the farm, disposal of dead animals, quarantine and isolation of new and sick animals, hygiene control by personnel, foot dips at the entrance of the farm and animal house and many other practices (Delabbio et. al., 2006). The type and frequency of biosecurity controls implemented on a farm varies based on the level of awareness of the importance of biosecurity by the farmer (Gillespie, 2000; Sanderson, 2000; Lee & O'Bryen, 2003) as well as the characteristics of the farm itself such as species grown, number of staff and adequate source of water on the farm (Delabbio et. al., 2003, 2004, 2005). A study done by Nooraisyah (2014) to measure the biosecurity status of small ruminant farms in Peninsular Malaysia found that only 40% of the total farms surveyed adopted proper bio security practices. The remaining 60% of farms practised poor biosecurity by not implementing proper isolation, traffic control or sanitation. The main causes of poor practice of biosecurity controls, especially among goat farmers in Malaysia, is the lack of time to maintain a biosecurity programme, the cost of running such a programme and the lack of technical knowledge about biosecurity programmes (Abdullah, 2015).

Implementation of biosecurity has been said to be able to reduce disease risk on a farm (Ganter, 2008), improve production efficiency and thus, increase farm revenue (Stott, 2003; Gunn, 2004). However, implementation of biosecurity controls on a farm should be seen as simple, practical and not burdensome or expensive (Ganter et al., 2008). Indeed, the effectiveness of biosecurity plans on a farm depends on the ability of the farmer to continuously adhere to the plan for the farm. As implementation of a biosecurity programme is very low among smallholder dairy goat farms, particularly in Malaysia, it is important for farmers to understand the essence of such a programme in preventing disease occurrence and to implement it efficiently on the farm.

Depending on the level of biosecurity planning to be implemented on the farm, the planning should start from proper selection of new animals on the farm. This includes purchasing animals from a known source that is free from any diseases, keeping proper health records for the animals and performing health screening in a quarantine pen once new a new herd arrives on the farm. This is important for ensuring the newly introduced animals are healthy and free from any transmittable disease. Besides that, proper farm planning is also a biosecurity measure that needs to be addressed, such as one-way entry to the farm, a vehicle dip with disinfectant at the entry of the farm and proper fencing surrounding the farm to ensure that the farm is restricted from outsiders who might harbour disease and affect animals on the farm.

Challenges and Problems Facing the Implementation of a Herd Health Programme Among Smallholder Farms

Despite the tremendous growth of dairy goat farming in Malaysia with the participation of many smallholders in this profitable venture, implementation of a herd health programme as the standard in goat rearing management of a farm in Malaysia is still low (Abdullah, 2015). One of the reasons is that this programme is seen among smallholders as being too ideal even though some are aware of the benefits of implementing the programme in terms of farm production and profit (Kristensen, 2008). A herd health programme is a new thing in dairy goat production in Malaysia as most dairy goat production depends on smallholders. Most smallholders perceive a herd health programme as being too ideal because their farm operation is small-scale, whereas implementation of such a programme will need a huge overhead in terms of medication, feed cost and restructuring of the farm for the addition of biosecurity controls, among other reasons. According to Esslemont (1992), farmers in the United Kingdom are not implementing a herd health programme on their farms due to the extra cost it will require. This is affirmed by Lievaatt and Noordhuizen (1999), who reported that high cost was the main reason that Dutch farmers did not implement a herd health programme and instead, stuck with the traditional farming system. Thus, it is important that the veterinarian be able to plan an effective herd health programme that is able to reduce disease incidence and mortality of goats on the farm; hence, it could successfully improve the productivity and profitability of the farm. Besides that, the veterinarian also needs to have vast knowledge of calculating costs and benefits of running a herd health programme.

Most smallholders think that a veterinarian's role is only to treat sick animals, control disease, support animal health and make welfare decisions (Hall et al., 2012). They seem unaware that veterinarians can also play an important role as advisor in optimising production and decreasing the overall cost of running their farms. Thus, the role of veterinarians on farms should be emphasised to farmers; they should be made aware that veterinarians can help by giving advice and guidance in every aspect of farm management.

A herd health programme is also difficult to implement in a family-orientated farm (Hall et al., 2012). This type of farm is passed down the generations of farming families, and farming skills are gained from the experience and knowledge passed from one generation to the next. It is indeed difficult to change the attitudes and ideas passed down in families; only an experienced veterinarian would be able to come against such entrenched thinking and habits (Jansen, 2010).

Good communication skills play a crucial role in developing a good relationship between farmers and veterinarians and enable effective delivery of knowledge (Derks, 2012). A strong relationship between

both parties indeed determines the success of a farm's herd health programme (Maister, 2000).

CONCLUSION

It is important for goat farmers, especially smallholders, to know the standard management practices needed to run a goat farm effectively and successfully, through implementation of a herd health programme. Such a programme can control the disease incidence and mortality rate on the farm, thus improving production and profitability of the farm. Veterinarians play a vital role in bringing new knowledge and technology to dairy goat farmers, especially smallholder farmers. It is hoped that the formulation of a standard herd health programme can guide dairy goat producers to proper herd management, increasing the productivity of their farms.

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Review Article

A Review Article of Biological Pre-Treatment of Agricultural Biomass

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ABSTRACT

Pre-treatment is a key step in the production of bioethanol from lignocellulosic biomass. Current pre-treatment techniques including physical, chemical and physico-chemical methods may increase the cost of production and produce inhibitors. In addition, they are not environmentally friendly. On the other hand, biological pre-treatment is mild, less costly, eco-friendly and consumes less energy. Despite all these benefits, several factors affect the biological pre-treatment process including microbial strain, the culture and environmental conditions as well as the type of lignocellulose material. To overcome these setbacks, different forms of biological pre-treatments such as microbial and ligninolytic enzyme pre-treatments as well as processes are studied. This review presents an overview of different forms of biological pre-treatment, various processes carried out with the aim of enhancing delignification and drawbacks of this pre-treatment process.

Keywords: Agricultural residues, biological pre-treatment, fungi, lignocellulosic biomass, ligninolytic enzymes

INTRODUCTION

Growing demand for energy worldwide is negatively affecting our environment due to the rise in fossil fuel combustion (Larran et al., 2015). The negative effects of fossil fuel combustion coupled with concerns about energy security, especially in growing economies, have resulted in the search for new sources of energy supply (Arora et al., 2016). Among the alternative sources of energy available, bioethanol produced from biomass has the potential to reduce dependence on petroleum products

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(Lopez-Abelairas et al., 2013a). Different types of biomass can be used for bioethanol production (Oke et al., 2016). Crops such as corn, sugar beets, sorghum and sugar cane are being used for the production of bioethanol (Lemée et al., 2012). However, the use of food crops to produce bioethanol contributes to high food prices globally (Larran et al., 2015).

Lignocellulosic biomass, a non-food source, can be transformed into useful products such as bioethanol, methane or other important chemicals (Cianchetta et al., 2014). Lignocellulosic biomass is rich in energy, less expensive and abundant all over the world (Okeke et al., 2015). This biomass comprises residue from crops and forest products that are not used. They are essentially made up of lignin, hemicellulose and cellulose (Horisawa et al., 2015) and are a good source for bioethanol production (Castoldi et al., 2014). However, access to the sugar component of lignocellulosic biomass is the major problem of bioethanol production from such materials (Garcia-Torreiro et al., 2016). The presence of lignin serves as a protective cover preventing enzymatic access to the hemicellulose and cellulose (Castoldi et al., 2014). A suitable pre-treatment technique which is able to eliminate and/or reduce or alter the lignin component; exposing the cellulose and hemicellulose for degradation and fermentation is, therefore, very important (Ma & Ruan, 2015).

Pre-treatment of lignocellulosic biomass can be physical, chemical, biological or a combination of these (Larran et al., 2015). Physico-chemical pre-treatment techniques, although effective, may produce substances that may impede sugar hydrolysis and the fermentation process (Salvachúa et al., 2013). These techniques are also costly and may not be environmentally friendly (Asgher et al., 2016). Biological pre-treatment is more eco-friendly and requires less energy as well as mild conditions (Garcia-Torreiro et al., 2016). It is a promising alternative to non-environmentally friendly physical and chemical pre-treatment methods (Arora et al., 2016). This review sought to discuss different forms of biological pre-treatment, its various processes carried out with the aim of enhancing delignification and drawbacks of this pre-treatment technique.

AGRICULTURAL BIOMASS

Different stages in crop production generate materials that may be used as feedstock for bioethanol production (Serna et al., 2016). Biomass from crop cultivation is rich in energy, less expensive (Okeke et al., 2015) and abundant in supply all over the world (Placido et al., 2013). The cell wall of plants is made up of a network of polysaccharides, structural proteins and phenolic compounds (Jenkins, 2014) that make the cell wall rich in chemicals and fermentable sugars for biofuel production (Guerriero et al., 2016). Although agricultural biomass has the same major components, there is variation in its composition from one species to another (Galbe & Zacchi, 2012).

The sustainable availability of agricultural residue is very important in the quest to increase the production and

utilisation of bioethanol as an alternative to fossil fuel (Cardoen et al., 2015). Searle and Malins (2016) reported that majority of European Union (EU) member states will have more than enough feedstock in 2020 to meet the directive of 0.5% advanced biofuel blending targets for transportation. Among the EU member states, France and Germany are the largest producers of agricultural residue. Cardoen et al. (2015) stated that 611 Mton/year of agricultural field residue is generated in India, out of which 158 Mtons (25%) are deemed to be surplus. Out of this surplus agricultural residue, 41 Mton/year are from sugarcane bagasse, 28 Mton/year from paddy straw, 21 Mton/year from wheat straw and 19 Mton/year from cotton stalk. Gao et al. (2016) reported that the overall agricultural residue in China is 10,818 PJ, out of which 8,419 PJ are available for the production of energy. The energy potential of rice residue (2,418 PJ), corn residue (2,334 PJ) and wheat straw (1,232 PJ) makes up 71% of the overall avaible energy supply. Rice husk, corn cob, sugarcane bagasse and peanut hull make up about 12% to 15% of the total available agricultural residue in China. Saini et al. (2015) indicated that worldwide production of the main agricultural residue is 354.34 million tons of wheat straw, 731.3 million tons of rice straw, 128.02 million tons of corn straw and 180.73 million tons of sugarcane bagasse. The largest amount of rice and wheat straw are produced in Asia, while corn straw and sugarcane bagasse are generally from America. Research into the effective utilisation of lignocellulose materials from agricultural biomass is on the increase (Wang et al., 2013). Different types of agricultural biomass including wheat straw, corn stover, corn cob, banana stalk and sugar cane bagasse have been biologically pre-treated for bioethanol production (Table 1 and 3).

PHYSICAL AND CHEMICAL PRE-TREATMENT

Physical, chemical and physico-chemical methods have been used to pre-treat lignocellulose materials (Maurya et al., 2015). Methods including milling, chipping, grinding and/or irradiation (gamma rays and electron beam) have all been applied to pre-treat lignocellulose material. However, these processes demand high energy, making them very expensive (Zhu, 2011).

Chemical pre-treatment is the most extensively used method. Chemicals such as acids, bases, ionic liquids and organic solvents are used for pre-treatment of lignocellulose materials (Aver et al., 2014). Sulphuric, nitric, hydrochloric and phosphoric acids have all been used (Nieves et al., 2016). However, high concentration of these acids is dangerous and corrosive (Kristiani et al., 2013). Partial hydrolysis of the cellulose and hemicellulose components (He et al., 2014) as well as production of inhibitors, including furfural derivatives, acetic acid, phenolic and other aromatic compounds, may also occur (Kim et al., 2016). Dilute phosphoric acid has been

reported as less corrosive and toxic compared to dilute sulphuric acid (Siripong et al., 2016), although very expensive (Nair et al., 2015). Organic solvents such as methanol, ethanol, acetone, ethylene, glycerol, acetic acid, glycols or phenols are also sometimes used to chemically pre-treat lignocellulose material (Hideno et al., 2013). Organic acids including maleic, oxalic, fumeric and citric acids are also used for pre-treatment (Lewandowska et al., 2016). Ionic liquids produce less inhibitory compounds but are expensive and can be toxic to hydrolytic enzymes (Ninomiya et al., 2013). Alkaline solutions including sodium, potassium or ammonium hydroxides can also break the bonds linking lignin to carbohydrates (Steffien et al., 2014).

Combinations of both physical and chemical methods have also been reported as an effective pre-treatment method. Steam explosion, hydrothermolysis, wet oxidation and ammonia fibre explosion (AFEX) are all types of physico-chemical pre-treatment methods. Steam explosion may produce inhibitors, while hydrothermolysis and wet oxidation requires consumption of large amount of energy. AFEX pre-treatment, however, is not suitable for biomass containing high lignin content (Galbe & Zacchi, 2012). Generally, physical, chemical and/or physico-chemical techniques may require special equipment and machines as well as harsh conditions, and this normally results in high energy demand and production of inhibitors that impede enzyme hydrolysis and fermentation processes.

BIOLOGICAL PRE-TREATMENT

Biological pre-treatment makes use of either microorganisms or the enzymes they produce to break down the lignin content of lignocellulose material (Ishola et al., 2014). Biological pre-treatment should be able to significantly reduce carbohydrate loss. It is, therefore, very important to choose a microorganism with high delignification potential but with less ability to break down cellulose during the pre-treatment process (Lopez-Abelairas et al., 2013b).

Microbial Pre-Treatment

A number of microorganisms such as bacteria, fungi and actinomycetes have the ability to reduce the lignin content in lignocellulose materials (Ma & Ruan, 2015). However, the ability of a microorganism to degrade only lignin is very important in preventing the loss of cellulose during the pre-treatment process. The best combination is a microorganism that will degrade low amounts of sugar and high amounts of lignin within the shortest possible time (Garcia-Torreiro et al., 2016).

Fungi are very important in the biological pre-treatment process as they are able to produce ligninolytic enzymes to break down lignin (Ghorbani et al., 2015). Lignin-degrading basidiomycetes (white-rot fungi) are the major decomposers in the forest ecosystem (Kamei et al., 2012) and are the most widely used microorganisms for biological pre-treatment of lignocellulose

materials (Pinto et al., 2012). The widespread use of white-rot fungi is due to their ability to produce high levels of ligninolytic enzymes (Cianchetta et al., 2014). Unlike other fungal groups, white-rot fungi are able to degrade all the major components (lignin, hemicellulose and cellulose) of lignocellulose materials. Some white-rot fungi may degrade lignin, hemicellulose and cellulose at the same time. However, others will selectively degrade lignin over the other components (Hatakka & Hammel, 2010). The degradation rate of white-rot fungi differs from one species to another (Castoldi et al., 2014). Although the major components of lignocellulose materials are the same, different fungi will act differently when cultured on these materials (Garcia-Torreiro et al., 2016). A number of white-rot fungi have been successfully used to reduce the lignin content of different lignocellulose materials (Table 1). Microscopic analysis of biologically pre-treated *Eucalyptus grandis* sawdust by Castoldi et al. (2014) revealed extreme changes in the structure of pre-treated sawdust, including separation of fibre and pore formation in much of the surface of the cell wall, compared to untreated sawdust. These changes were reported to be clearly related to the growth of the fungi studied. Pores formed in material pre-treated with *Pleurotus pulmonarius*, *Trametes* sp. and *Ganoderma lucidum* were more visible compared to the others although pre-treatment was done under the same conditions. This resulted in differences in total cellulose composition after pre-treatment (Table 1). Variations in growth and degradation rates of the fungi were stated as the possible cause for the structural and total cellulose differences observed (Castoldi et al., 2014). Similarly, Lopez-Abelairas et al. (2013) reported different sugar yields of 46 ± 2% and 65 ± 2% after pre-treating wheat straw with *Pleurotus erynii* and *Irpex lacteus,* respectively for 21 days under the same optimised conditions. Saha et al. (2016) observed great variations in the rate of delignification (1.5 ± 0.0% to 51.4 ± 2.9%) of corn stover by various fungi under the same pre-treament conditions of 74% moisture level and 28ºC temperature for 30 days (Table 1). Wang et al. (2014) also reported variations in delignification rate for *Trametes velutina* and *Trametes orientalis* after pre-treating *Populus tomentosa* with the two microorganisms for 12 weeks to enhance enzyme hydrolysis (Table 1).

Pre-treatment of corn stalk with *Phanerochaete chrysosporium* (Zhao et al., 2012) and *Pleurotus ostreatus* (Saha et al., 2016) under different conditions resulted in differences (35.3% and 54.7%, respectively) in the rate of delignification between the two processes. These differences may be attributed to the fungi variation and/or differences in the pre-treatment conditions. Environmental as well as nutritional conditions greatly affect microbial growth and hence, delignification. An effective lignin degrading microorganism and an efficient process of culturing are very important for ensuring high rate of delignification (Chang et al., 2014). Saha et al. (2016) reported that under optimum culture conditions of 84% moisture, 28ºC

temperature and 42 days of incubation, *Phlebia brevispora* was able to increase the sugar yield of corn stover by 15.4% after pre-treatment. The metabolic activities of microorganisms may be influenced by the type of substrate they are cultured on. The composition of agricultural residues may vary from one species to the other (Galbe & Zacchi, 2012). *Irpex lacteus* pre-treatment of different agriculture residue under the same conditions resulted in differences in the rate of delignification among the various residues (Table 1). This was attributed to variations in the structure of agricultural residues with respect to species, tissue, origin and growth period (Garcia-Torreiro et al., 2016).

Brown-rot fungi have the ability to degrade wood. However, brown-rot fungi degrades mainly the hemicellulose and cellulose components of lignocellulose materials, leaving behind a chemically-modified lignin residue. They are able to degrade hemicellulose and cellulose without the removal of lignin, or remove only a very small part of it. Their ability to degrade both hemicellulose and cellulose without lignin removal has been attributed to both oxidative and hydrolytic attacks. It has been suggested that the oxidative non-enzymatic attack by these fungi is through the use of low molecular weight chemicals that are able to diffuse and degrade cellulose (Hatakka & Hammel, 2010). Several brown-rot fungi produce low molecular weight chemicals, including hydrogen peroxide and oxalic acid, which are used during the non-enzymatic degradation process (Schilling et al., 2012). Schilling et al. (2012) reported the use of the brown-rot fungi *Gloeophyllum trabeum* and *Postia placenta* to break down aspen, spruce or corn stover for a period of 16 weeks before saccharification with enzymes. Generally, pre-treatment with the brown-rot fungi increased sugar yield threefold. *Gloeophyllum trabeum* pre-treatment of aspen for two weeks provided the best yield i.e. 72% glucose.

Ascomycetes and mitosporic fungi are responsible for soft-rot decay in wood. These soft-rot fungi predominantly degrade carbohydrates in lignocellulose material, causing extensive reduction in the carbohydrate content. Some soft-rot fungi can, however, partly degrade lignin. Compared to the white-rot fungi, not much study has been done on the degradation of lignocellulose material by soft-rot fungi (Hatakka & Hammel, 2010).

Certain bacteria have been effectively used to pre-treat lignocellulose material. The bacteria *Bacillus* sp. isolated from forest soil in Japan have been used to degrade alkali lignin. Initial concentrations of 0.05-2.0 g/l cell culture were able to degrade not less than 61% alkali lignin within 48 h. Pre-treament of rice straw by B*acillus* sp. also resulted in 20% degradation of Klason lignin, with 3.2% cellulose degradation (Chang et al., 2014). Pourcher and Peu (2016) isolated and identified five strains of lignin-degrading bacteria from soil and sediments. The isolates included *Serratia* sp. JHT01, *Serratia liquefacien* PT01, *Stenotrophomonas maltophilia* PT03, *Mesorhizobium* sp. PT04 and *Pseudomonas*

chlororaphis PT02. All the isolates were able to significantly grow and degrade lignin. Pre-treatment of rice straw with lactic acid bacteria produced a total sugar concentration of 30% compared to 16% for untreated rice straw (Chang et al., 2014).

Unlike white-rot fungi, pre-treatment with actinomycete results in the easy recovery of degraded lignin. Saritha et al. (2013) reported the use of *Streptomyces griseorubens* ssr 38 for degradation of lignin in paddy straw. A large amount (25%) of acid-precipitable polymeric lignins (APPLs) was recovered from pre-treated paddy straw. Delignification helped to increase the carbohydrate content, and this resulted in an increase in saccharification efficiency (97.8%).

Table 1
Delignification effect of different microorganisms on Lignocellulose material

Organism	Substrate	Incubation Time (days)	Effect of Delignification	Reference
Phanerochaete chrysosporium	*Eucalyptus grandis*	30	2.8% total cellulose	(Castoldi et al., 2014)
Pleurotus ostreatus				
Pleurotus pulmonarius			16.7% total cellulose	
Trametes sp			15.4% total cellulose	
			10.1% total cellulose	
Irpex lacteus	Wheat straw	21	42.3 ± 2.3% delignification	(Garcia-Torreiro et al., 2016)
Pleurotus eryngii (ATCC 90787)	Corn stover		45.8 ± 3.5% delignification	(Lopez-Abelairas et al., 2013)
Irpex lacteus (Fr. 238 617/93)	Corn cob		17.1 ± 5.3% delignification	
			46 ± 2% sugar yield	
P. chrysosporium NRRL-6370	Wheat straw	21	65 ± 2% sugar yield	(Saha et al., 2016)
P. sanguineus NRRL-FP-103506	Corn stover	30	51.4 ± 2.9% delignification	
I. lacteus FP-101234			51.0 ± 1.2% delignification	
C. stercoreus NRRL-6573			46.7 ± 1.8% delignification	
P. compactus NRRL-A-2351			46.2 ± 0.8% delignification	
P. brevispora NRRL-13108			45.4 ± 1.5% delignification	
A. bisporus NRRL-20762			39.6 ± 1.6% delignification	
C. cinereus NRRL-20638			7.1 ± 0.6% delignification	
B. fumosa FP-135285-T			6.6 ± 0.8% delignification	
F. velutipes NRRL-2367			4.4 ± 0.0% delignification	
R. crocatus MJL-1465			3.6 ± 0.2% delignification	
Panaeolus sp.FP-102035			2.4 ± 0.1% delignification	

Table 1 *(continue)*

C. pannocincta FT-100624			2.2 ± 0.0% delignification	
			1.5 ± 0.0% delignification	
Trametes orientalis	*Populus tomentosa*	84	47.3% delignification	(Wang et al., 2014)
Trametes velutina			58.1% delignification	
P. chrysosporium	Corn stalk	15	35.3% delignification	(Zhao et al., 2012)
Pleurotus ostreatus	Corn stalk	30	54.7% delignification	(Chen et al., 2016)
Phlebia brevispora NRRL-13108	Corn stover	42	15.4% increase in sugar yield	(Saha et al., 2016)

Process of culturing microorganisms for delignification. For effective and efficient delignification, microbial growth and activities should be optimum. The culturing process as well as the environment should lead to optimum growth and activities. Understanding the behaviour of microorganisms and the conditions under which they grow best will help to optimise the conversion of lignocellulose material into bioethanol (Shi et al., 2014). Different culturing techniques and processes have been employed with the aim of optimising microbial growth and activities for enhancement of delignification.

Solid state fermentation (SSF) process has been reported to offer several benefits compared to submerged fermentation (SmF) including higher yields, lower cost, easy recovery of products and absence of froth in medium (Asgher et al., 2016). Pinto et al. (2012) reported that fungi pre-treatment under solid state fermentation resulted in higher saccharification compared to when using a liquid medium. Addition of a source of metabolic energy to the culture medium such as glucose was reported by Castoldi et al. (2014) to improve microbial growth. The glucose helped to decrease the breakdown of the carbohydrate content of lignocellulose materials. However, Ghorbani et al. (2015) reported that a lower amount of glucose supplement is more effective for improving the process of delignification compared to larger amounts. Surfactants have also been reported to enhance delignification. Ghorbani et al. (2015) revealed that addition of a surfactant (Tween 80) to the culture medium used in their study increased the rate of delignification from 31% to 42%. Salvachúa et al. (2013) reported that adding 0.3 mM Mn(II) increased glucose yield up to 68% compared to 62% and 33% for pre-treated biomass without Mn(II) and the non-treated biomass, respectively. Saha et al. (2016) indicated that enhancement of pre-treatment conditions including the incubation period (42 days) and moisture content (84%) led to a higher sugar yield of 442 ± 5 mg/g from 383 ± 13 mg/g of *Phlebia brevispora* NRRL-13018 pre-treated corn stover, an increase of 15.4%. Ghorbani et al. (2015) revealed that low biomass-to-liquid ratio increases delignification efficiency.

However, very low ratios result in a decrease in the efficiency of delignification. The optimum biomass-to-liquid ratio in their study was 0.041 g/L.

Naturally, different microorganisms form associations and depend on each other for growth and survival. On the other hand, the presence of some microorganisms might inhibit the growth and survival of others (Wang et al., 2014). Effective and efficient breakdown of lignin in lignocellulose materials may be achieved using consortia of microorganisms. Investigations into the biological pre-treatment of Napier grass by three different groups of microorganisms designated, WSD-5 (*Coprinus cinereus* and *Ochrobactrum* sp.), MC1 (thermophilic bacteria) and XDC-2 (mesophilic bacteria in the genera Clostridium, Bacteroides, Alcaligenes and Pseudomonas) revealed that the lignin degradation efficiency of all the microbial groups was above 30% after 21 days (Wen et al., 2015). However, the nutritional as well as environmental conditions might not be favourable to all the microorganisms carrying out the processes (Berlowska et al., 2016). Wang et al. (2014) reported that monoculture of *Lenzites betulinus, Trametes orientalis* and *Trametes velutina* generally showed higher delignification efficieny compared to their respective co-cultures. The poor performance of co-culturing in their study was attributed to the inability of these microorganisms to co-exist. After microscopic observation, it was revealed that the hyphae of different microbial species do not enter each other's zone. For effective and efficient delignification, the best combination of microorganisms and biomass is very significant (Cianchetta et al., 2014). It is very important therefore to screen for species that can co-exist and support each other's growth.

Enzymatic Pre-Treatment

Biological pre-treatment with lignin degrading enzymes might help to prevent the loss of hemicellulose and cellulose that occurs during microbial pre-treatment processes (Wang et al., 2013). The use of ligninolytic enzymes is on the increase because of their ability to act on specific reactions (Chen et al., 2012). The enzyme set-up for delignification is determined by the type of microorganism, substrate as well as environmental conditions during culturing. Different microbial strains produce different types of these enzymes at varying rates (Ma & Ruan, 2015).

Ligninolytic enzymes. Different types of enzymes including laccases, peroxidases and oxidases that produce hydrogen peroxide are involved in the breakdown of lignin in lignocellulose materials. However, laccase (EC 1.10.3.2), manganese peroxidase (MnP, EC 1.11.1.13) and lignin peroxidase (LiP, EC 1.11.1.14) are the most widely used ligninolytic enzymes (Daâssi et al., 2016). The type of ligninolytic enzyme(s) responsible for delignification of a particular process is dependent on the fungi species, substrate and culture conditions. Among these enzymes, laccase is the most widely studied. Laccase is a copper-containing

enzyme that uses molecular oxygen as oxidant. Majority of white-rot fungi have the ability to synthesise laccase (Ma & Ruan, 2015). The production of laccase by white-rot fungi can be induced by adding copper, xenobiotic compounds or dyes. Laccase has the ability to degrade numerous compounds that have a phenolic structure because of its low substrate specificity. This has resulted in the use of laccase in a wide range of areas including biomass delignification and degradation of xenobiotic compounds (Wong, 2009). MnP and LiP are heme-proteins and they require hydrogen peroxide as an oxidant. They also have low substrate specificity, hence they are capable of oxidising various nonphenolic lignin model compounds as well as phenolic aromatic substrates. They were first identified in cultures of *Phanerochaete chrysosporium*. MnP is the most common lignin degrading peroxidase found in ligninolytic fungi (Hatakka & Hammel, 2010). Despite being a peroxidase, LiP is able to oxidise substrate at high redox potential (Wong, 2009).

Ligninolytic enzyme production. Inadequate production of ligninolytic enzymes by white-rot fungi has been reported as an important factor preventing the use of ligninolytic enzymes in biotechnology (Asgher et al., 2016). The slow activity of synthesised enzymes also hinders the commercial use of these biological agents for pre-treating lignocellulose material (Hyeon et al., 2014). Optimisation of the nutritional and environmental conditions of the production medium helps to increase enzyme production and activity. However, extreme nutritional and environmental conditions might inactivate enzymes, leading to a decrease in their activity (Asgher et al., 2016). Changing the fermentation medium or culture conditions can help to increase the production of these enzymes. Lignocellulose material containing large amount of lignin may be ideal in enhancing the production of ligninolytic enzymes (Rastogi et al., 2016). Mediators such as $MnSO_4$ also influence ligninolytic enzyme production by increasing the surface area for microbial growth (Asgher et al., 2016).

Different microorganisms have been used for the production of ligninolytic enzymes under different nutritional and environmental conditions (Table 2). Under optimum conditions of 50% moisture, 5 g substrate, pH 5.5, 30°C temperature, 2% fructose as carbon source, 0.02% yeast extract as nitrogen source, 25:1 carbon-to-nitrogen ratio, and 5 ml fungal spore suspension for four days, *Ganoderma lucidum* produced higher MnP activity followed by LiP and laccase (Asgher et al., 2014). Asgber et al. (2016) reported that culturing *Schizophyllum commun*e on rice straw under solid state fermentation recorded the highest ligninolytic enyme production after 144 hours. Saritha et al. (2012) revealed that *Trametes hirsute* cultured on Reese's mineral medium with 1% paddy straw as the sole carbon source in a submerged culture yielded higher activity of laccase compared to LiP and MnP after seven days. However, enzyme activities decreased with further incubation beyond

seven days. Ma and Ruan (2015) indicated that laccase was the key enzyme produced when *Coprinus comatus* was cultured in a liquid fermentation medium comprising agricultural waste materials. The highest activity of laccase was recorded on Day 6 (1520 U/ml). Cultivation of *Trichoderma reesei*, on the other hand, showed very low laccase activity (<10 U/ml) after seven days of incubation. Co-culturing of the two fungi, however, resulted in the highest laccase activity (2180 U/ml) after five days. Rastogi et al. (2016) cultivated *Pyrenophora phaeocomes* on different types of agricultural residue moistened with five parts of salt solution for the production of a ligno-hemicellulolytic enzyme cocktail. Wheat straw had the highest (25413.23 ± 35.06 IU/gds) laccase activity compared to all the other material (Table 2). Production of ligninolytic enzymes can be significantly enhanced by changing the fermentation medium. The presence of lignin and/or lignin-related compounds in the substrate can activate the production of these enzymes (Mann et al., 2015). Culturing of *Coridus versicolor* CV-1 at 28°C to 30°C on a shaker at 150 rpm for seven days resulted in laccase, Lip and MnP activities of 2066 ± 15, 0.21 ± 0.05 and 0.25 ± 0.03 U/ml, respectively. *Phanerochaete chrysosporium* PC-1 also cultured at 37ºC to 39°C statically for a period of nine days yielded 0.54 ± 0.07 (LiP) and 20.52 ± 1.36 (MnP) U/ml enzyme activity (Wang et al., 2013).

Table 2
Microbial production of Ligninolytic enzymes under different conditions

Microorganism	Amt. of Culture	Substrate	Incubation Time (Days)	Ligninolytic Enzyme	Enzyme Activity	Reference
Ganoderma lucidum	5 ml	Wheat straw	4	LiP	532 ± 4.2 U/ml	(Asgher et al., 2014)
				MnP	882 ± 13.3 U/ml	
				Laccase	340 ± 6.4 U/ml	
Schizophyllum commune IBL-06	4 ml	Rice straw	6	LiP	1347.2 U/gds	(Asgher et al., 2016)
				MnP	1846.7 U/gds	
				Laccase	316.28 U/gds	
Coprinus comatus	5 ml	LEPBM	6	LiP	1347.2 U/gds	(Ma & Ruan, 2015)
				MnP	1846.7 U/gds	
				Laccase	316.28 U/gds	
Trichoderma reesei	5 ml	LEPBM	7	Lip	0 U/ml	
				MnP	0 U/ml	
				Laccase	1520 U/ml	

Table 2 *(continue)*

Microorganism	Amt. of Culture	Substrate	Incubation Time (Days)	Ligninolytic Enzyme	Enzyme Activity	Reference
C. comatus & *T. reesei* (1:1)	7 ml	LEPBM	5	Lip	0 U/ml	
				MnP	0 U/ml	
				Laccase	<10 U/ml	
				Lip	0 U/ml	
				MnP	0 U/ml	
				Laccase	2180 U/ml	
Pyrenophora phaeocomes S-1	5 dics (7 mm)	Wheat straw	4	LiP	25413.23 ± 35.06 IU/gds	(Rastogi et al., 2016)
		Corn stover		MnP	22305.79 ± 128.55 IU/gds	
		Dried grasses		laccase	16669.42 ± 3.00 IU/gds	
		Rice straw			10859.51 ± 46.74 IU/gds	
Phanerochaete chrysosporium PC-1	3 loops of spores	Peroxidase medium	9	LiP	0.54 ± 0.07 U/ml	(Wang et al., 2013)
				MnP	20.52 ± 1.36 U/ml	
				Laccase	0 U/ml	
Coridus versicolor CV-1	3 dics (12 mm)	Laccase medium	7	LiP	0.21 ± 0.05 U/ml	
				MnP	0.25 ± 0.03 U/ml	
				Laccase	2066 ± 15 U/ml	
Trametes hirsute	5 discs (6 mm)	Reese's medium	7	LiP	35.2 ± 0.86 IU/ml	(Saritha et al., 2012)
				MnP	6.67 ± 0.69 IU/ml	
				Laccase	57.9 ± 1.08 IU/ml	

Note: Lignocellulolytic enzyme production basal medium (LEPBM)

Delignification of lignocellulose material with enzymes. Compared to purified ligninolytic enzymes, crude ligninolytic enzymes used for delignification offer several benefits in the existence of factors such as protein and mediators in the medium that helps to improve the activities of the enzyme (Asgher et al., 2016). The amount of crude enzyme extract as well as length of pre-treatment can affect the process of delignification. Different ligninolytic enzymes or combinations of enzymes play

a key role in the process of delignification depending on the type of microorganism they are produced from (Ma & Ruan, 2015).

Various studies have been conducted on delignification of lignocellulose materials from agricultural residus using crude enzyme extract (Table 3). The lignin content of wheat straw was decreased by 39.6% after pre-treatment with 25 ml of ligninolytic enzyme extract (Asgher et al., 2014). Asgher et al. (2016) reported a significant reduction (72.3%) in the lignin content of sugarcane bagasse after pre-treatment of different agricultural residue under the same conditions with 25 ml of crude ligninolytic enzyme extract from *Schizophyllum commune* for 48 h. Delignification rates in the other residue were also encouraging (Table 3). The differences in the rate of delignification may be attributed to variations in the structure and composition of the various agricultural residue. After ligninolytic enzyme pre-treatment of sugarcane bagasse for 48 hours at 35ºC, maximum delignification of 33.5% was recorded with 25 ml enzyme extract (Asgher et al., 2013). As stated earlier, different microorganisms will produce different types of ligninolytic enzymes (Ma & Ruan, 2015). Crude ligninolytic enzymes from co-culturing of different microorganisms may enhance the delignification process. Delignification of corn stover with crude enzyme extract from the co-culturing of *Coprinus comatus* and *T. reesei* resulted in the highest lignin removal (45.1%) compared to the extract from monocultures of *C. comatus* and *T. reesei* after 72 h (Ma & Ruan, 2015). Using complex enzyme systems is also another way of biologically breaking down the lignin component in lignocellilosic biomass. Hyeon et al. (2014) genetically engineered an efficient laccase complex by combining the laccase CueO of *E. coli* and the dockerin domain of a cellulosome system. The system was then fused with the scaffoldin miniCbpA to form a laccase-miniCbpA complex, which was used to pre-treat barley straw. The pre-treated barley straw was effectively fermented by cellulase, allowing *S. cerevisiae* to produce 2.34 g/L ethanol after 72 h, 2.1-fold higher than without laccase complexes.

CHALLENGES IN BIOLOGICAL PRE-TREATMENT

Although biological pre-treatment yields less waste and uses small amount of energy in addition to being eco-friendly, there are major setbacks hindering the success of this technique (Aver et al., 2014). Several factors affect the biological pre-treatment process, including the microbial strain, culture and environmental conditions as well as the type of lignocellulose material (Gai et al., 2014). Biological pre-treatment has been described as a very slow process that needs an aseptic environment. Problems associated with contamination can hinder the biological pre-treatment process; hence, the need for aseptic conditions. It requires a longer incubation time, normally several weeks to months. Various microbial strains differ with the rate at which they are able to carry out the delignification process (Tian et

al., 2012). Insufficient ligninolytic enzyme production by white-rot fungi is a major problem contributing to the long incubation time (Asgher et al., 2016). In addition, the slow activities of enzymes produced prolong the delignification process (Hyeon et al., 2014). Biological pre-treatment may produce low sugar yield as some fungi break down and utilise sugars from cellulose (Larran et al., 2015). Chen et al. (2012) reported that microbial pre-treatment may result in significant loss in the amount of dry matter. Furthermore, the large amount of space required for this process makes it industrially unfeasible.

Optimisation of biological pre-treatment processes can, however, help to increase the microbial and enzymatic activities. Culture conditions such as incubation time, pH and temperature should be varied to obtain the optimum conditions for microbial growth. This will help to enhance the growth and metabolic activities of ligninolytic fungi. Supplementing the culture medium with sources of carbon and nitrogen in addition to mediators, can also stimulate enzymatic activities and improve yield (Castoldi et al., 2014). Carbon (glucose, starch, molasses) and nitrogen (peptone, yeast extract, urea) sources as well as mediatiors such as veratryl alcohol, oxalate, hydrogen peroxide and manganese (II) sulphate can be added to the culture medium (Asgher et al., 2016).

Various ligninolytic fungi species have been studied and recommended for the production of ligninolytic enzymes (Duangporn & Siripong, 2015). *P. chrysosporium* is an efficient producer of LiP and MnP, while *Phlebia radiate* secrets laccase, LiP and MnP. *Ceriporiopsis subvermispora, Phlebia tremelosa, Phellinus pini* and *Pleurotus ostreatus* have all been reported as having high delignification efficiency (Hatakka & Hammel, 2010). Under optimum culture conditions and with a suitable substrate, these fungi species can effectively degrade lignin. Isolation and study of new ligninolytic fungi species from the natural environment will also help to solve the problems associated with biological pre-treatment.

Ligninolytic enzyme production can be induced by the presence of a wide range of substrates. Choosing a suitable substrate is, therefore, very important to ensure efficient production of ligninolytic enzymes. Lignocellulose materials containing a large amount of lignin may be ideal for the production of ligninolytic enzymes (Rastogi et al., 2016). Agricultural, forestry and agro-industrial wastes are all potential substrates for the production of ligninolytic enzymes. Spent mushroom substrate (SMS) is an organic medium obtained from the cultivation of mushrooms. It is made up of agricultural residue and mushroom mycelium that remains after harvesting of mushrooms. It is very rich in extracellular hydrolytic and oxidative enzymes synthesised from the mushrooms that were growing on it (Phan & Sabaratnam, 2012). SMS may be a potential source of ligninolytic enzymes for delignification of lignocellulose materials. The Solid-State Fermentation (SSF) process provides an environment similar to the natural habitat

of fungi and helps to enhance fungi growth and increase their metabolic activities (Asgher et al., 2016). Culturing ligninolytic fungi by SSF process will, therefore, help to increase the rate of delignification and enzyme production.

Table 3
The effect of enzyme delignification under different conditions

Microorganism	Amount of Crude Enzyme (ml)	Substrate	Incubation Time (h)	Delignification Effect	Reference
Ganoderma lucidum	25	Wheat straw	48	39.6% delignification	(Asgher et al., 2014)
Schizophyllum commune IBL-06	25	Banana stalk	48	61.7% delignification	(Asgher et al., 2016)
		Corn cob		47.5% delignification	
		Sugarcane bagasse		72.3% delignification	
		Wheat straw		67.2% delignification	
Coprinus comatus	100	Corn stover	72	41 ± 1.6% delignification	(Ma & Ruan, 2015)
Trichoderma reesei	100			0.5 ± 0.2% delignification	
C. comatus & T. reesei (1:1)	100			45.1 ± 0.7% delignification	
P. ostreatus IBL-02	25	Sugarcane bagasse	48	33.5% delignification	(Asgher et al., 2013)

CONCLUSION

Biological pre-treatment is a mild process that is less costly and eco-friendly and it consumes less energy compared to other pre-treatment processes. Ligninolytic fungi or enzymes are used for biological pre-treatment. Ligninolytic enzyme pre-treatment has been identified as a potential alternative process to overcome the drawbacks of microbial pre-treatment including long pre-treatment time as well as utilisation of sugars in some cases. However, inadequate production of ligninolytic enzymes coupled with low activity has been reported as an important factor impeding the use of ligninolytic enzymes in biotechnology. The biological pre-treatment processes are continuously optimised by varying the culture conditions to enhance microbial growth as well as enzyme production and activities. Despite the different optimisation processes of biological pre-treatment, an extremely effective biological pre-treatment technique with satisfactory level of delignification is yet to be established.

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Review Article

Kedah Water Resources Enactment 2008 for Sustainable Agriculture Development

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ABSTRACT

The agriculture sector in Malaysia contributes significantly to the country's economic growth and to national development. The series of Malaysia Development Plans (Plans) or 'Rancangan Malaysia', has set out strategies to ensure that productivity and growth of the agricultural industry become a mainstay of the five-year development programmes. The link between sustainable agriculture and sustainable water resources is acknowledged in the plans, and in several policy documents. In Kedah, Malaysia's rice bowl, the Kedah Water Resources Enactment 2008 provides nine key regulatory aspects that can help realise the goals of sustainable agriculture. This paper briefly discusses the enactment, particularly the role it can play in ensuring sustainable development of the agriculture sector through efficient water-resource management.

Keywords: Sustainable water resources, sustainable agriculture, Kedah Water Resources Enactment 2008

INTRODUCTION

Since the nation's independence in 1957, the agriculture sector has become one of the key contributors to Malaysia's development. The trends that chart the growth as well as the direction for the development of the sector are encapsulated in the Malaysia Plans. The First Malaysia Plan, spanning a period from 1966 to 1970, took note of the need to expand the sector and made provisions for strategic policy directions for intensive investment and the opening of new agricultural areas. In the Second Malaysia Plan (1971-1975), it was noted that the strategic push enforced in the First Malaysia Plan had helped generate up to one

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third of the Gross Domestic Product (GDP), provided employment and accounted for about 50% of Malaysia's foreign exchange earnings. In the Eleventh Malaysia Plan (2016-2020), it was noted that the sector had contributed RM455 billion to the nation's GDP, with a 2.4% growth per annum during the years of the Tenth Malaysia Plan, 2011 to 2015.

The Eleventh Malaysia Plan, which is currently in implementation, lays further emphasis on transforming and modernising the agro-food and industrial commodity sector so as to ensure that it will yield higher revenue and become a sustainable sector (Eleventh Malaysia Plan, 2015). Seven strategies were proposed in an effort to support the transition of the agriculture sector into an agribusiness sector i.e. improving and increasing the income of agroprenuers; promoting the training and development of agropreneurs, particularly among the youth; strengthening institutional support and extension services; building capacity of agricultural cooperatives and associations along the supply chain; improving market access and logistical support; scaling up access to agricultural financing; and intensifying performance-based incentives and certification programmes (Eleventh Malaysia Plan, 2015).

This echoes the directions put into place in the previous Third National Agricultural Policy spanning the period from 1998 to 2010 (NAP3) that outlined the strategies and approaches, which among others, focussed on optimising productivity as well as securing and enhancing food security (Third National Agricultural Policy, 1998). With the aspiration of becoming a high income country by the year 2020, the Government has taken the initiative to include the sector in its Economic Transformation Programme (ETP) as the sector has been identified as one of the 12 National Key Economic Areas (NKEAs) given its potential to contribute to the nation's Gross National Income (GNI) (PEMANDU, 2013).

Following on from this, the National Agro-Food Policy (2011-2020) has been adopted to replace the NAP3, focussing on ensuring food availability, security and safety; competitiveness and sustainability of the agrofood industry; and income increase of agropreneurs (National Agro-Food Policy, 2011). Measures relating to the export of agricultural commodities have been further strengthened with the adoption of the National Commodity Policy 2011-2020, with strategic directions set to increase the development of the plantation and commodities industry towards meeting the aspirations of achieving Vision 2020 (Economic Planning Unit, 2013).

The sustainability of the sector is dependent on many factors, one of which is the availability and secure supply of water. Water-resource management is a critical aspect in the Eleventh Malaysia Plan 2016-2020, and both water resources and agriculture have been given specific focus, particularly to aid efforts to mitigate the impact of climate variability and change (Eleventh Malaysia Plan, 2015). This is echoed in the National Agro Food Policy 2011-2020, which recognises the

importance of improving irrigation and drainage infrastructure to help manage water resources efficiently to support the productivity of crop yields and secure food supply through measures such as the introduction of paddy seed varieties that use less water (National Agro Food Policy, 2011).

A dedicated National Water Resources Policy adopted in 2012 ('2012 Policy') also sets out policy direction and strategic thrusts to ensure sustainable use of water resources. The 2012 Policy emphasises the need to ensure the security and sustainability of water resources, which is made a national priority to ensure adequate and safe water for all. The 2012 Policy takes on a complementary two-pronged approach i.e. the sustainable use, conservation and effective management of water resources enabled by a mechanism of shared partnership involving all stakeholders. The sixth policy thrust of the 2012 Policy makes specific mention of the need for conservation and protection of water resources setting out specific targets, where priority use and users are determined at the outset before an allocation and management plan is developed based on demand priority and resource availability (National Water Resource Policy, 2012).

The state of Kedah, in the north of Peninsular Malaysia, is given focus in this paper, as it is historically known as the country's 'Rice Bowl' or 'Jelapang Padi', with paddy farming areas making up 14.4% of the state's agricultural area (Kedah Structure Plan 2020, 2011). Areas under the Muda Scheme, the Department of Irrigation and Drainage irrigation scheme in the district of Yan and west of Kubang Pasu will continue to remain as paddy farming areas and will continue to receive further attention to spur optimal production to help meet food security targets (Kedah Structure Plan 2020, 2011). Kedah was also chosen because it has a specific water-resources related enactment that can be used as a means to translate the national policy directions stated earlier.

The Kedah Structure Plan 2020 also sets out policy measures for controlled development so as to ensure that water supply, quality and yield are not affected so as to negatively impact paddy production. This sits well with the Kedah Water Resources Enactment 2008 ('2008 Enactment'), which seeks to provide for controlled use, development and protection of water resources in a more integrated manner. As the agriculture sector is the main growth sector in Kedah, the management of its water resources for multiple and equitable use is critical, and options to translate ideal solutions for sustainable agriculture and water development will have to be made within the remits of the law. This would ensure that proper measures are instituted and a chain of responsibility as well as accountability is established.

The following sections of this paper briefly discuss the role of the Kedah Water Resources Board as established under the said 2008 Enactment and the statutory provisions therein on the role it plays and can play in ensuring sustainable agriculture development through efficient water-resource management. In order to identify

the provisions that fit the options for balance, the basic elements that are essential for sustainable agriculture are looked at, albeit, cursorily.

WATER AND SUSTAINABLE AGRICULTURE DEVELOPMENT

The sustainable development goals of 2015 (SDG 2015) place great emphasis on food security and promotion of sustainable agriculture (United Nations, 2014). At The World Food Summit 1996, food security was defined as the condition in which all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life. The FAO (2015) looks at sustainable agriculture as being that which involves increasing and generating adequate crop yield and livestock products without compromising the equal status of people, natural resources and ecosystem services.

There is a strong connection between sustainable agriculture, food systems, and agri-food value chains, as noted by the United Nations, that can help address issues relating to hunger and food insecurity, thus reducing cases of malnourishment. It also has the potential to help balance and meet the growing demand for food, feed, fuel and fibre, as this link will ensure agricultural systems become more productive and less wasteful (United Nations, 2013). In order to sustain food supply for the future, the United Nations (2014) recommended the adoption of ecologically and socially sustainable agriculture through better investments, enhanced legal frameworks, provision of secure access to information, particularly in land and water management, and better agricultural practices as well as land use planning, which can help build resilience to impact from climate change.

Velten et al. (2015) noted that there are multiple takes on the definition or concept of sustainable agriculture, given that there are various viewpoints grounded on different types and sectors of agriculture. Abubakar and Attanda (2013) suggested that the concept of sustainable agriculture should be closely linked to environmental changes and their impact on society, the environment and economic value. Pretty (2008) proposed that sustainability in agricultural systems encompasses the concepts of resilience and ability to address economic, social and environmental outcomes. Among the key elements identified are the integration of biological and ecological processes into food production processes, minimisation of the use of non-renewable inputs, better use of existing resources, minimisation of human capital costs and also cooperation from various stakeholders in addressing and solving agricultural problems (Pretty, 2008). The United Nations (2013) noted that sustainable agriculture and food security would require specific actions that are comprehensive in application. These actions should take into account the need to increase agricultural productivity and improve efficiency of resource use; increase the income of farmers and employment opportunities; conserve ecosystems and water as well as improve land use management;

improve the access and distribution of food supply; take into consideration value-added aspects of primary commodities and agri-food value chains; and institute measures to ensure resilient food production systems and promotion of food security. These considerations will also require recognition of indigenous and local knowledge in developing agricultural policies.

The need to consider ecological and environmental changes and to ensure sustainability highlights water as the key component in agricultural activity that requires specific focus. The agriculture sector is the biggest consumer of water; several studies have noted that the sector consumes about 70% of water resources (Braimoh, 2013; FAO, 2013; Lal, 2015; UNESCO, 2015). The utilisation of water resources for agriculture is expected to be adequate up to 2050 globally; however, due to the unsustainable use of water resources, there will be water scarcity in many regions (FAO, 2015; Lenton, 2014). Furthermore, various publications have also cautioned that unsustainable agricultural practices can lead to pollution and degradation of the environment, which in turn will have a big impact on the sector (Braimoh, 2013; Lenton, 2014). Other potential impacts will also have to be looked at, including salinisation and waterlogging (Heuperman et al., 2002; Cai et al., 2011) as well as city expansion and urbanisation, which can lead to stress to existing and future availability of water (Cai et al., 2011).

There is also a need to take into account the potential threats and impact from climate change and extreme climate events; despite assurances of adequacy, the situation could be made worse in light of emerging threats and the ensuing risks (Braimoh, 2013; Bakkes et al., 2009). Extreme climate events have great bearing on the sustainability of the sector. A case in point would be the prolonged drier climate or the 'Big Dry' in the Murray Darling Basin, Australia from 1997 to 2009 that impacted farmers greatly, resulting in a decrease in the yield of crops and deterioration of the environment (Wei et al., 2011).

Braimoh (2013), for example, proposed that agriculture should be pinned to climate-smart agriculture (CSA). CSA looks to address the challenges of food security and climate change, including agriculture, which is a source of greenhouse gas emission (FAO, 2015). Assouline et al. (2015) proposed the use of desalinated water (DS) as an alternative to freshwater for irrigation. The application of treated effluents (TE) for irrigation may have an adverse impact on the quality of soil over the long term (Assouline et al., 2015).

Country examples were also noted, such as Australia, which adopted water trading as one of the measures to help address the problem of water scarcity (Ashton et al., 2009). Furthermore, as in the case of Australia, the prolonged critical drought led farmers to change their water application methods as well as to enhance the irrigation infrastructure (Ashton et al., 2009).

Another option to deal with water scarcity is to put into place measures for water productivity assessment, with a focus

on improving yields, with less water used in securing food supply and maintaining livelihood (Cai et al., 2011). This was also echoed by Chartzoulakis and Bertaki (2015), who described sustainable water management in agriculture as an approach that facilitates meeting water supply and demand both in quality and quantity in time and space, at reasonable cost and without compromising the quality of environment.

The legal and institutional framework plays an important role in sustainable water resource management in agriculture. An institution or an agency should be set up for water resource planning, management, operation and decision making so as to avoid conflict with various authorities (Chartzoulakis & Bertaki, 2015). Tilman et al. (2002) stated that policies for sustainable agriculture should be outlined in a way that gives priority to environmental consideration. The Australian National Water Commission supported the idea of environmental needs becoming a priority for consideration in water allocation as reflected in the National Water Initiative (NWC, 2009).

In order to tackle the conflicting issues between different water users, engagement and consultation between different stakeholders should be included in water resource planning and management (Wei et al., 2011). Lenton (2014) suggested that in developing policies for irrigated agriculture, several issues will need to be addressed such as groundwater resource sustainability; water storage management; use-conflict between agricultural purposes and environmental needs; and incentives for water-efficient application. In developing any agricultural or food security policies, the need to consider water resources is critical as these policies are strongly interlinked with one another (FAO, 2012). This will enable the issues related to water scarcity to be identified and tackled (FAO, 2012).

Traditionally, water management for agriculture has focussed on the irrigation system and farming (Cai et al., 2011). This segmented approach to management can cause further problems, as water resources need to be managed holistically, taking into account the entire river basin, as the issues do not stem only from agricultural problems, but are interlinked, particularly the hydrological aspects and its people (Cai et al., 2011). Multidisciplinary approaches are required in order to address the issues for sustainable use of water resources (Assouline et al., 2015) even in soil application, which requires comprehensive monitoring strategies of soil health as soil properties, and their application is closely related to irrigated agriculture and sustainable use of water resources.

Due to water scarcity, disputes can arise among users of water resources, making institutional and legal frameworks key in helping to resolve existing and emerging issues (Vaidyanathan & Jairaj, 2009). Salman and Bradlow (2006) noted that water-related legislation is relevant and important in managing water resources. This includes addressing ownership and responsibility (Iyer, 2010). There is also the idea of water resources being a public trust,

where the State acts as public custodian of water resources (Salman & Bradlow, 2006).

In guiding the decisions pertaining to water resources, principles and priorities pertaining to conservation, protection, equitable allocation and sustainable use including development of plans for water resources should be specified (Salman & Bradlow, 2006). Others elements suggested by Salman and Bradlow (2006) for water legislation include the regulation of water use, which relates to the requirement of permits and licences; protection of water resources from non-point and point sources; regulation pertaining to wastewater discharge, land use and procedures for enforcement of water quality standards; regulation of water infrastructure; institutional and financial arrangements; enforcement of the regulations and elements of dispute settlement.

Based on the selected literature referenced here, there are several aspects that will have to be considered in determining the role that can be played by the Kedah Water Resources Board through the 2008 Enactment. There is a need for measures and mechanisms that can look at and address environmental change and its impact on society; environmental and economic value of multiple and related resources; direction of development; and the means to improve land use management. It should also look at establishing assessment measures that will look at the state and condition as well as productivity of the sector.

In addition, the measures and mechanisms should also look at existing, emerging and potential threats and impact of different hazards or environmental impact, including the impact of climatic hazards. Planning options should also be looked at so as to optimise what is available and ensure its sustainability in addition to addressing potential conflict between use and users.

LAW, WATER RESOURCES AND AGRICULTURE IN KEDAH

There are five key regulatory instruments that are directly related to water resources and agriculture in the state of Kedah. These five instruments have bearing on different aspects of water and agriculture. The Kedah Water Resources Enactment 2008 ('2008 Enactment') serves as the primary legal instrument where water resources is concerned for the State of Kedah. The 2008 Enactment makes provisions for the establishment of a Kedah Water Resources Board ('Board') with a mandate to provide for the integrated management of use, development and protection of water resources in Kedah (State of Kedah Legislative Assembly 2008).

The second instrument that has bearing on water would be the National Land Code 1965 (NLC), which provides a comprehensive administrative framework for dealings related to land, including water resources (see Section 5 of the NLC). The NLC does not set out to control State resources but serves to aid the governing of land resources through processes and procedures enforced through rules and regulations. This is crucial, as if water is understood as it is defined in the NLC's

definition of land, which is provided for in Section 5, then the State Authority, in lieu of a specific regulatory instrument over water resources, can still regulate water resources using the NLC. Section 5 interprets land as:

(a) The surface of the earth and all substances forming that surface;

(b) The earth below the surface and all substances therein;

(c) All vegetation and other natural products, whether or not requiring the periodical application of labour to their production, and whether on or below the surface;

(d) All things attached to the earth or permanently fastened to anything attached to the earth, whether on or below the surface; and

(e) Land covered by water.

Section 40 of the NLC vests the State Authority with the entire property of all State land within the territories of the State, including all mineral and rock material not disposed of. This would include water as defined in Section 5 above.

The NLC empowers a State Authority to divide territories, districts or sub districts as well as to vary or alter boundaries, which in turn affects the administration and control of water bodies and resources. Rules in relation to objects and purposes pertaining to land and any dealings related to it can be issued, which includes access to, permission and licences as well as regulation and control of dealings related to a particular land concerned that also includes any water body on it. Section 58 of the NLC for example, addresses matters pertaining to rights of access to and use of alienated lands, whereby a State Authority may carry out works to facilitate the passage of water. In addition, under Section 62, a State Authority can reserve land for any public purposes; this includes land with water bodies in or on top of it, indicating what cannot be separated from water resources and vice versa. This does seem to mean that water can be understood here to fall within the definition of land.

The Muda Agricultural Development Authority Act 1972 sets out the mandate for the promotion, planning and management of MADA-gazetted areas. In addition, it also makes provision relating to matters pertaining to drainage and irrigation infrastructure; management of water resources for granary areas; and improving the economic and social status of farmers.

MADA areas can only be determined, delineated and gazetted after the Ministry of Agriculture and Agro-Based Industries (MOA) consults with the State government of Kedah and Perlis. Urban development is restricted in MADA areas. This legal instrument applies only in areas that have been gazetted under MADA, but as far as water resources that course through these areas are concerned, the 2008 Enactment prevails.

The Irrigation Areas Act 1953 (revised 1989) provides the mandate related to the declaration of irrigation areas for

paddy cultivation (Sections 3 and 4) and classification of land within an irrigation area (Section 6). Any area that has been declared an irrigation area cannot be used, nor activities planned or developed there except for paddy cultivation purposes (Section 5). Section 7 and Section 8 look at payable water rates and their collection.

Under the Irrigation Areas Act 1953 the power to manage irrigation areas by a Drainage and Irrigation Engineer or an officer appointed by the Ruler in Council or Yang DiPertua Negeri in Council of the particular state (Section 9). The person appointed is obliged to provide reports to the Council or Yang DiPertua Negeri in Council of the particular State on matters related to the area in his charge, furnish annual balance sheets showing the receipts on account of water rates and disclose the expenditure on construction, management, supervision and maintenance of the works in his charge. Similar to MADA areas, as far as water resources are concerned the 2008 Enactment prevails.

Sections 10 to 24 of the Irrigation Areas Act 1953 spell out the powers of the officer appointed, which also includes control of matters related to filling up obnoxious water ways, removal of trees or refuse, damage to irrigation works, obstruction or damage, a penalty for wasting water, passage of water, pollution, use of vehicles as well as tampering with irrigation works. The power of arrest for offences against provisions in a number of sections of the Act is also provided for under the said Act.

Land use planning and development has had a great impact on water resources in Kedah. The Kedah Town and Planning Department holds the mandate to oversee, in as far as land use planning for the whole State in concerned, provided for under the Town and Country Planning Act 1976. There are two levels of land use planning and development that are documented into detailed plans and gazetted by the State Authority. They are the Kedah State Structure Plan 2020, endorsed and gazetted in 2011, which is to be used for the whole State, and several separate city, municipal and district area local plans that translate into actions, policies and strategies in the Structure Plan. These plans help set the direction that will guide physical development but does not in any way control land or water.

There are also special area plans that are prepared to facilitate micro planning and land use control at specific levels in specific areas either within district boundaries on inter-district boundaries. In Kedah, water resources and water bodies as well as overall use, particularly for irrigation areas and conservation of water bodies are given much focus in the Kedah Structure Plan 2020. The policy emphasis of the Structure Plan revolves around three key factors i.e. conservation, sustainable use and protection of environmentally sensitive areas. In the preparation of such plans, be it State, Local or Special Area Plans, public participation is mandatory under the Town and Country Planning Act 1976.

SUSTAINABLE AGRICULTURE DEVELOPMENT AND THE KEDAH WATER RESOURCES ENACTMENT 2008

Water resources in the 2008 Enactment are interpreted to include any river, river basin, ground water or water body, and the term 'water body' is taken to mean "any river, lake, pond, wetland, coastal waters, ground water and other bodies or water whether natural or artificial, including its banks and bed or any part thereof or its surroundings" (Section 3). The preamble states that the 2008 Enactment is intended to provide for the integrated management of the use, development and protection of water resources in the State of Kedah. It serves as the encompassing legal instrument that will serve as the management instrument for water resources in Kedah.

In order to manage water resources in Kedah, a Water Resources Board is established, made up of membership from related government departments, agencies and statutory bodies as well as experts who are appointed by the State Authority. In addressing multiple use of water resources in Kedah, subject to Section 6, the Board functions to ensure, maintain and facilitate the integration of water-resource management in order to support the continuous role of ecosystems and maximise the social, economic and environmental benefits; to regulate the transfer of water resources; to promote coordination and cooperation between various stakeholders; to coordinate the development and exploration of additional water resources; to improve their capacity and expertise as an integrated water-resource management centre; and to advise the State in terms of water transfer between states.

The Board has the power to approve river basin plans; formulate or review legislation, directives and procedures aimed at promoting or facilitating the implementation of integrated water-resource management ('IWRM'); approve development projects related to IWRM; and divide the State into river basin districts and designate and determine boundaries for proper management by a River Basin Committee. It can also approve guidelines, performance standards, methods and procedures pertaining to management, utilisation and conservation of water resources and issue directives (Section 7).

These functions provide an ideal platform to set out measures towards ensuring sustainable agriculture development in Kedah, as it looks at management from an integrated perspective, grounded in maintaining the balance between the role of ecosystems and the benefits gained from development. It also recognises at the outset that there are multiple stakeholders, and three steps are offered to help bring together the many stakeholders i.e. through a River Basin Committee, the development of plans and promotion of cooperation.

A Water Resources Director is appointed by the Board, with specific functions, which includes preparation of river basin plans for approval of the Board and State Authority. The Director also ensures that each plan contributes to the integrated approach in river basin management; prepares and publishes

annual reports on the state of the water environment; prepares and publishes reports on Board activities and implementation of duties and the function of relevant agencies provided for under the 2008 Enactment; prepares development proposals for IWRM; and looks into the administration and management of the Board. The Director also holds the responsibility of ensuring the flow and exchange of information on projects, plans and activities that have bearing on IWRM and keeps the Board informed and updated on Federal Government initiatives on IWRM that have implications for the State (Section 11). In order to carry out his functions, the Director may issue a directive requiring any person or body to provide assistance or data as well as to make inquiries (Section 11).

The functions and roles of the Board and the Director are useful in setting out the platform for collective action, direction and measures relevant for ensuring sustainable agriculture. In addition, with the delineation of river basin areas, issues pertaining to allocation and measures to address issues that may affect availability and quantity of water required for agriculture, including measures to address threats and risks to agriculture and water resources can be looked into. Nine aspects are considered in determining the best measures to help ensure water-resource sustainability for agriculture.

River Basin Committees

The Board has powers to identify and publish in a gazette delineated river basin districts, with each district having a River Basin Committee responsible for investigating matters that affect management of the area; assisting the Water Resources Director in the preparation of river basin plans and other related reports; and devising programmes stipulated in the river basin plans (Section 15). In carrying out their functions, committee members are required to consult relevant stakeholders from government agencies and members of the public.

Section 15 of the 2008 Enactment does provide an opportunity, where sustainable water resources and agriculture are concerned, to firstly engage all stakeholders, be they mandate holders, users or beneficiaries of the plan. This will allow for concerted action to ensure that each plan devised is able to balance different needs as well as meet as far as practicable the different expectations of different users and mandate holders. This platform can help ensure that the point of conversation will be centred on the river basin, and that a collaborative approach is taken.

The committee will also have an opportunity to consolidate all relevant indicators and benchmarks that will be brought together from a river basin perspective. This will allow discussions on development of indicators that have bearing on water and agriculture, such as scarcity indicators, particularly during incidences of drought. It can also address issues that may arise in relation to determining appropriate volumetric control, including rate of intake, that will aid effective allocation during rainy and dry seasons, as well as weigh options to

put into effect better storage and recharge of catchment areas.

River Basin Plans

Section 7 of the 2008 Enactment provides the Board with powers to approve river basin plans and development projects related to integrated water-resource management, guidelines and procedures; to advise and assist the State Authority in formulating or reviewing legislations; as well as to designate and divide the State according to its river basin districts. Section 20 makes provisions for the preparation of river basin plans in consultation with relevant agencies that have a stake in the river basin. A platform is also made available in Section 21 for the public to provide comments and objections to any plans to be approved in Section 20. The approved plans will be translated through the mandate of respective key agencies and stakeholders (which includes the private sector and community groups).

The plan should include, as outlined in detail in Section 20, three key components that look at policy implications; state of water, including activities and impacts that have influence, with measures to address them; and measures for conservation, protection, development and use. This would require setting out a statement of objectives; references to related policies and plans; identification of water resources; state of quality and quantity including conditions and development trends; activities that influence quantity and quality with impact assessment; water quality objectives for each water body; strategies and measures for protection, conservation, development and use including quality improvement; inclusion of indicators; identification of water reserve areas; variation of river reserves; and areas of extraction of sand and rock material. Each plan is subject to comments and objections from the public.

This provides a great opportunity for complementing the existing physical plans, and to an extent, influence existing structural and physical development plans that have been gazetted by the State. The plan can be framed to include six keys aspects that can help ensure water resources and agricultural sustainability i.e.:

- Identify priority use and users according to seasonal weather changes, and anticipated climatic hazard events, such as extreme drought or El Nino;
- Develop use-based allocation, to ensure that the rate of extraction will remain within acceptable thresholds that are set by the River Basin Committee that is specific to different areas and conditions;
- Develop specific measures to address environmental needs to help reduce risks, threats and impact arising from changing climatic conditions or incidences of hazards such as landslides and riverbank erosion;
- Identify and develop specific measures that will allow for adaptation to climatic changes and hazard incidences, taking into account multiple needs, conditions and users (human and environment),

particularly environmental needs, source availability including man-made sources (e.g. recycled rain water or treated wastewater) and rate of recharge;

- Develop specific response and management plans in times of drought, flooding or other hazard events or incidences of environmental impact;
- Explore and set out options for on-site (paddy areas) water storage and capture.

Abstraction and Irrigation

Abstraction of water from any water body will require a license, as per Section 24 of the 2008 Enactment, which will only be granted if it does not pose any threat to or have an undue adverse impact on the quality, quantity or flow of the water, or the water environment or conflict with any river basin plan that has been adopted. The licence for abstraction, however, does not apply for irrigation for paddy cultivation in declared irrigation areas (Irrigation Areas Act 1953) or under the Muda Agricultural Development Authority Act 1972.

Exploration too is regulated, where abstraction or use of drilled ground water or enlargement of existing wells or drilling of land for the purposes of ground water exploration will require a licence (Section 26 of the 2008 Enactment). A copy of the licence is to be transmitted to the Director General of the Minerals and Geosciences Department within 30 days of issuance. The Director General, under the Geological Survey Act 1974, regulates aspects related to information and methods related to the drilling of wells. Exemption is also given for abstraction for households and subsistence agricultural use from ground water on his premises or from any water body with frontage to the premises owned or occupied by him, provided that the amount abstracted in a day does not exceed 10 m^3 or serve more than 20 persons. In the case of ground water, the amount abstracted in a day must not exceed 2500 L and the abstraction does not require a well to be drilled (Section 27).

Section 28 of the 2008 Enactment provides that no one can divert or disrupt the natural flow of water in a water body or put or cause or permit to be put or to fall or to flow into any water body any object that may interfere with the natural flow of water. Anyone who wishes to do any of the two prohibited acts can only do so if they are permitted by a licence issued by the Water Resources Director.

These sections provide a good opportunity for the Board to develop clear measures that will provide the means:

- to control and gather information pertaining to all manner of abstraction, irrigation and diversion, based on licence application, that in return will allow for better understanding of the rate, frequency and types of use, not just those for agricultural purposes;
- to provide measures to determine the rate of abstraction and scale of diversion;
- to establish measures to set volumetric control and rate of intake;

- to develop strategic policies for abstraction and diversion, taking into account environmental and social factors;
- to explore options for setting quotas during drought seasons or hazard events; and
- to explore options for deficit irrigation.

Control of Contaminants and Sewage Discharge

Part VI of 2008 Enactment addresses aspects related to protection of water resources. In Section 29, the discharge of contaminants into any water body or land is prohibited except when a licence has been granted according to the requirement of the provisions. Under Section 31, effluents may be permitted to be discharged if licensed for under the Environmental Quality Act 1974 or the sewage is discharged into a sewerage system in a sewerage services area, with added provisions that the person doing so is required to avoid, remedy or mitigate any adverse effects on the water body or environment arising from the licensed activities. This allows the Board to set the threshold for helping to control elimination or total removal of contaminants or effluent discharges. This also allows the setting of timelines for phasing out discharge.

Control of Activities

The Board is also responsible for certain aspects of control of land use activities in Kedah, as provided for in Section 32, where activities related to water bodies are regulated on land, particularly earthwork. An application will have to be made to relevant authorities for approval, and an earthwork plan will have to be sent to the Water Resources Director, who will make recommendations on measures relating to erosion mitigation and sedimentation control.

The 2008 Enactment also looks at the protection of special areas, river reserves and water conservation areas. Section 35 makes provisions for the control of activities in water reserves, which involves the removal of natural vegetation or material, felling of trees, erection of structures or buildings, carrying out of agricultural activities or alteration to or interference with a water body. The State Government of Kedah, on advice of the Board shall gazette areas of land or water bodies as water conservation areas and the Director of the Kedah Water Resources Board may direct the occupier or the owner of the premises within those water conservation areas to take measures or action to conserve and protect the area.

Section 39 makes provision for the extraction of sand and other rock material. According to the provisions, no person shall extract sand or rock material unless that extraction is carried out at a sand mining site in an approved river basin management plan or at a site located in an area not covered by a river basin plan, provided that application is accompanied by studies by experts showing that the sedimentation rate is sufficient even though there is an extraction, or removal is required for flood mitigation purposes or for maintenance of navigation channels.

Recreational activities are also controlled under the 2008 Enactment in Section 40, where owners or operators of all recreational or leisure activities on or in water bodies have to take appropriate steps to avoid causing damage to the bed, banks or shore of a water body; or contamination of a water body; or obstruction to navigation; or danger or being a nuisance to any person or property. The Water Resources Director can from time to time issue directives spelling out measures to help avoid or reduce any of the impacts arising from recreational use.

These provisions, when read with the larger river basin management plan, can actually address issues arising from multiple use and users, as well as conflicting interests, particularly during extreme weather conditions. It will allow for measures and procedures to be set out so as to ensure that limits are set and risks to water resources minimised.

Protection of Special Areas

Two key areas are addressed in the 2008 Enactment, river reserves and water conservation areas. The 2008 Enactment states that river reserves are areas (Section 34):

- within 50 m of the top of the bank of a river, including its estuary, where the river channel is 40 m or more than 40 m in width;
- within 40 mof the top of the bank of a river, including its estuary, where the river channel is 20 m or more than 20 m but less than 40 m in width;
- within 20 m of the top of the bank of a river, including its estuary, where the river channel is 10 m or more than 10 m but less than 20 m in width;
- within 10 m of the top of the bank of a river, including its estuary, where the river channel is 5 m or more than 5 m but less than 10 m in width; or
- within 5 m of the top of the bank of a river, including its estuary, where the river channel is 1 m or more than 1 m but less than 5 m in width.

The Board can recommend to the State Authority to either reduce or increase the distances in respect of specified river reserves in existing built-up areas, particularly for flood mitigation purposes. Section 35 makes provisions for activities within river reserves that require a licence. These activities include removal of natural vegetation, felling of trees or the removal or deposition of any material; erection of a structure or building; operation of a commercial or agricultural activity; and alteration to, obstruction of or interference with any water body. This is to ensure that the activities do not cause a reduction in the volume or flow of water or degradation to the quality of water or the water environment. If they do, the owner or occupier is required to either modify or cease the activity; or modify, relocate or remove the structure or building; or restore the river reserve or water body to the condition in which it was immediately prior to the carrying out of the activity or the erection of the structure or building.

In order to protect water bodies or even land areas so that they are adequately protected from change in flow, contamination or degradation or to ensure they can serve as a water catchment area for an impounding reservoir or water supply intake, the 2008 Enactment in Section 36 makes provisions for the declaration of such areas, setting out:

- the limits of the area and the purpose of declaring such areas as a water conservation area;
- the types or classes of activity or development and the specific measures or work that apply;
- the types of activities that are prohibited;
- the terms, conditions and restrictions that apply to activities, measures undertaken or development within the area respectively; and
- the body empowered to manage the area.

The Water Resources Director also has additional powers in Section 37 to direct owners or occupiers in a water conservation area:

- to take measures to slow down, reduce or prevent water from running off the premises into a water body;
- to plant specific types of vegetation;
- to relocate structures;
- to undertake an activity, including an agricultural activity, in a specific way;
- to take such other measures as the Water Resources Director may specify to prevent degradation of water resources; and
- to restore the river reserve or water body within the area to the condition in which it was immediately prior to the commencement of the activities or development in the area.

These sections provide the Board with powers that can ensure the development and adoption of special measures to protect and conserve special areas that can help balance the needs of different users and ensure water-resource sustainability. This can be adopted in river basin management plans to ensure that not only are allocation and distribution strategically planned, but conservation measures are put into place and carried out by owners or occupiers of these areas. It does seem to suggest that a partnership can be formed between the authority and the public so as to ensure that water bodies and their land area are protected. This is critical for sustainable agriculture, as it will allow for clear direction and procedures to ensure balance between multiple use and users.

Minimising Threats, Impact or Risks in Extreme Events

Sections 41 to 43 of the 2008 Enactment makes provision for emergency measures in cases of acute contamination, drought and flood. In case of accidents or events resulting in significant undue contamination of any water body, or which gives rise to the risk of such contamination, the person involved has a duty to promptly take such

measures as are necessary to minimise the impact or risk to the environment and to promptly inform the police or the Water Resources Director (Section 41). The Water Resources Director in turn may issue directives setting out actions required to add any threats to public health or safety or to the environment. The power to redress harm is spelt clearly, and this is useful in cases where immediate action is required.

In cases of drought (Section 42), the Board may, by reason of an exceptional shortage of rain, where a serious deficiency of supply of water in any area exists or is threatened, abstract or direct any person to abstract water from any water body or discharge water to a place specified in a specific directive, or prohibit or limit the abstraction by any person of water from any water body or the supplying of water to any person. This provision allows for the establishment of an allocation plan for cases that will need help to ensure recharge of water bodies or minimisation of usage of any water body. This will require a set of strategies for action to be outlined in a river-basin management plan.

Section 43 of the 2008 Enactment sets out measures to control and manage floods that includes the formation, organisation and operation of flood defence committees for river basins; the adoption of mitigative measures to lessen the impact of flooding; and steps necessary for the proper management of flood defence. This sets the premise for an opportunity to develop a comprehensive plan to address events that give rise to threats and risks to water bodies and their surrounding land area.

Licensing

Section 44 of the 2008 Enactment sets out a detailed description of what is required in licences. Licences require information that states the nature of the relevant activity and all the works and measures necessary to undertake the activity and the measures to control and mitigate any adverse effects of the activity on the water environment. The Water Resources Director can also request information on the use of the best available techniques pertaining to measures that are proposed or should it not include such techniques, the applicant will have to include information on the efficiency and effectiveness of the alternative techniques.

What is critical here is that Section 45 mandates that the Water Resources Director publish details of any significant activity requiring a licence in at least two local newspapers, and objections are invited from the public. This form of public engagement is positive, as it allows for better feedback and to an extent, is a platform for informed consideration. Depending on the objective lodged, the Water Resources Director may require an applicant to also conduct a public forum. Significant activities are defined as (Section 45):

- any activity that in the opinion of the Water Resources Director is likely to have a significant impact on the quality, quantity or flow of water or on the environment;

- water abstraction at a rate of more than 100 m^3 per day from ground water or from a river of less than 5 m in width or any other maximum rate stipulated in directives under this Enactment;
- water abstraction at a rate of more than 500 m^3 per day from a river of 5 m or more in width, or any other maximum rate stipulated in directives under this Enactment;
- waste water discharge at a rate of more than 10 m^3 per day or any other maximum rate stipulated in directives under this Enactment;
- river works and structures that may obstruct navigation; or
- any other activity prescribed by the Board as significant.

It can be said that licence issuance is not merely an administrative process, particularly for significant activities, where the public has a role to play. In cases where planned development is underway with activities that may impact a water body, a public forum may result, and in the spirit of sustainable agriculture, public engagement is one of the tenets that has been pushed forward for consideration.

Licences are also subject to variation (Section 51) if there has been a significant change in circumstances, which include the following:

- new information on adverse effects on human health or the environment caused by contamination is obtained, or legislation laying down environmental quality standards have been changed;
- new techniques have become available that make it possible to reduce the environmental impact of the activity significantly;
- the operational safety of the process requires other techniques to be used; or
- reconsideration is required by other legislation.

CONCLUSION

As agricultural activities are heavily dependent upon water resources, the need to restructure water-resources management and changes of agricultural practices are required. Present agricultural practices require a large quantity of water and can be the main polluter of water resources. The concept of sustainable agriculture can increase productivity and yields of agricultural products without compromising the need to sustain ecosystem functions as well as to conserve water resources, both in quantity and quality. In terms of resolving disputes among water users for multiple use and for better decision making, a regulatory framework is the best option for managing water resources.

The Kedah Water Resources Enactment 2008 provides the regulatory means that can help push the sustainable agriculture agenda through sustainable use of water resources. What is critical is the formation of a River Basin Committee to bring together all key stakeholders and the

development and adoption of a river basin plan that includes directions and measures related to priority use and users; allocation, abstraction and extraction; ways to reduce environmental risks, threats and impacts and incorporate environmental needs in decision making, planning and licensing; adoption of measures to address and allow for adaptation to climatic changes and hazard incidences; addressing environmental needs, particularly environmental flow and recharge of water bodies; and development of specific management and response plans to address droughts, floods, environmental impacts and other hazard events.

The plans could also look at the adoption of measures as well as long-term strategies for water storage and capture, recharge and water-resource augmentation, focusing on water resources security. An allocation plan for multiple use of water resources would also be beneficial, particularly in times of crisis. The 2008 Enactment also makes room for public engagement, which would allow for better resource planning and agriculture development, and this should be capitalised on to ensure informed planning and development of the agricultural sector in Kedah. Examples drawn from this can perhaps be considered in other states in Malaysia that have specific enactments for water regulation.

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Review Article

Sperm DNA Impairment in the Bull: Causes, Influences on Reproduction and Evaluations

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ABSTRACT

Conventional semen examination involving sperm motility, viability and morphology remains the backbone of assessing the fertility status of a sire. However, there remains instances where these semen parameters appear normal but cases of low conception rates or failure of pregnancy occur. This review highlights the causes of sperm DNA damage and the effectiveness of techniques designed to evaluate the contribution of sperm DNA damage to lowered fertility in bulls. Among the many causes of sperm DNA impairment are imperfect spermatogenesis, faulty apoptosis, reactive oxygen species, *in-vitro* handling, impact of environment, radiography and the stress of cryopreservation processes. Furthermore, DNA impairment impairs fertilisation, interferes with embryonic development and implantation and blocks blastocyst formation. The most frequently used tests to determine DNA damage are the acridine orange test (AOT) using acridine orange stain with examination under a fluorescence microscope and the sperm chromatin structure assay (SCSA) using the same stain but examined with flow cytometry.

Keywords: Sperm, DNA impairment, sperm DNA evaluation

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INTRODUCTION

The integrity of sperm DNA of bulls is critical for assisted reproductive technology in cattle. It permits agriculturalists to improve and develop their breeds in

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response to rural development. Sperm are haploid cells and the chromosomes in them are mono-chromatid structures. The sperm cell nucleus is mainly occupied with DNA, which is responsible for approximately 40% of its dry matter. Bull sperm comprise 3.44 pg DNA/nucleus (Bochenek et al., 2001). The cell nucleus is the most vital component of sperm ultrastructure, as fertilisation efficiency is influenced by the presence of a normal structure. Chromatin of sperm is systematised in toroids that are steady and solid structures that are attached to nuclear matrix through toroid linker regions. These linker regions are the most vulnerable to DNA injury (Sotolongo et al., 2003) with single (ssDNA) or double strand breaks (dsDNA; Aitken et al., 2013).

In both bull and human spermatozoa, there is a high quantity of chromatin heterogeneity (Evenson et al., 1980a; Takeda et al., 2015). Even so, conventional semen examination comprising examination of sperm motility, viability, sperm count and morphology has remained the pillar of examining semen-associated male factor of infertility. However, there remain some conditions where these parameters are all in the 'normal' range but the male has low or reduced fertility (Dietrich et al., 2005). Simon and Lewis (2011) found that from all conventional parameters such as those stated above there was only one negative correlation (r=0.21) between sperm DNA impairment and progressive motility. In addition, Venkatesh et al. (2011) found out that 15.5% of idiopathic infertile males had normal semen parameters. Hence, sperm features are essential for considering not only the conventional parameters but also for assessment of DNA, acrosome and the fertilising ability of sperm. This review aimed to discover the causes and also the effectiveness and evaluations of sperm DNA impairment in bulls.

Causes of Sperm DNA Impairment

Imperfect spermatogenesis. DNA deficiency in sperm could largely be due to imperfect spermatogenesis (Manicardi et al., 1995; Sailer et al., 1995). One example of imperfect spermatogenesis is the presence of vacuoles in the sperm head. This vacuole appearances could be associated with chromatin destruction (Oliveira et al., 2010; Franco et al., 2012). Furthermore, the percentage of fertility rate was correlated negatively with sperm vacuoles (Berkovitz et al., 2006). The sperm head comprises DNA almost fully and it has been reported that in case of alterations in DNA structure, morphologic abnormalities are expected (Enciso et al., 2011). Nevertheless, several studies have revealed that sperm containing DNA dicondensation do not essentially present morphologic alterations (Beletti, et al., 2005; Soares & Beletti, 2006). An earlier study by Beletti and Mello (2004) showed a great positive relationship between primary sperm defects and DNA alteration, suggesting that sperm DNA structure affects the morphology of sperm head. Furthermore, Kipper et al. (2017) found a strong relationship between compaction of DNA and the morphometry of the sperm head in Nelore bulls.

In the course of spermiogenesis, histones, the dominant protein in spermatocyte nucleus, are replaced by protamines in mature sperm (Zhao et al., 2004). The protamines of sperm are essential for providing structural rigidity and maintaining highly condensed sperm DNA packing (Miller et al., 2010). In fact, it is now known that a reduction in sperm protamine content could lead to increased sperm DNA impairment (Fortes et al., 2014). Thus, modification in chromatin rebuilding during the process of spermatogenesis might result in DNA impairment (Marcon & Boissonneault, 2004).

Faulty apoptosis. Programmed cell death (apoptosis) is an important factor offered to remove unnecessary or damaged sperm cells throughout spermatogenesis. In the development of the sperm germ cell through spermatogenesis, Sertoli cells take control to induce apoptosis in 50% of sperm cells that enter meiosis I (Mahfouz et al., 2009); however, due to poorly understood mechanisms, this procedure might not operate well and some imperfect germ cells that have evaded apoptosis might progress on to the process of spermiogenesis (Burrello et al., 2004). In the process of apoptosis, anti-apoptotic protein (BCl-2) and pro-apoptotic protein (BAX) deliver a signalling pathway that supports and maintains balance in a cell. The prorated levels of these proteins are important for a feedback mechanism. In fact, in the course of spermatogenesis, pro-apoptotic protein acts as a checkpoint for keeping the quality of sperm (Oltvai & Korsmeyer, 1994; Dogan et al., 2013).

Reactive oxygen species production before ejaculation. It is clear that excessive reactive oxygen species (ROS) induces DNA impairment (Moustafa et al., 2004). The source of ROS before ejaculation can be from immature sperm or from epididymal epithelial cells. Studies showed that immature sperm in *cauda epididymis* produce a high amount of ROS and these could affect negatively on the DNA of mature sperm (Ollero et al., 2001). Furthermore, epithelial cells of epididymis, which could be affected by environmental factors, play an important role in increasing the amount of ROS. This would cause an increase in the antioxidant intake and thus, could reduce the harmful effect of ROS on sperm DNA (Sakkas & Alvarez, 2010). Although the internal anti-oxidant enzyme capacity of Karan Fries bulls was increased in hot dry and hot humid seasons, the ROS and malondialdehyde were significantly higher in these seasons compared to in winter or spring (Soren et al., 2016) and significantly correlated with seminal quality.

Impact of environment. Fluctuating environmental temperatures induce sperm DNA impairment (Karabinus et al., 1997). Bovine semen quality alterations due to season have been recorded even though bulls are not considered seasonal breeders (Menegassi et al., 2015; Malama et al., 2017). In dairy bulls there is a strong decline in semen output owing to the stress of

temperature and humidity (Al-Kanaan et al., 2015). Lucio et al. (2016) revealed that scrotal heat stress, which is induced by scrotal insulation in crossbred bulls, led to moderate to strong alterations in all sperm head morphometric measures and DNA integrity. Moreover, the roundness of the sperm head is a major reflection of heat stress. The adverse effects of rising testicular temperature might have an effect on meiotic stages of spermatogenesis and can result in remodelling of sperm DNA (Rahman et al., 2011; Lucio et al., 2016). In contrast, Malama et al. (2017) found that DNA integrity seemed to be stable over the course of the seasons. Values of DNA impairment of frozen-thawed semen of Holstein-Friesian bulls did not change in winter and summer. Whether extremes of temperature significantly affect sperm DNA is still not fully understood as Michael et al. (2013) found that 91% of bulls from tropical environments that are characterised by high environmental temperature and humidity do have stable sperm DNA. It should be stressed here that males fed based on sub-optimal feeding requirements have higher sperm DNA impairment and reduction in testicular mass, sperm motility and total sperm count in ejaculates (Aitken et al., 2012; Guan et al., 2014). The common DNA impairment in males fed sub-optimally is incomplete development of spermatid during spermiogenesis. This could be due to the positive relationship between DNA impairment and poor chromatin packaging as a result of under protamination concentration in complete sperm (Gorczyca et al., 1993; Guan et al., 2014).

***In-vitro* handlin**g. Sperm DNA can undergo impairment through handling and shipment after collection (Bollwein et al., 2008; Jenkins et al., 2015). Imperfect handling of fresh, chilled or frozen-thawed semen might lead to a change in pH, decrease or increase in temperature and increase in the amount of ROS (Jenkins et al., 2015). Therefore, proper handling of semen is priceless. Collecting tubes, sperm handling, light exposure, washing, semen processing and any sub-optimal condition of ejaculated semen can increase the risk of DNA impairment (Drevet, 2016) because spermatozoa are exposed to environments that are different from the physiological.

Radiotherapy and chemotherapy. It has been stated that exposure to radiotherapy or chemotherapy could cause sperm DNA impairment. This is highlighted in a study by O'Flaherty et al. (2008), who found that the integrity of sperm DNA was affected in patients with testicular cancer after they were given any chemotherapy (O'Flaherty et al., 2008).

Chilling and cryopreservation of semen. Storage of semen in chilled temperatures results in excessive production of ROS (Crespilho et al., 2014; Daramola & Adekunle, 2015), and this directly affects sperm DNA (Morte et al., 2008). The processes of thawing frozen semen could

also lead to DNA impairment (Holt, 2000; Métayer et al., 2002; Gadea et al., 2008; Kumar et al., 2011; Papa et al., 2015; Ezz et al., 2017), which could greatly affect the fertility status of the semen.

In fact, the mechanisms accountable for freezing and chilling induced DNA impairment are not properly understood. Data suggest that the consequence of lipid peroxidation (LPO) in injurious sperm chromatin (Kasimanickam et al., 2007; Kumar et al., 2011). Simões et al. (2013) indicated that there was a negative relationship between intact DNA and the amount of ROS in bull semen. On the other hand, Gürler et al. (2016) revealed that not all kinds of ROS are harmful to sperm DNA as only H2O2 is related to DNA impairment. Moreover, sperm nuclei exposure to high ionic strength in the course of cryopreservation instigates deterioration of chromatin assembly and sequentially, makes post-thawing nuclear DNA available to oxidative occurrence through extra or intracellular ROS (Gadea et al., 2008; Makker et al., 2009; Simões et al., 2013). Additionally, perhaps, unrestrained post-thawing influx of wondering calcium ions in frozen sperm (Holt, 2000) may possibly encourage additional splitting of nucleoprotein and DNA through endogenous protease and nuclease stimulation (Métayer et al., 2002).

Influence of Sperm DNA Impairment on Reproduction

To date, there is no dependable data on the effects of DNA impairment in bulls during embryo development or pregnancy product (Kipper et al., 2017). Spermatozoa lack a DNA repair mechanism, and this could be due to their unique structural differentiation. In fact, spermatozoa are metabolically silent with high condensed chromatin that is unable to self-repair damaged DNA (Smith et al., 2013). On the other hand, oocytes have the ability to repair both its own DNA and the DNA sperm, and this repair occurs after fertilisation prior to cleavage. However, sometimes, this repair is possible because of a high level of sperm DNA impairment or owing to low oocyte repair activity (Drevet, 2016). Likewise, Evenson et al. (1980a) revealed that sperm with denatured ssDNA could have decreased fertilisation efficiency both *in vivo* and *in vitro*. Around 15% of sperm population with impaired DNA is usually considered normal, whereas a value between 15% and 25% will result in decreased fertility, and much higher values such as 25% and above signify a higher chance of infertility (Larson-Cook et al., 2003; Michael et al., 2013). Despite these figures, some studies have reported decreased fertility in bulls having 10% injured sperm in their semen (Bochenek et al., 2001). Kipper et al. (2017) found that 4% to 16.15% of sperm with DNA impairment did not reduce in-vitro embryonic development until Day 8. Similarly, Fatehi et al. (2006) confirmed that DNA impairment in bull sperm did not prevent fertilisation and early embryonic development. However, it can lead to induced apoptosis after the first cleavage.

The presence of any damage(s) to the sperm ssDNA can reduce the success of fertilisation (Simon & Lewis, 2011; Ribas-Maynou et al., 2012), while damage(s) to the sperm dsDNA could lead to a disturbance in embryonic development (Lewis & Aitken, 2005), commonly resulting in cases of miscarriage (Lewis & Simon, 2010). A contrary opinion to this has been presented by Fatehi et al. (2006), who revealed that sperm DNA impairment does not cause any problems in fertilisation of the oocyte or in first, second or third cleavage achievement, though it can stop formation of blastocysts through apoptosis induction i.e. low sperm DNA impairment can be successfully repaired by either the ovum or the embryo leading to the birth of normal offspring. Early embryonic death or abortion is only linked with higher assault to sperm DNA (Wyrobek et al., 2006). The involvement of sperm DNA damage in reducing breeding effectiveness of bulls after artificial insemination (AI) (Bollwein et al., 2008) permits consideration of factors that could lead to deviation in the chromatin structure in the course of *in-vitro* semen processing. Spermatozoa with DNA impairment have the potential to interrupt genetic and epigenetic rule of embryonic growth (Aitken & De Iuliis, 2007). Recent studies revealed that higher proportions of intact sperm chromatin correlates with improved fertilisation success and normal embryonic growth (Fatehi et al., 2006; Khalifa et al., 2008). Fatehi et al. (2006) induced impairment for sperm DNA by exposure to irradiation with X- or Gamma rays. The results showed that embryonic growth was totally blocked at Day 7 and the blastocyst percentage reduced from 28% in non-irradiated sperm to less than 3% in irradiated sperm.

Sperm exposure to traumatic situations such as cryopreservation may compromise the mechanism, resulting in inhibited fertilisation or embryonic growth (D'Occhio et al., 2007; Kasimanickam et al., 2007; Khalifa et al., 2008). Kasimanickam et al. (2007) and Lymberopoulos and Khalifa (2010) showed that a rising proportion of sperm DNA impairment was related to declining field fertility of cryopreserved bull semen. Similarly, Khalifa et al. (2008) indicated that a substantial negative association exists between the occurrence of sperm DNA impairment in cryopreserved semen and the developing capability of bovine embryos *in vitro*. It is likely that the procedure of semen thawing could have a negative or positive effect on chromatin uncertainty. For instance, fast thawing of cryopreserved semen straws at a high temperature (45°C for 30 s), is measured by an empirically resultant procedure in minimising intracellular hyper-osmotic trauma at the course of re-warming of sperm handled at a high freezing rate (Hammerstedt et al., 1990). Furthermore, a 240-min incubation of cryopreserved semen at 25°C or 39°C doubled sperm DNA impairment frequency, with the degree of increase being intense at 39°C and advancing the biologically important limit (Bollwein et al., 2008).

It has been suggested that antioxidants safeguard frozen spermatozoa from DNA disintegration (Gadea et al., 2008; Kumar et al., 2011). The defence mechanism of sperm cell against nuclear DNA injury depends on the effectiveness of their chromatin compaction, stoppage of endogenous nucleases and degree of extra and intracellular antioxidants (Aitken & De Iuliis, 2007; D'Occhio et al., 2007). A notable decrease in sperm DNA impairment occurrence was seen after frozen-thawed semen centrifugation and extracellular milieu elimination, the main cause of post-thawing production of ROS in the existence of egg yolk and dead sperm cells (Vishwanath & Shannon, 1996).

Research shows that bull semen cooled in egg yolk extenders could not upset sperm chromatin steadiness (Waterhouse et al., 2006; Khalifa et al., 2008) and that there was no relationship between sperm movement, DNA injury incidence, agglutination and LPO (Lymberopoulos & Khalifa, 2010). DNA impairment was negatively related to the fertility of the bull sperm (García-Macías et al., 2007). Moreover, there was negative association of sperm DNA injury with capacitation status, viability and membrane reliability in buffalo (Pawar & Kaul, 2011). It should be noticed that fresh semen may contain a huge number of dead and degenerated spermatozoa with DNA impairment (Liu & Liu, 2013). Thus, the consequence of DNA injury of ejaculated sperm does not correctly replicate DNA position of motile sperm portion. Consequently, clinical evaluation of sperm DNA impairment ought to be done in the motile sperm portion and not the entire ejaculated sperm using, for instance, the swimming up procedure (Madrid-Bury et al., 2003), self-migratory method (Makler et al., 1984) or the discontinuous Percoll gradient procedure (Berger et al., 1985) to collect solely the motile sperm before doing a DNA integrity test.

Evaluation of Sperm DNA Integrity

Evaluation of sperm DNA integrity has a stronger capability to predict the fertility rate of semen than other conventional parameters such as percentage progressive motility (Simon & Lewis, 2011). A number of tests have been used for the evaluation of sperm DNA integrity including the acridine orange test, sperm chromatin structure assay, TUNEL assay, single-cell gel electrophoresis, sperm Bos-Halomax assay and sperm chromatin dispersion test.

Acridine orange test (AOT). The most regularly used test instrument for DNA impairment identification is the acridine orange staining test, which permits sperm chromatin steadiness detection in an acidic environment. The dye has metachromatic characteristics. Spermatozoa having double-stranded DNA discharges fluorescence in the green band, whereas those having RNA and single-strand DNA discharges red fluorescence.

DNA-intercalating dyes such as acridine orange have proved useful for examining alterations in chromatin packaging. The composition of normal double-stranded

DNA results in the spatial separation of acridine orange molecules, causing them to act like the monomeric form of the dye and emit green fluorescence. When excited by a 488-nm light source, in the presence of denatured (single-stranded) DNA, the dye molecules bind electrostatically to the strands and to each other to form aggregates, whereby dye-dye interaction causes a concentration-dependent loss of absorbed energy and a subsequent metachromatic shift to red fluorescence (Evenson et al., 1980b). The microscopic assessment of sperm chromatin integrity classifies acridine orange-stained spermatozoa as normal if fluorescing green and abnormal if red. However, this visual classification introduces some subjectivity to the assessment, since the emission spectrum from individual spermatozoa is often a mix of wavelengths, with stained spermatozoa appearing yellow to brown and not clearly identifiable with either category.

The microscopic AOT has demonstrated a significant relationship with male infertility and with fertilisation and pregnancy rates in IVF, independent of other sperm characteristics including sperm zona binding and morphology (Tejada et al., 1984; Liu & Baker, 1992).

Sperm chromatin structure assay (SCSA). Automation of the detection and analysis of AO fluorescence is provided by flow-cytometry (FCM) and employed in the sperm chromatin structure assay (SCSA), which was initially developed by Evenson et al. (1980b) as a potential measure of livestock fertility. Flow-cytometry provides a powerful statistical advantage over manual microscopic methods through objective and rapid multi-parametric analysis of large numbers of cells. The SCSA assay assesses the integrity of sperm chromatin structure, using AO as an enquiry to quantify the vulnerability of sperm DNA to in-situ-induced denaturation. The original assay measured heat-induced denaturation, but this has been replaced with the acid-detergent treatment (pH 1.2, Evenson et al., 1986). Spermatozoa with a normal chromatin structure appear impervious to treatment, while spermatozoa with an abnormal chromatin structure undergo partial denaturation (Evenson et al., 1980a). The SCSA is a flow-cytometric technique, while AOT is a microscopic method, and the two quantify the metachromatic shift of acridine orange fluorescence from green (native DNA) to red (denatured DNA), and the two methods mostly determine the DNA impairment with the toroid linker region. The SCSA evaluation has been commonly used to assess sperm DNA excellence in bulls through FCM (Januskauskas et al., 2001, 2003; Waterhouse et al., 2006; Fortes et al., 2012; Michael et al., 2013; Serafini et al., 2015).

TUNEL assay. Another method for DNA fragmentation assessment is using the TUNEL assay. The TUNEL assay identifies double- and single-stranded DNA discontinuities by identifying a free 3-OH terminus with altered nucleotides in an enzymatic response with terminal

deoxynucleotidyl transferase (TdT) and may be examined using a microscope or through flow cytometry (Sharma et al., 2013) or fluorescence microscope (Takeda et al., 2015).

Single-cell gel electrophoresis (COMET) assay. In this assay, sperm DNA breaks move apart in the head region forming 'comets' after electrophoresis, while complete DNA is intact in the normal head location. The COMET assay includes embedment of sperm in agarose using a glass slide, electrophoresis and assessing DNA movement in the comet tails using a specific software programme in a computer. The COMET assays (i.e. alkaline and neutral) apparently permit stain admittance to the toroid linker and toroid regions (Shaman et al., 2007) for documentation of ssDNA and dsDNA disruption. Evenson et al. (2002) and Baumgartner et al. (2009) suggested that the neutral COMET assay recognises dsDNA disruption and closely related ssDNA disruption, while the alkaline COMET assay recognises ssDNA disruption only. In bulls, the neutral COMET assay identified higher DNA breakdown (i.e. higher tail moment) in non sex-sorted spermatozoa rather than in sex-sorted spermatozoa (Boe-Hansen et al., 2005).

Sperm chromatin dispersion test (SCD). SCD is a moderately new technique that is introduced to evaluate spermatozoa DNA disintegration (Fernandez et al., 2003). The SCD test is centred on the belief that spermatozoa having disjointed DNA fail to give the distinctive halo of discrete DNA loops that are witnessed in spermatozoa with non-disjointed DNA after acid denaturation and nuclear protein elimination. The sperm tail was removed during the process of the SCD test (Fernandez et al., 2003). Fernández et al. (2005) and Pawar and Kaul (2011) modified the test's protocol, so the tail was unbroken from the sperm head. Furthermore, the scoring patterns were four different categories in the modified method vs. five in the non-modified method (Pawar & Kaul, 2011).

Sperm Bos-Halomax assay (SBH). The Sperm Bos-Halomax (SBH) assay was established for evaluation of spermatozoa DNA reliability in bulls, and centres on the Spermatozoa Chromatin Dispersion Test (SCDt) for humans (Fernandez et al., 2003). The SBH assay is comparable to the COMET assay with the exemption that sperm treated are not visible in an electrophoretic field. Higher DNA disintegration gives rise to more halos, while less DNA disintegration produces fewer halos (García-Macías et al., 2007).

Sailer et al. (1995), Aravindan et al. (1997) and Chohan et al. (2006) detected a solid association between SCSA and TUNEL outcomes for spermatozoa DNA disintegration, while Simões et al. (2013) indicated that a positive correlation between sperm DNA impairment and susceptibility to ROS in two different methods, the SCSA and the COMET assay, but there was no correlation between the COMET assay and the SCSA assay. Furthermore, there was a

strong relationship between SCD and AOT in the experiment that was conducted by Pawar and Kaul (2011).

CONCLUSION

Sperm chromatin is arranged in toroids, which are steady and solid structures; these linker regions are the most susceptible to DNA damage with single- (ssDNA) or double-strand (dsDNA) breaks. DNA defects are most likely due to to defective spermatogenesis (genetic disorder), faulty apoptosis, extreme reactive oxygen species production, *in-vitro* treatment, impact of the environment, exposure to radiography and chilling and cryopreservation procedures. A level of approximately 15% sperm with injured DNA is considered to be quite normal. DNA damage impairs fertilisation, interferes with embryonic development and implantation and also blocks blastocyst formation. During fertilisation the damage on the ssDNA of the sperm can be repaired by the oocyte, while the dsDNA damage leads to early embryonic death or abortion. Antioxidants present in the semen may guard frozen-thawed sperm from DNA disintegration. There is no relationship between spermatozoa motility, agglutination, LPO and DNA impairment, while DNA impairment was found to be negatively correlated with the viability, membrane integrity, capacitation and fertility of the bull sperm. Hence, clinical evaluation of sperm DNA impairment ought to be performed in motile spermatozoa fraction than in the entire ejaculated spermatozoa. The most frequent tests that are used to determine DNA damage are the AOT and SCSA tests, as they enable sperm chromatin steadiness determination in an acidic environment. AOT and SCSA have demonstrated a significant relationship with male infertility and with fertilisation and pregnancy rates in IVF.

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Simple Net Rainfall Partitioning Equations for Nearly Closed to Fully Closed Canopy Stands

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ABSTRACT

Many net rainfall models have been developed, but they are often complex, data demanding and usable only for a specific vegetation type. The focus of this study was to develop and validate two simple equations (a two- and a three-coefficient equation) for nearly full canopies of oil palm, rubber and pine trees. Throughfall and stemflow data from seven past studies were used to determine the best-fit coefficients for the two equations. The three-coefficient equation was $Pn = Pg \times \exp [- \{0.3443 – (Pg / (58.9748 + Pg)\} \times 0.1639)]$ and the two-coefficient equation was $Pn = 0.7724 \times Pg – 0.5845$ ($R^2 = 0.91$), where Pn and Pg are the net and gross rainfall, respectively. To validate these two equations, field data collections were started. Thirteen rain gauges fit with data loggers were used for rainfall measurement. Three sampled trees were selected randomly for stemflow measurement and one rain gauge was installed at a nearby open area. Two error indices were used as a goodness-of-fit measure for equation accuracy: index of agreement and normalised mean absolute error. The results showed that the two- and three-equation equations performed nearly equally well. They predicted the net rainfall with an error of between 12 to 23% (ranked as "Fair" to "Good" in terms of overall equation accuracy) and with an index of agreement of more than 90%. The results showed that these two equations can be used fairly accurately to estimate throughfall and net rainfall, and, to a lesser degree, stemflow. Estimation errors occurred most probably because canopy and rainfall characteristics were not taken into account in the two equations.

Keywords: Interception loss, oil palm, pine, rainfall, rubber, stemflow, throughfall, water balance

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INTRODUCTION

Water balance refers to water input (precipitation and snowmelt) and output (evapotranspiration, ground water recharge and stream flow). The major input of water is from precipitation and output from evapotranspiration (Kerkides et al., 1996). Knowledge of water balance is useful in managing water supply; this includes irrigation, water erosion, flood and pollution control and water shortage control.

In some areas, the amount of rainfall is adequate for crop water requirements. However, some places experience insufficient rainfall to meet crop water requirements; therefore, irrigation is required (Odhiambo & Murty, 1996). Crop development, soil water movement and agricultural water management would be highly affected by the amount of irrigation during the crop growing season. This is true especially in arid regions, where water is scarce (Jalota & Arora, 2002).

The daily soil water balance within a plant's rooting system can be described in the following equation:

$$Pn + I + CR = P + ET_a + \Delta\Theta + OF \qquad [1]$$

where, Pn is net rainfall, I is irrigation, CR is capillary rise, P is deep percolation, ET_a is actual evapotranspiration, $\Delta\Theta$ is the change in soil water content and OF is surface runoff or overland water flow. All the above components are in the unit mm day^{-1}.

Tree canopies partition gross rainfall (rain above canopies) can be divided into throughfall, stemflow and interception. A portion of the rainfall is intercepted and temporarily retained by the canopies and would subsequently evaporate. This process is known as interception loss. Canopy interception loss is influenced by canopy architecture and meteorological properties (Crockford & Richardson, 1990). Canopy interception loss ranges from 10% to 40% of gross rainfall in natural forests (Zinke, 1967) and may even exceed 50% (Calder, 1990). Redistribution of throughfall and stemflow by canopies modifies evaporation, which plays an important role in water balance on local and catchment scales (Herbst et al., 2006, 2007). Both throughfall and stemflow have an important influence on the hydrological budget of forest ecosystems. The solute composition of rain also affects soil chemistry, nutrients and pollutants, soil moisture gradients, ground water recharge, soil erosion and the location of epiphytes (Ahmadi et al., 2009).

Other than influencing the hydrological budget, rainfall interception also contributes to weather pattern (Amell et al., 2002). Evaporation rates, for instance, are higher in forests than in short vegetation due to the former's higher aerodynamic conductance compared to that of the latter (Rutter, 1967; Stewart, 1977; Calder, 1979). Therefore, knowledge of the rainfall partitioning process is needed to predict the hydrological effects of a site. The importance of hydrology has received more attention after the mid-20^{th} century (Ward & Elliot, 1995).

Many interception models, such as the numerical, analytical and stochastic simulation models, have been developed

to predict interception loss (Herbst et al., 2006) to understand the water balance in a particular location. Muzylo et al. (2009), who reviewed several rainfall interception models, remarked that only three models out of 15 models are widely used today. This is because most rainfall models are data demanding and have intensive and complex calculations.

Hence, one of the questions that shaped this study was: *Can net rainfall be predicted by using only a single equation for different types of closed canopy without requiring detailed or intensive measurement and information?* The main objective of this study was to develop and validate a two- and a three-coefficient equation for rainfall partitioning parameters (throughfall, stemflow and net rainfall) under three nearly closed canopies of pine, oil palm and rubber.

MATERIALS AND METHOD

Development of the Three-Coefficient Equation for Net Rainfall

Net rainfall is assumed to decrease exponentially with increasing values of an empirical coefficient called G-factor. This can be described as:

$$Pn = Tf + Sf = Pg * \exp(-G) \quad [2]$$

where, Pn is the net rainfall (mm), Tf is the throughfall (mm), Sf is the stemflow (mm) and Pg is the gross precipitation (mm). The smaller the G, the greater the increase of the Pn, following an exponential function (Figure 1). Furthermore, it is assumed that G is related to gross rainfall, Pg, by a rectangular hyperbola relationship (Figure 1). G ranges between two extremes, G_{min} and G_{max}, so that with increasing gross rainfall (Pg), G decreases according to Eq. [3]:

$$G = G_{max} - \frac{Pg}{(C+Pg)} * (G_{max} - G_{min}) \quad [3]$$

where, G_{max} , G_{min}, and C are empirical coefficients obtained by minimising the error between fitted and observed values using the Microsoft Excel add-in called Solver (Microsoft Corp, Redmond, Washington, USA).

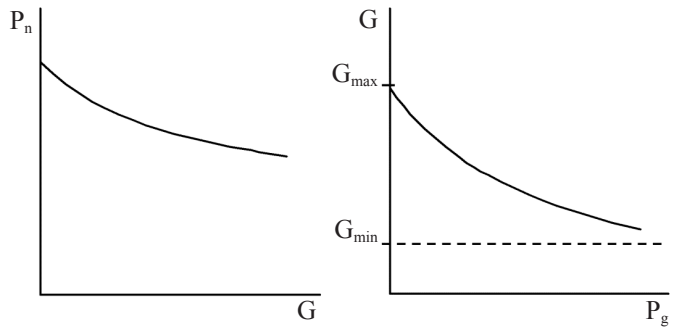

Figure 1. Three-coefficient equation: Net rainfall (Pn) is assumed to decrease exponentially with increasing value of a parameter called G which, in turn, follows a rectangular hyperbola relationship with gross rainfall (Pg). G varies between two extreme values denoted by G_{min} and G_{max}

The Two-Coefficient Equation for Net Rainfall

In addition to the three-coefficient equation for net rainfall, a two-coefficient equation, which is a linear regression equation, was also derived. The purpose of this derivation was to check whether a single-linear regression equation could be used for estimating net rainfall for various canopies.

Assumptions and Limitations of the Equations

These two equations do not take into account detailed information such as age of tree, leaf area index, canopy characteristics, stem characteristics, rainfall characteristics (amount, intensity and duration), wind speed and direction, temperature and other meteorological parameters. The two equations in this study are applicable only for nearly- to fully-closed canopy stands. The only input required is the daily gross precipitation.

Validation of Equations

Equation [2] and [3] were tested on seven studies selected from literature. These studies were selected because they provided raw data on rainfall partitioning (Table 1). Two error indices were used to measure the goodness-of-fit of these two equations. These indices were the index of agreement, d (Willmott et al., 1985; Legates & McCabe, 1999), and the normalised mean absolute

Table 1
Seven studies used to derive the two- and three-coefficient equations

Reference	Tree	Age (years)	LAI (m^2m^{-2})	Location	No. of Rain Days	Range for Pg (mm)	% I of Pg	% Tf of Pg	%Sf of Pg
Bentley (2007)	Oil palm (*Elaeis guineensis*)	8	NA+	Skudai Johor, Malaysia (1°43´N; 103°32´E)	55	2.50-98.40	41.08	56.93	1.99
Damih (1995)	Oil palm	NA	NA+	Skudai Johor, Malaysia	31	0.50-39.50	33.29	63.81	2.90
Lubis (unpublished data)	Oil palm	15	6.0	Pekan baru, Indonesia (0°32′0″N; 101°27′0″E)	252	0.50-153.00	29.62	68.82	1.56
Zulkifli et al. (2006)	Oil palm	8	NA+	Skudai Johor, Malaysia	21	0.20-36.32	32.17	65.10	2.73
Germer et al. (2006)	Tropical rainforest	NA	5.4	Rondonia, Brazil (10°18´S; 62°52´W)	97	0.50-78.23	2.38	89.81	7.81
Loustau et al. (1992)	Maritime Pine (*Pinus pinaster*)	22	3.0	Bordeaux, France (0°46´W; 44°-42´N)	32	0.30-62.70	17.38	79.07	3.55
Zulkifli et al. (2003)	Rubber (*Hevea brasiliensis*)	35	NA+	Skudai Johor, Malaysia	28	2.55-54.43	12.13	86.73	1.14
						Average	24.00	72.90	3.10

+ Mature trees, NA = not measured, I = Interception loss, Tf = Throughfall, Sf = Stemflow, Pg = Gross rainfall

error, NMAE. The second error index used was a modified form of MAE index (mean absolute error) from Legates and McCabe (1999). The estimated Pn from Eq. [2] and [3] were compared with field measurements and validated by these two error indices.

Normalized Mean Absolute Error, NMAE index is given by

$$NMAE = \frac{\frac{\sum_{i=1}^{N}|P_i - O_i|}{N}}{M_o} * 100 \quad [4]$$

where, P_i and O_i are the predicted and observed values, respectively; N is the number of observation, and; M_o is the mean observed values. NMAE is given in percentage. The lower the NMAE value, the more accurate the model's estimations. According to Jamieson et al. (1991), the overall prediction accuracy can be defined as "Great" when the NMAE ranges from 0 to 10%, "Good" from 10% to 20%, "Fair" from 20% to 30% and "Poor" for greater than 30%.

Index of agreement, d, is given by

$$d = 1 - \frac{\sum_{i=1}^{N}|y_i - \hat{y}_i|}{\sum_{i=1}^{N}\left(|\hat{y}_i - \bar{y}| + |y_i - \bar{y}|\right)} \quad [5]$$

where, y_i, $\hat{y}_i$ and $\bar{y}$ are the value of measured, value of estimated and average of measured, respectively. The error index ranges from 0 (worst fit) to 1 (perfect fit). The higher the d value, the lower the overall prediction error.

Field Studies

Field data collections were carried out at three sites; each site had different tree crops. The tree canopies were nearly closed. The trees were pine (*Pinus caribaea*), oil palm (*Elaeis guineensis*) and rubber (*Hevea brasiliensis*). A description of the sites is given in Table 2.

Table 2
Description of the three field sites in this study

	Pine	Oil Palm	Rubber
Location	Serdang, Selangor (03°00.067'N, 101°43.392'E)	Jengka, Pahang (03°53.882'N, 102°31.972'E)	Sungai Buloh, Selangor (03°09.502'N, 101°33.479'E)
Age of Trees, Years	28	12	26
Hill Slope, %	15	0-1	0-1
Mean Elevation, m	58	61	41
Planting Density, Trees ha^{-1}	1736	136	450
Planting Distance, m	2.4 x 2.4	8 x 8 x 8	5.5 x 2.8
Mean Height of Tree, m	26	6	12
Mean Canopy Diameter, m	4.4	14.0	13.0
Leaf Area Index (LAI), m^2m^{-2}	4.4	4.2	3.1
Mean Trunk Circumference (at breast height), m	0.9	2.6	1.0

Rain gauges (Spectrum Technologies, Inc., USA) based on the tipping-bucket concept with resolution of 0.254 mm of rain were used for rainfall measurement. The rain collector had an opening diameter of 205 mm. To avoid rain water splashes from the ground, the rain gauge was screwed on a metal rod, which was hammered into the ground. The distance between the gauge and the ground was about 1 m. The rain gauge was connected to a data logger, which gave data in 1 decimal point, for the recording of rainfall parameters (throughfall, stemflow and gross rainfall) at five-minute intervals.

For throughfall measurement, 10 rain gauges were arranged along a straight line in North-South direction at a 10-m distance between every two gauges, while for stemflow measurement, three sampled trees were selected randomly. The bark of selected trees were gently removed to fix a rubber collar and sealed with nails and bitumen to direct stemflow into the rain gauge. Finally, for collecting gross precipitation above the canopies, another rain gauge was installed in a nearby open area that was not hindered by tall plants and buildings. It was taken as representative of the gross precipitation (above canopies) at the experiment sites.

RESULTS AND DISCUSSION

Derivation of the Coefficient Values for the Two Equations for Net Rainfall

Table 1 reports the rainfall partitioning of seven studies. The number of rain days ranged from 28 to 252 days and daily rainfall from as low as 0.30 to as high as 153 mm was recorded. This provided data with a good range from low to heavy rainfall.

An average of 63.67% from gross rainfall contributed as throughfall and 2.30% as stemflow for oil palm. At the forest sites, throughfall contributed about 79.07% and 89.81% at maritime pine and tropical rainforest, respectively, whereas stemflow was 3.55% and 7.81%, respectively. Lastly, throughfall was 86.73% and stemflow, 1.14% at the rubber site.

Results from the seven studies were used to fit the three-coefficient equation of Eq. [3]. The coefficients were fit using the Solver add-in (a component of Excel) in Excel by minimising the mean differences between the estimated and measured values. Those data were compiled and analysed, and the values for G_{max}, G_{min}, and C were found to be 0.3443, 0.1804 and 58.9748, respectively. The equations were thus:

$$Pn = Pg * \exp(-G) \quad [6a]$$

$$G = 0.3443 - \frac{Pg}{58.9748 + Pg} * (0.3443 - 0.1804) \quad [6b]$$

$$\therefore Pn = Pg * \exp\left[-\left(0.3443 - \frac{Pg}{58.9748 + Pg} * 0.1639\right)\right] \quad [6c]$$

A two-coefficient equation (linear regression) was also derived from the same set of data (seven studies combined). The equation was:

$$Pn = 0.7724Pg - 0.5845 \quad (R^2 = 0.91) \qquad [7]$$

Accuracy of the Two- and Three-Coefficient Equations for Net Rainfall

The accuracy of the two equations, Eq. [6] and [7], was tested on the individual data set from the seven studies (Table 1). The error indices, NMAE and d in Eq. [4] and [5], were used as a goodness-of-fit measure for both equations.

Figure 2 and 3 show the overall prediction accuracy for the two equations for all the seven studies. The NMAE of the three-coefficient equation was 19.86%, which is in the "Good" prediction accuracy range, and the d value 0.88 (Figure 2). The NMAE of the two-coefficient equation was 20.10%, which was in the border between "Good" and "Fair", and the d value was the same as that of the three-coefficient equation, 0.88 (Figure 3). The two net rainfall equations' errors were similar, but the three-coefficient equation was slightly more accurate than the two-coefficient equation. This is because the three-coefficient equation for net rainfall is more flexible in representing the distribution of data, as it has three coefficients, whereas the other equation had only two coefficients.

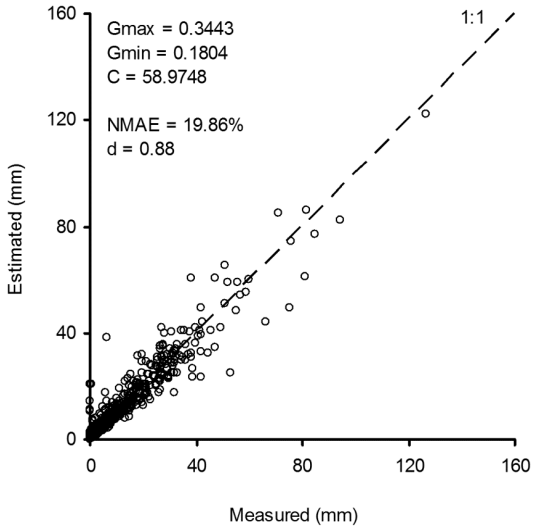

Figure 2. Derivation of the three-coefficient equation, Eq. [3], for net rainfall, where G_{max} = 0.3443, G_{min} = 0.1804 and C = 58.9748. NMAE and d are the normalised mean absolute error and the index of agreement, respectively. The dash line (1:1) is the line of agreement

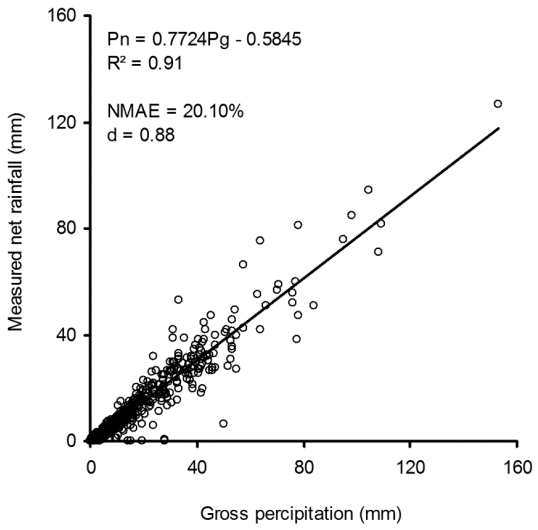

Figure 3. The two-coefficient equation between measured net rainfall (Pn) and gross rainfall (Pg). NMAE and d are the normalised mean absolute error and the index of agreement, respectively. The solid line is the linear regression

Table 3 shows the degree of accuracy when Eq. [6] and [7] were tested on the individual seven studies. The mean errors (NMAE and d) for the two- and three-coefficient equations were nearly the same, showing that the performance of the simpler two-coefficient equation was equal to that of the slightly more complex three-coefficient equation.

Table 3
Average errors for the two- and three-coefficient equations for estimating the net rainfall (Pn)

Reference	Tree	Three-Coefficient Equation		Two-Coefficient Equation	
		NMAE, %	d	NMAE. %	d
Bentley (2007)	Oil Palm	30.70	0.81	30.16	0.82
Damih (1995)	Oil Palm	21.44	0.89	19.86	0.90
Lubis (Unpublished data)	Oil Palm	15.58	0.91	15.96	0.91
Zulkifli et al. (2006)	Oil Palm	33.72	0.83	34.19	0.83
Germer et al. (2006)	Tropical Rainforest	27.26	0.85	27.90	0.84
Loustau et al. (1992)	Maritime Pine	15.16	0.88	15.47	0.87
Zulkifli et al. (2003)	Rubber	15.14	0.86	15.54	0.86
	Average	22.72	0.86	22.73	0.86

NMAE = Normalised Mean Absolute Error, d = Index of agreement

The average NMAE and d value for the three-coefficient equation at the oil palm sites were 25.36% and 0.86, respectively. However, the average NMAE for the two-coefficient equation at the oil palm sites was slightly better at 25.0%, while the d value was 0.87. For forest sites (Maritime pine and tropical rainforest), the three-coefficient equation performed slightly better than the two-coefficient equation, giving a reading of was 15.16% versus 15.47% for Maritime pine and 27.26% versus 27.90% for tropical rainforest. However, this was not the case for the d value. For the rubber sites, same as for the forest sites, the NMAE for the three-coefficient equation was slightly better at 15.14% than for the two-coefficient equation, 15.54%; the d values for both equations were the same.

Estimation of Throughfall and Stemflow Component:

This study also attempted to estimate Tf and Sf separately using the general equation form of Eq. [6] and [7] based on the methods described previously. Both the Tf and Sf equations are summarised below.

The three-coefficient equation:

$$Tf = Pg * \exp\left[-\left(3.9100 - \frac{Pg}{0.1089 + Pg} * (3.9100 - 0.3016)\right)\right] \quad [8]$$

$$Sf = Pg * \exp\left[-\left(4.0771 - \frac{Pg}{2.3328 + Pg} * (4.0771 - 3.8837)\right)\right] \quad [9]$$

The two-coefficient equation:

$$Tf = 0.7511Pg - 0.6790 \quad (R^2 = 0.91) \quad [10]$$

$$Sf = 0.0213Pg + 0.0946 \quad (R^2 = 0.28) \quad [11]$$

Most of the R^2 for the stemflow linear regression equation was high in the seven individual studies. However after combining the studies, the two-coefficient equation for stemflow gave a low value of R^2 (Eq. 11). This might have been due to the difference in tree morphology (trunk texture, trunk diameter, branch inclination degree, bark roughness, leaf architecture and leaf zenith angle distribution) that influenced the production of stemflow at the same gross precipitation amount received (Ward & Robinson, 1990; Xiao et al., 2000).

Accuracy of the Throughfall and Stemflow Equations

The equations above were tested again on the previous studies to check the accuracy of estimating throughfall or stemflow itself. Table 4 shows the error indices for Tf and Sf for the two- and three-coefficient equations for each individual study. The estimation of Tf for oil palm had the highest error (highest NMAE and lowest d), while Sf had a more varied error range. However, the average NMAE for both Tf equations were in the range of "Fair" and the d values

Table 4
Average 4rrors for the two- and three-coefficient equations for estimating throughfall (Tf) and stemflow (Sf)

Reference	Tree	Three-Coefficient Equation				Two-Coefficient Equation			
		NMAE, %		d		NMAE, %		d	
		Tf	Sf	Tf	Sf	Tf	Sf	Tf	Sf
Bentley (2007)	Oil Palm	31.24	9.70	0.81	0.93	30.55	26.58	0.82	0.80
Damih (1995)	Oil Palm	22.46	44.30	0.87	0.72	20.60	39.35	0.88	0.73
Lubis (Unpublished Data)	Oil Palm	16.07	110.70	0.78	0.30	16.49	128.56	0.74	0.45
Zulkifli et al. (2006)	Oil Palm	34.19	49.87	0.83	0.72	34.67	67.68	0.83	0.59
Germer et al. (2006)	Tropical Rainforest	25.15	74.24	0.86	0.55	25.97	65.21	0.86	0.58
Loustau et al. (1992)	Maritime Pine	13.56	54.74	0.89	0.60	14.78	55.41	0.88	0.56
Zulkifli et al. (2003)	Rubber	16.38	77.80	0.85	0.61	17.40	129.99	0.84	0.43
	Average	22.72	60.19	0.84	0.63	22.92	73.26	0.84	0.59

NMAE = Normalised Mean Absolute Error, d = Index of Agreement

showed low average estimation errors. These Tf equations were further tested on field experiments. For Sf equations, both NMAE and d values showed high average estimation errors. These Sf equations would be further tested to check their accuracy using the field experiments.

Field Studies

A total of 47, 33 and 28 rain days were recorded at the pine, oil palm and rubber sites, respectively. Table 5 indicates that interception loss ranged from 18 to 23%, throughfall from 65 to 81% and stemflow up to 3% of total gross rainfall for these three crops.

Table 5
Total precipitation, throughfall, stemflow and average rainfall under three test crops for duration of study

Parameter	Pine	Oil Palm	Rubber
Duration of Study (day)	60	85	57
No. of Rain Days Used in Study (day)	47	33	28
Total Gross Precipitation (mm)	623.90	291.40	335.40
Total Throughfall (mm)	506.68	203.21	217.94
Total Stemflow (mm)	4.22	8.53	1.30
Throughfall as % of Rainfall	81.21	69.74	64.98
Stemflow as % of Rainfall	0.68	2.93	0.39
Interception Loss as % of Rainfall	18.11	27.33	34.63
Maximum Precipitation (mm)	74.7	57.4	44.7
Minimum Precipitation (mm)	0.3	0.3	0.2
Average Rainfall per Rain Day (mm)	13.3	8.8	12.0

Rainfall Partitioning at Pine, Oil Palm and Rubber Sites

Pine had the lowest interception loss among the three crops, 18% of total gross precipitation. This was similar to that reported by Loustau et al. (1992), who obtained 13-21% interception loss for maritime pine in Bordeaux, 17% for a pine forest in central Portugal (Valente et al., 1997), 19% for a pine forest in Mexico (Cantu-Silva & Rodriguez, 2001), 17.6 and 22% for a pine plantation and mature pine forest, respectively, in the US (Bryant et al., 2005). In Portugal, a sparse pine forest recorded 17% interception loss, which was higher compared to the 11% recorded at a sparse eucalyptus forest. This may have been due to the higher canopy storage capacity and the larger aerodynamic conductance resulting from the greater height of the ground at the pine forest (Valente et al., 1997).

Throughfall for pine was 81% of total gross precipitation, which was similar to that obtained by Bryant et al. (2005) (77 to 81%) and within the range of the study of Loustau et al. (1992), 77-83%. For stemflow, the study of Bryant et al. (2005) on maritime pine obtained 0.5%, which was close to this study of 0.7%, but slightly lower than that obtained at the pine plantation (1.96%) and by Loustau et al. (1992), (1-6%). These differences may have been due to tree age and tree spacing. Teklehaimanot et al. (1991) reported that Sitka spruce, in a 2-m tree spacing treatment (close stands), had higher stemflow (17%) than in 4-m (2.9%), 6-m (10%) and 8-m spacing (0.5%). The larger number of trees per unit ground area in the 2-m spacing treatment resulted in the overlapping of the tree crowns; thus, when the rain was intercepted by the upper branches of tree, there were several layers of canopy for the rain to drip through, resulting in a higher chance of water being conducted towards the trunk as stemflow. Similar tree sizes may receive up to three times the stemflow amount at 2-m spacing than at 8-m spacing. Teklehaimanot et al. (1991) further clarified that their lower stemflow was only 17% compared to that obtained by Ford and Deans (1978), 27%, because of tree age. The younger trees in Ford and Dean's study meant that the branches were steeper, leading to a larger volume of stemflow.

The rubber site in this study recorded the highest interception loss with 35% of total gross precipitation. This was different from the results reported by Teoh (1977) and Zulkifli et al. (2003), whose studies reported that interception loss was only 12% and about 15-16%, respectively, of total gross precipitation.

Dinata (2007) studied net rainfall under rubber trees at three ages, 10, 15, and 25 years old, and with planting distance 3 x 3.3 m in Sumatera. He reported that interception loss was 31.5, 40.7 and 51.8% at age 10, 15 and 25 years old, respectively. The study showed that canopy storage capacity can be estimated from canopy area and canopy density. Age of tree is the main factor that influences canopy density. In the study, the author cited Pramono and Ginting (1997) that the denser the canopy, the higher the intercepted amount of rain. The 10-year-old

canopy area was small, 52.9 m^2, compared to that of the 15-year-old (95.2 m^2) and 25-year-old (126.9 m^2). Therefore, canopy storage capacity at age 25 years old is expected to be higher than that at 15 and 10 years old, and interception loss is expected to be higher for rubber trees at age 25. The age of tree and planting distance may explain the reason interception loss in this study (35%) was closer to that in Dinata's study for 10-year-old rubber trees (31.5%) and 17% lower for the same age range (25 years old) and higher than the figures recorded in Zulkifli (12%) and Teoh's (15-16%) studies.

Throughfall at the rubber site obtained 65% of total gross precipitation, which was close to that obtained for Dinata's 10-year-old trees (60.6%) but higher than for the 25-year-old trees (43.8%) and lower than recorded in Zulkifli's study (87%, 36 years old). According to Dinata (2007), throughfall amount is inversely related to tree age. This means that when a tree becomes older, canopy storage capacity increases with increasing dimension canopy, and as such, the throughfall amount decreases. However, when older trees reach a certain threshold, they tend to leave larger canopy gaps due to their having more branches and their higher leaf death rate; as such, the throughfall amount increases instead (Pypker et al., 2005). Stemflow in this study (0.4%) was similar to that recorded by Zulkifli (1.1%) but different from that obtained by Dinata's study on 25-year-old trees (4.4%).

In this study, the rubber site showed the highest interception loss compared to the other tree sites probably due to differences between tree types in terms of canopy storage capacity (Loustau et al., 1992). The rubber trees had a storage capacity of 0.682 mm, the pines, of 0.656 mm and the oil palms, of 0.515 mm. Other possible explanations could be the difference between tree types in terms of their vegetal morphology, leaf arrangement along the branches and stem surface area (Ward & Robinson, 1990; Xiao et al., 2000).

Interception loss at the oil palm site was 27% of total gross precipitation. Kee et al. (2000) reported interception loss by 11-17% in the oil palm study in Malaysia (estimated by difference between gross rainfall and net rainfall). However, some Malaysia studies indicated interception loss of 32-41% and 29.6% in Indonesia, readings that were more similar to those obtained in this study. Banabas (2007) remarked that these differences may have some relevance to the acutely-angled leaves in redistribution of rainfall during high and low crop seasons. In low crop season, fronds are generally at an acute angle as palms go through a male phase, resulting in generating more stemflow. In contrast, in high-crop season, fronds are pulled down by heavy fruit bunches, causing a less acute angle between the fronds and trunk. This was where intercepted rain water was mostly intercepted, held up by the bunches, frond buds and the trunk, although the frond

pinnae intercepted only a small amount of rain water. Therefore, more rain water was intercepted, generating less rain as stemflow (Banabas, 2007).

Throughfall accounted for 70% of Pg, similar to the findings of a study by Kee et al. (2000), who found that 70-78% of rainfall would turn to throughfall in oil palm in Malaysia and 72-104% in Papua New Guinea (Banabas, 2007); this was slightly higher compared to findings of other oil palm studies, which ranged between 57 and 69%. On the other hand, stemflow was also slightly higher (2.9%) than 2.0-2.7% in Malaysia studies. However, Kee et al. (2000) reported stemflow at 11-13% in Malaysia and Banabas (2007), at 10-14% in Papau New Guinea. As mentioned earlier, the variation in interception, throughfall and stemflow fractions could be linked to oil palm fruit bunch production seasons.

Validation of Throughfall and Stemflow Equations, NMAE and d

The three-coefficient equation [Eq. 8] and two-coefficient equation [Eq. 10] for throughfall were tested on the data obtained from the three field sites (Table 6). NMAE for the three-coefficient equation for throughfall was in the range of "Good" for the three data sets. The same rankings were obtained for the two-coefficient equation for throughfall except for oil palm, which was in the range of "Fair". The d value for both equations represented the same agreement. The three-coefficient equation showed slightly better results compared with the simpler two-coefficient equation. The use of the three-coefficient, Eq. [9], and the two-coefficient, Eq. [11], equations for stemflow, both seemed to register doubtful readings for NMAE and d for stemflow estimation. As mentioned earlier, the R^2 for Eq. [11] was low after the results of the seven studies were combined; this was probably due to the difference in tree morphology, rainfall intensity and the macro and microclimate. When these stemflow equations were further tested on field experiments, high error and low confidence levels were recorded.

Validation of Net Rainfall Equations, NMAE and d

Table 6 and Figure 4 indicate that the three-coefficient equation's NMAE for pine, oil palm and rubber were 12.13, 19.18 and 20.54%, respectively. This classified the three-coefficient equation's accuracy as "Good" for pine, oil palm and rubber. For the two-coefficient equation, NMAE was 12.10, 22.65 and 19.99% at the pine, oil palm and rubber sites, respectively. These readings were close to those obtained by the three-coefficient equation's NMAE. The d values for both equations were the same at the respective sites. The NMAE and d for the two- and three-coefficient equations used for the three crops were not that much different from one another.

Table 6
Accuracy of throughfall (Tf), stemflow (Sf) and net rainfall (pn) for the two-coefficient equation (2CE) and the three-coefficient equation (3CE) at pine, oil palm and rubber site

	Pine	Oil Palm	Rubber	Average
Tf				
NMAE (3CE)	13.48	19.18	19.49	17.38
NMAE (2CE)	14.42	23.32	19.88	19.21
d (3PE)	0.93	0.92	0.91	0.92
d (2PE)	0.92	0.90	0.91	0.91
Sf				
NMAE (3CE)	197.87	94.33	419.21	237.14
NMAE (2CE)	320.23	109.87	651.65	360.58
d (3PE)	0.49	0.62	0.31	0.47
d (2PE)	0.32	0.61	0.19	0.37
Pn				
NMAE (3CE)	12.13	19.18	20.54	17.28
NMAE (2CE)	12.10	22.65	19.99	18.25
d (3PE)	0.94	0.92	0.91	0.92
d (2PE)	0.94	0.90	0.91	0.92

Accuracy class for NMAE: Great (0-10%); Good (10-20%); Fair (20-30%); Poor (>30%)

Figure 4 shows a tight clustering of points along the line of agreement, especially for low to medium rainfall events. At heavier rainfall events, the three-coefficient equation, however, tended to be underestimated. This was similar to situations using the two-coefficient equation.

Equations for Oil Palm and Rubber

In Malaysia, oil palm and rubber are major crops. Table 7 shows the percentage of rainfall partitioning and the equation coefficients and error indices for oil palm and rubber. Those equations were derived using data from previous studies (oil palm and rubber in the seven previous studies) and field experiments (oil palm and rubber). For oil palm, 63% of Pg was throughfall, 5% was stemflow and about 68% was net rainfall. For rubber, 66% of Pg was throughfall, 0.5% was stemflow and net rainfall was 66%. The two- and three-coefficient equations estimated both oil palm and rubber in the rank of "Good" (with an index of agreement of about 90%). The two- and three-coefficient equations performed nearly equally well.

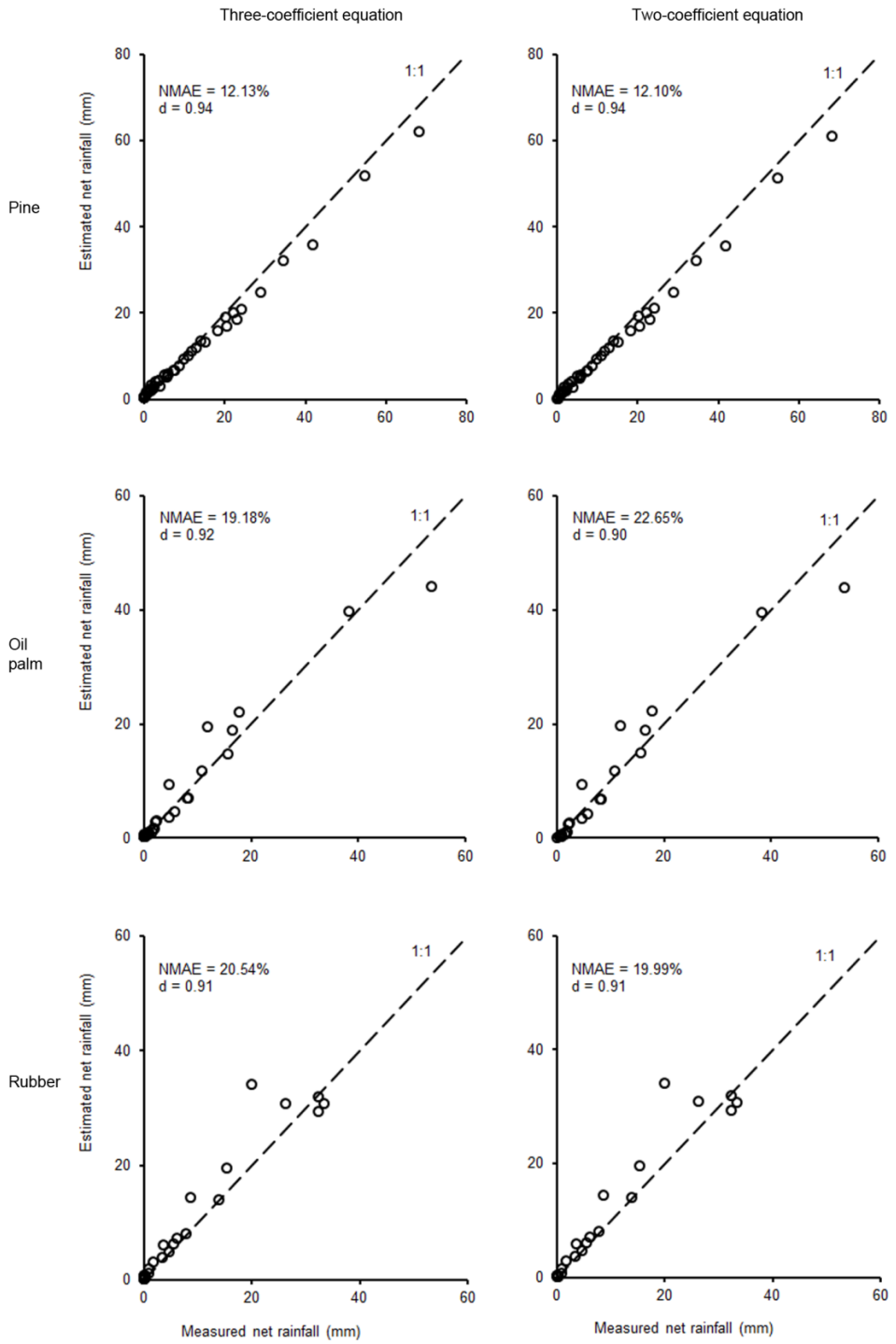

Figure 4. Error indexes for the two-coefficient equation (2CE) and the three-coefficient equation (3CE) for net rainfall at (a) pine, (b) oil palm, and (c) rubber sites. NMAE and d are the normalised mean absolute error and the index of agreement, respectively. The line of dashes (1:1) is the line of agreement

Table 7
The two- and three-coefficient equations for oil palm and rubber

		Oil Palm			Rubber		
		Tf	Sf	Pn	Tf	Sf	Pn
Two-Coefficient Equation	% of Pg	62.81	5.11	67.92	65.70	0.51	66.21
	bo	-1.023	0.117	-0.906	-0.377	-0.038	-0.415
	b1	0.718	0.013	0.731	0.809	0.011	0.820
	NMAE	19.66	93.80	19.06	13.96	57.48	14.16
	d	0.89	0.46	0.89	0.92	0.73	0.91
Three-Coefficient Equation	Gmin	0.362	3.925	0.370	0.160	3.983	0.101
	Gmax	2.149	14.448	4.478	4.136	7.281	2.930
	C	0.094	0.213	0.010	0.184	9.387	0.908
	NMAE	18.69	85.29	18.54	12.88	48.23	13.76
	d	0.89	0.58	0.89	0.92	0.77	0.92

Accuracy class for NMAE: Great (0-10%); Good (10-20%); Fair (20-30%); Poor (>30%)

CONCLUSION

Two- and three-coefficient equations for measurement of rainfall were successfully developed based on seven studies recorded in the literature and validated for each individual study against measured data from three field data collections. Two error indices (NMAE and d) were used in the goodness-of-fit measure for equations. Both net rainfall equations estimated the studies to have an average of NMAE=23% (Fair) and d=0.86; both throughfall equations estimated an average of NMAE=23% (Fair) and d=0.84; and the stemflow equations estimated an average of NMAE=60 and 73% (Poor) and d=0.63 and 0.59. In field experiments, the three-coefficient equation for net rainfall and throughfall performed slightly better than the two-coefficient equations in NMAE (12-21%) and were similar in d value. However, the two-coefficient equation was fairly accurate in estimating net rainfall for closed to nearly closed canopies with an error of NMAE=12-23%. Equations for estimating stemflow had high error. However, stemflow only contributed a small portion of the gross precipitation.

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Effect of Mevalonic Acid (MVA) and Linalool as a Precursor in Enhancement of Limonene in *Citrus grandis* Osbeck Albedo Tissue Culture

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ABSTRACT

The effects of melavonic acid (MVA) and linalool as precursors in the production of limonene and linalool on *Citrus grandis Osbeck* callus tissues were investigated. MVA and linalool were used as precursors to stimulate limonene production in the biosynthetic pathway. This study proved that low concentrations of MVA (0.077 mM to 1.557 mM) and linalool (0.056 mM to 1.117 mM) were able to produce limonene when tested on callus tissues for 7 to 35 days. The aim was to determine the highest accumulation of both limonene and linalool. The highest production of limonene obtained was 0.97 ppm on day 28 when the tissues were treated with linalool and 1.50 ppm on day 35 with the addition of 0.077 mM MVA. On the other hand, linalool concentration reached a maximum of 2.88 ppm on day 7 with tissues treated with 0.077 mM MVA. As the culture period lengthened, the limonene level increased from 0.76 ppm at day 7 to 1.82 ppm on day 28, whereas linalool concentration decreased steadily from 2.88 ppm at day 7 to 1.55 ppm at day 35. This is due to the bioconversion of linalool to limonene. The best result for precursor-treated tissues was at 0.838 mM linalool, where the limonene level achieved was 0.97 ppm on day 28. The production of limonene and linalool using low precursor concentrations within a short period of time is favourable as it has good market value.

Keywords: Mevalonic acid (MVA), linalool, precursor, limonene production, *Citrus grandis Osbeck*, albedo tissue culture

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INTRODUCTION

A precursor is a compound that participates in a chemical reaction that produces another compound. Precursors are naturally-occurring compounds, intermediates originating from biosynthetic pathways or related synthetic compounds (Orlita et al., 2008). Exogenous addition of a precursor to a culture medium may enhance the production of secondary metabolites, especially alkaloid accumulation, where production is limited due to the lack of a particular precursor (Vijaya et al., 2010; Yoshida et al., 1988). This approach is advantageous if the precursors are inexpensive. Precursor feeding at low levels, especially intermediates of the biosynthetic pathway, would prove beneficial to the production of a desired product. Naturally-occurring as well as related synthetic compounds can be used as precursors. However, synthetic compounds are expensive and not safe for consumption; therefore, they should not be used in in food and drinks. Only a few studies have been found to use mevalonic acid (MVA) and linalool as precursors to increase secondary metabolite accumulation (NikNorulaini et al., 2003; Zarina, 2005). Likewise, the use of MVA and linalool as precursors for limonene and linalool production on citrus albedo tissue culture has not been widely published. The manipulation of culture conditions with precursor supplements may potentially promote limonene accumulation in *Citrus grandis* cell cultures.

Evidence from the literature suggests that limonene and linalool are biosynthesised from mevalonic acid (MVA), which is the primary precursor of all terpenoids generated from the acetate-mevalonate pathway predominantly localised in the cytosol (Zarina, 2005). Juice vesicles of Citrus sp. have an enzyme system, which is capable of phosphorylating MVA and forming both isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMPP) (Figure 1) (Mayakrishnan, 2008).

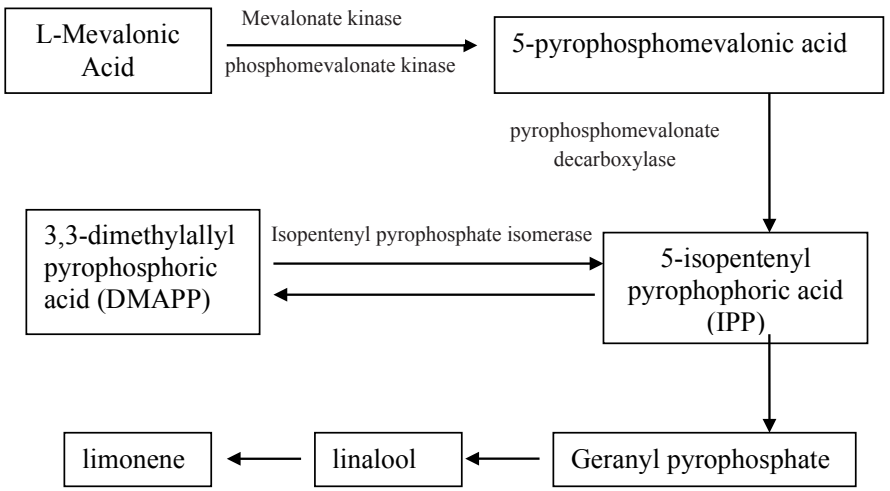

Figure 1. Schematic diagram of mevalonic acid (MVA) pathway in plants

The final product from the phosphorylation and decarboxylation-dehydration of MVA is linalool. Enzyme systems that can be found in intact fruit and leaves are responsible for the conversion of linalool to limonene (Karoui et al., 2010). Linalool thus can be considered as an immediate precursor that is directly converted to limonene. The feeding of MVA and linalool is expected to enhance the production of linalool and limonene, respectively.

Essential oil of citrus species consists of 1.85% of the total volatile oils extracted. Linalool is among the important compounds that are found in the essential oils of *Anibarosae odora* (rosewood), *Coriandrum sativum* L, *Bursera delpechiana, Citrus* spp, *Citrusa urantium sub* sp. *amara* L, *Laurus nobilis* L, *Cinnmamomun camphora, Cinnmamomun verum* L, *Matricaria chamomilla* L, *Salvia sclarea* L., *Lavandula officinalis Chaix* and *Ocimum basilicum* (Maia et al., 2006). The linalool content in the peel oils of oranges and tangerines decrease markedly in relative concentration as the fruits mature (Tounsi et al., 2011).

Limonene is the main compound in the essential oils of citrus fruits, where it occurs in concentrations of more than 90% and in pure form. It produces a pleasant aroma, which results in the essential oil being in high demand for the manufacture of perfumes and soaps, among other scented and flavoured products. Natural sources such as plants are the best alternatives for producing limonene in order to fulfil the global demand and at the same time reduce the cost of limonene production. Limonene production using citrus fruit cell cultures can be enhanced by applying a precursor in the culture media. The present study was conducted to determine the effect of precursor (MVA and linalool) concentrations on limonene and linalool accumulation in tissue culture at a certain culture period.

METHODOLOGY

Chemicals and Standards

Chemicals (analytical grade) for the Murashige and Skoog (MS) medium preparation were purchased from Sigma Chemicals, St. Louis, the United States; Koch-light Laboratory, Colbrook, Bucks, England; Fluka, Japan and Merck, Darmstadt, German. Agar was bought from Bacto Difco Laboratories, Detroit, USA. Plant growth regulators such as 2, 4-dichlorophenoxyacetic acid (2, 4-D), 6-furfurylaminopurine (kinetin), abscisic acid (ABA) and standards such as limonene and linalool (liquid form with ~97 % purity) were obtained from Fluka, Japan. Yeast extract was purchased from Becton Dickinson & Co, USA.

Cell Culture

The fruit, pomelo, (*Citrus grandis*) was obtained from a plantation in Sungai Gedong, Perak. Young fruits 4-7 cm in diameter were chosen. The spongy white rind surrounding the juice vesicle, called albedo, was used in all the studies conducted. The albedo was soaked in Clorox® (5.75%

sodium hypochlorite) in a sterile Petri dish and then cut into pieces measuring 1 cm × 1 cm × 0.5 cm before being placed in Petri dishes containing MS medium.

Precursor Preparation

Effect of linalool on tissue growth and limonene accumulation. The amount of linalool used was 10, 50, 100, 150 and 200 µL. Each sample was added to another 1 L, modified MS medium (Zarina, 2005) to obtain media with different final concentrations of linalool (0.056 mM, 0.279 mM, 0.559 mM, 0.838 mM and 1.117 mM). The linalool-added MS modified media were autoclaved at 121°C, 15 psi for 15 min after pH adjustment (pH 5.7). The media were poured into Petri plates. Albedo tissues were placed on the modified MS media once the media had cooled and hardened.

Each albedo tissue was cut into 1 cm × 1 cm × 1.5 cm, and five pieces were planted onto the modified MS medium with different linalool concentrations. Cultures were maintained in the dark at room temperature (25 ± 2°C). The five albedo tissues were pooled and analysed as a single sample.

Effect of mevalonic acid (MVA) on tissue growth and limonene accumulation. Aqueous solution of MVA was prepared by dissolving mevalonic acid lactone (Fluka, Japan) in distilled water. The solutions were prepared in concentrations of 5, 10, 50, 100, 150 and 200 mg/L and were filter-sterilised in Nalgene® disposable sterile filterware and kept as stock stored in the refrigerator at 4 ºC. Final concentrations of MVA were calculated as 0.038, 0.077, 0.384, 0.768, 1.152 and 1.537 mM. These solutions were prepared by adding the appropriate amount of sterile stock solution into different flasks of sterilised modified MS medium before the medium solidified. Each flask was swirled to mix the solution without creating any bubbles and the solution was carefully poured into Petri plates in a sterile laminar flow hood.

This study was conducted using a factorial combination of different concentrations of MVA at 0.077, 0.384, 0.768, 1.152 and 1.537 mM and culture periods of week 1 to week 5. Albedo tissue was cut into 1 cm × 1 cm × 1.5 cm pieces and planted in the medium containing MVA at the various concentrations mentioned above. Cultures were maintained in the dark at room temperature (25 ± 2°C). Cultures were pooled from five tissues and analysed as a single sample.

Tissue Growth Determination

The growth of the tissue was determined by subtracting the final weight and the initial weight of tissue grown on media within the studied incubation period. The initial weight was measured once calli were removed from the parent tissue prior transferring to modified MS media and incubated for 7, 14, 21, 28 and 35 days. After the respective incubation period, the calli were removed from the media and cleaned of excess media using tissue paper before weighing for the final weight. Five calli were measured as replicates for each incubation period.

Extraction of Limonene and Linalool

After recording the wet weight, the same tissues were used to extract limonene and linalool. A mass of 2 g of tissue was cut and chopped in preparation for Soxhlet extraction. Extraction was carried out for 2 h using methanol as the solvent. The liquid sample obtained was concentrated to 2 mL using a rotary evaporator and was also used for limonene and linalool determination.

Analysis of Limonene and Linalool

Limonene and linalool concentration was determined using gas chromatography (GC) (Brand Shimadzu, GC-17A, Japan). The operating system of the gas chromatography was carried out under several conditions. The carrier gas used was helium. The column used for chromatographic separation was Phase –HP-5 (30 m × 320 µm × 0.25 µm). The system was left to reach equilibrium state for 30 min before the samples were injected. The temperature of the transfer line and ion source was maintained at 280ºC and 250ºC, respectively. Injector temperature was set at 250ºC. The column temperature was programmed at between 60ºC and 104ºC at 4 °C/min, while the rate was changed to 6°C/min for 6 min when it reached 104°C up to 182°C.

Statistical Analysis

The statistical analysis was carried out using the Analysis of Variance (ANOVA) using the MINITAB 17 statistical software.

RESULTS AND DISCUSSION

Effect of Linalool on Tissue Growth

The change in fresh weight of the callus tissue grown in culture treated with varying concentrations of linalool can be observed in Table 1 below.

Table 1
Effect of tissue fresh weight when added with different concentrations of linalool as precursor. mean ± s.d, n=3. (No limonene was detected in control)

Culture Period (days)	Linalool Concentration in mM					
	Control	0.056	0.279	0.559	0.838	1.117
	Wet Weight of Tissue (g)					
7	0.23 ± 0.05	0.21 ± 0.08	0.11 ± 0.05	0.15 ± 0.06	0.10 ± 0.08	0.07 ± 0.04
14	0.12 ± 0.04	0.21 ± 0.04	0.18 ± 0.05	0.18 ± 0.05	0.18 ± 0.01	0.24 ± 0.04
21	0.18 ± 0.01	0.23 ± 0.06	0.20 ±0.03	0.20 ± 0.02	0.19 ± 0.06	0.28 ± 0.03
28	0.91 ± 0.03	0.25 ± 0.05	0.22 ± 0.05	0.16 ± 0.07	0.27 ± 0.05	0.22 ± 0.05
35	1.12 ± 0.02	0.21 ± 0.01	0.25 ± 0.05	0.20 ± 0.02	0.28 ± 0.01	0.14 ± 0.04

*Control - Without Additional Linalool

The change in tissue wet weight with relation to linalool concentration in the media depends on the number of days in culture. Early culture period, at 7 days, showed that the control culture promoted growth, while linalool inhibition increased with concentration. With progressive days in culture up to 21 days, the tissue samples without linalool experienced a decrease in wet weight; this was in contrast to the increase in weight that was evident in cultures with linalool. However, feeding the callus with a high concentration of linalool did not induce any significant tissue growth ($p>0.05$). It was apparent that the presence of high linalool (1.117 mM) exerted a negative effect on tissue growth as the culture days extended beyond 21 days. The wet weight of the control culture was more than triple that of the linalool-treated cultures at 28 and 35 days in culture, after a drop at 14 days that later rose again. Tissue in cultures with 0.279, 0.559 and 0.838 mM linalool saw its wet weight steadily increase with more days in culture but the net weight did not meet the higher weight of the control tissue (without linalool). Adding linalool as a precursor led to an inhibition of tissue growth. The inhibition of tissue growth in the presence of linalool was consistent for all the tested concentrations. Statistically, no significant ($p>0.05$) difference in tissue growth was observed for the cultures added with different concentrations of linalool (Appendix 1A).

Slow growth of precursor-treated tissue may reflect an unadapted culture prone to necrosis, leading to stressed conditions suitable for the onset of secondary metabolism (Oswald et al., 2007). Although no necrosis was observed in the studies, the albedo tissue samples showed slow or retarded growth rates indicating stressed conditions suitable for a switch from primary to secondary metabolism.

Linalool in this experiment was shown to be a tissue growth suppressor or a cell division suppressor. The treated albedo tissue samples appeared to be under a prolonged lag phase, unlike untreated or control tissue. During lag phase, little or no cell division takes place. However, this does not mean that the cells are dormant, as the cells are undergoing a period of intense metabolic activity involving DNA and enzyme synthesis (Tortora et al., 1995).

The control tissue growth followed a typical lag phase up to 21 days in culture, followed by a 7-day log or exponential growth from 21 to 25 days; after that, the growth slowed down. Cellular production is most active during the log phase, when cells are metabolically active. Since only the control tissue reached this stage, it can be concluded that only untreated tissue undergoes primary metabolism or cell division.

After the 28th day, cell growth slowed as it reached the stationary phase due to nutrient exhaustion, accumulation of waste products and harmful changes including pH of the culture media. Removing tissue from spent media and providing fresh media can overcome this problem. Thus,

the albedo tissue samples were transferred to fresh media after 28 days to move past the stationary phase in this study.

The findings on hampered growth of linalool-treated albedo tissue were consistent with a study conducted by Engels et al. (2008) on anthraquinones production, where high levels of secondary metabolite production inhibited culture growth.

Effect of Linalool on Limonene Accumulation

Linalool is an immediate precursor to limonene and its presence in a medium is expected to trigger the conversion of linalool to limonene under favourable conditions as reported by NikNorulaini et al. (2003). As evident from Table 2 below, the presence of linalool influenced the production of limonene in the tissue samples.

Table 2
Effect of various concentrations of linalool on the accumulation of limonene in albedo tissue culture. mean ± s.d, n=3. (No limonene was detected in control)

Culture Period (days)	Linalool Concentration in mM				
	0.056	0.279	0.559	0.838	1.117
	Limonene Concentration in mM				
7	0.42 ± 0.06	0.49 ± 0.05	0.34 ± 0.01	0.36 ± 0.03	0.54 ± 0.04
14	0.48 ± 0.09	0.59 ± 0.06	0.45 ± 0.02	0.47 ± 0.03	0.62 ± 0.01
21	0.68 ± 0.10	0.64 ± 0.02	0.63 ± 0.05	0.68 ± 0.04	0.72 ± 0.05
28	0.81 ± 0.10	0.83 ± 0.05	0.72 ± 0.05	0.97 ± 0.05	0.66 ± 0.02
35	0.70 ± 0.11	0.88 ± 0.01	0.65 ± 0.04	0.72 ± 0.05	0.43 ± 0.06

*Control - Without Additional Linaloo1

Different concentrations of linalool acting as precursor to limonene showed an increase of limonene concentration in all the tissues except for the control, which did not produce any limonene at all. The increase was steady for all the samples up to day 21. On day 28, further increase was obtained for all the cultures, except for the culture with 1.117 mM linalool added. The highest limonene accumulation, 0.97 ppm, was obtained on the 28th day of the tissue samples cultured in 0.838 mM linalool. Tissue samples induced with 0.838 mM linalool showed an increase from 0.68 ppm (day 21) to 0.97 ppm (day 28) before decreasing slightly to 0.72 ppm on day 35. The difference in linalool concentration feeding showed a statistically significant ($p<0.05$) value in the limonene accumulation (Appendix 1B). Adding a precursor to the MS media induced the production and accumulation of limonene up to a certain period before the amount showed a decline. Table 2 also shows the presence of limonene in all the tissue samples grown in media supplemented with linalool for all the concentrations used.

The fresh tissue weight of the albedo tissue did not increase in linalool-added media as much as in the control. Conversely, the limonene concentration rose steadily, while no limonene was detected in the control tissue samples (Table 2).

Zarina (2005) demonstrated that adding linalool at 0.838 mM to callus cultures of *C. grandis* increased limonene production rate from less than 5 x 10^{-5} mg/g/day at day 21 to more than 80 x 10-^{5} mg/g/day at day 49. The rate increased rapidly around the 45th day of the culture period. However, there was no significant increase in callus fresh weight within the same culture period. Supplementing metabolic precursors has proven to be effective in increasing concentrations of desired secondary metabolites. For example, an immediate precursor of vanillin, ferulic acid, was known to affect accumulation of flavour compounds of cultured *Vanilla planifolia* cells (Karoui & Marzouk, 2013). In contrast, Sarfaraj et al. (2012) found that *C. grandis* callus cultures that were not supplemented with any precursor produced limonene only after 10 months of the culture period; as this study revealed, no limonene was detected in untreated albedo tissue up to day 35.

Secondary metabolites, such as limonene in this study, are not products of single genes but are the by-product of metabolism due to multistep and multi-enzyme processes. Changes in the activity of one enzyme or intermediate like exogenous linalool often results in the simultaneous up-regulation of some enzymes and down-regulation of other enzymes in the same and parallel pathways. The production of other metabolites may be enhanced or reduced and thus may alter the overall product profile (Federica, 2012).

Effect of MVA on Tissue Growth

Table 3 compares the growth of tissue grown on modified MS media using different concentrations of MVA. Comparison of tissue fresh weight was determined every 7 days from day 7 till day 35. Exogenous MVA caused a decrease in tissue growth for all the tissues studied. As the days increased, tissue weight also increased steadily. The highest tissue weight of 0.64 g was achieved without the addition of MVA at day 35. The highest tissue fresh weight added with MVA was obtained with 0.077 mM of MVA after 35 days. Addition of 1.537 mM of MVA increased the tissue fresh weight slowly for 2 weeks before the weight surged drastically. The feeding of different MVA concentrations did not give any significant ($p>0.05$) change to tissue wet weight (Appendix 1C).

Table 3
Effect of tissue fresh weight when added with different concentrations of mva as a precursor. mean ± sd, n=3

Culture Period (days)	MVA Concentration in mM					
	Control	0.077	0.384	0.768	1.152	1.537
	Wet Weight of Tissue (g)					
7	0.09 ± 0.02	0.12 ± 0.04	0.09 ± 0.02	0.12 ± 0.07	0.13 ± 0.07	0.07 ± 0.03
14	0.25 ± 0.05	0.14 ± 0.05	0.16 ± 0.02	0.19 ± 0.03	0.24 ± 0.09	0.11 ± 0.02
21	0.38 ± 0.06	0.24 ± 0.08	0.23 ± 0.03	0.24 ± 0.05	0.32 ± 0.12	0.36 ± 0.13
28	0.51 ± 0.08	0.31 ± 0.07	0.32 ± 0.05	0.33 ± 0.08	0.38 ± 0.05	0.39 ± 0.14
35	0.64 ± 0.08	0.46 ± 0.04	0.44 ± 0.04	0.45 ± 0.08	0.39 ± 0.13	0.39 ± 0.12

*Control - Without Additional MVA

The highest increase in tissue wet weight was observed after 28 days of culture for all the tissues. The greatest increase was observed in tissue cultured in 0.077 mM MVA, which was 0.02 g/day during the last 7 days in culture (from day 28 to day 35). However, this was equivalent to reduced wet weight by 18% in tissue cultured without MVA (control). All the tissue cultured in the presence of MVA was subject to slower growth and the least increase of weight was in tissue cultured with the highest MVA concentration, 1.537 mM. Primary metabolism occurred during the whole culture period for MVA-added tissue. A study by Vanisree et al. (2004) claimed that protein synthesis took place during primary metabolism, thus increasing tissue growth. Imbault et al. (1996) also found that addition of MVA led to cytokinin synthesis, which functions to promote cell division and elongation. They treated *Catharanthus roseus* with 1 mM MVA and found that this concentration improved culture growth prior to inhibition with pravastatin.

Effect of MVA on Limonene and Linalool Accumulation

The effect of MVA on limonene production was measured by the accumulation of limonene in callus tissue grown in media supplemented with different concentrations of MVA. The data are shown in Table 4. Concentration of MVA played a defined role in the final accumulation of limonene. The concentration of 0.077 mM MVA produced the highest accumulation of limonene throughout the culture periods, achieving 1.50 ppm after 35 days in culture. The second most abundant accumulation of limonene was seen in tissue grown with the addition of 0.384 mM of MVA at day 35, which was 1.27 ppm. Statistical analysis using the Analysis of Variance (ANOVA) test revealed that there was statistically significant ($p<0.05$) accumulation of limonene in cultures (Appendix 1D), with 0.077 mM of MVA producing the highest limonene accumulation compared to the other concentrations used.

Table 4
Effect of various concentrations of mva on the accumulation of limonene in tissue culture. mean ± sd, n=3. (No limonene was detected in control)

Culture Period (days)	MVA Concentration in mM				
	0.077	0.384	0.768	1.152	1.537
	Limonene Concentration in ppm				
7	0.59 ± 0.08	0.50 ± 0.05	0.32 ± 0.04	0.32 ± 0.03	0.17 ± 0.07
14	0.83 ± 0.04	0.79 ± 0.05	0.30 ± 0.06	0.34 ± 0.07	0.30 ± 0.06
21	1.12 ± 0.12	0.90 ± 0.06	0.48 ± 0.06	0.35 ± 0.08	0.38 ± 0.05
28	1.23 ± 0.09	1.12 ± 0.09	0.64 ± 0.03	0.5 ± 0.09	0.45 ± 0.07
35	1.5l ± 0.07	1.27 ± 0.09	0.7 ± 0.07	0.66 ± 0.14	0.71 ± 0.06

*Control - Without Additional MVA

Generally, limonene was detected from day 7 in tissue culture and it increased in production for all the MVA concentrations studied. Cultures without exogenous MVA did not produce any detectable amount of limonene.

Table 5 shows linalool concentrations gradually decreased in all the cultures after 7 days added with MVA. The results demonstrated that linalool production in *C. grandis* was triggered by the introduction of MVA at low concentration. This finding was supported by that of Zarina et al. (2005), who reported that precursor feeding had triggered limonene production in *C. grandis* cultures earlier than in the non-fed cultures. In comparison, the linalool concentrations slowly increased to about 1.25 ppm after a month in the control culture (not supplemented with MVA). The presence of MVA in the medium was able to first trigger a hike in the amount of linalool compared to in the untreated media as early as day 7. However, the amount was reduced for all MVA treatments a week in culture; this was in contrast to the limonene profile (Table 4). Using 0.077 mM of MVA resulted in the highest linalool accumulation, showing statistically significant ($p<0.05$) (Appendix 1E) readings throughout the culture period even though the linalool concentration was seen to decrease from day 7 to day 35 for all the MVA concentrations studied. Tissue samples without the addition of exogenous MVA (control) showed a different trend, where linalool was observed to increase from 0.55 ppm on day 7 to 1.25 ppm on day 35. The rest of the tissue samples all seemed to decline in linalool production for all the MVA concentrations added.

Table 5
Effect of various concentrations of mva on the accumulation of linalool in tissue culture. mean ± sd, n=3

Culture Period (days)	MVA Concentration in mM					
	Control	0.077	0.384	0.768	1.152	1.537
	Linalool Concentration in ppm					
7	0.55 ± 0.12	2.88 ± 0.23	2.44 ± 0.31	2.23 ± 0.31	1.97 ± 0.24	1.53 ± 0.13
14	0.7 ± 0.06	2.32 ± 0.27	2.12 ± 0.23	1.90 ± 0.18	1.55 ± 0.10	1.15 ± 0.12
21	0.85 ± 0.09	2.07 ± 0.24	1.87 ± 0.24	1.76 ± 0.28	1.52 ± 0.13	0.86 ± 0.10
28	0.9 ± 0.11	1.97 ± 0.12	1.58 ± 0.31	1.56 ± 0.21	1.21 ± 0.43	0.94 ± 0.08
35	1.25 ± 0.08	1.55 ± 0.23	1.31 ± 0.12	1.46 ±0.13	0.83 ± 0.10	0.79 ± 0.14

*Control - Without Additional MVA

Linalool accumulation in tissue grown in cultures with MVA gradually decreased after day 7 (Table 5). The decrease of linalool happened when MVA concentration was increased because only low concentrations of MVA feeding promoted linalool accumulation. High concentrations of MVA caused linalool accumulation in cells, and this increased toxicity and led to cell fatality (Brown et al., 1987; Charlwood & Brown, 1987). A small amount of secondary metabolite formation in the cultures despite the presence of MVA may have been due to alternative biosynthetic pathways present, as found by Oswald et al. (2007). They also noted that a complete absence of or low enzyme activity before MVA (for example HMG-coenzyme A reductase) was added might have ceased the biosynthetic pathway for limonene/linalool production. For both treatments, it could be concluded that limonene production was not dependent on the amount of precursors added. It showed that only a small amount of MVA and linalool was needed as a stimulant for limonene production.

CONCLUSION

Mevalonic acid (MVA) and linalool proved to be good precursors in triggering limonene production in the callus tissues of *Citrus grandis Osbeck*. However, tissue culture cell growth prevailed over the secondary metabolite production in untreated tissue. Tissue cultured on linalool-added media displayed increased production of the secondary metabolite limonene, despite lower gains in wet weight that was indicative of slower cellular growth. A similar observation was made on tissue cultured on exogenous MVA-added media. Adding MVA to the media raised the concentration of MVA in the tissue cells, and this triggered the subsequent series of reactions in the pathway, leading to higher linalool concentration and eventually, limonene synthesis. The outcome of precursor addition on linalool or limonene accumulation depended on the age of the culture, which is inadvertently linked to the precursor uptake period and assimilation into the metabolic pathway. Accumulation of linalool precedes limonene production since

linalool is the intermediate to limonene. As the culture ages, limonene has a tendency to be converted to other substances, and this reduces the limonene concentration in the tissue. The highest linalool production in culture for limonene, 2.88 ppm and 1.5 ppm, were obtained using 0.077 mM of MVA at day 7 and day 35, respectively. Manipulation of the medium by addition of exogenous precursors to stimulate relatively high productivity of the secondary metabolites showed a successful example of the plant cell culture technique. Limonene and linalool have good market value, so reducing their production by addition of a low concentration of precursor is very beneficial. In addition, monoterpene hydrocarbon limonene has been widely used as a starting product for bioconversions into flavour and scented compounds, thus increasing its demand.

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APPENDICES

Appendix 1A
Tissues wet weight versus linalool concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
Linalool Conc.	5	0.4185	0.08369	2.15	0.094
Error	24	0.9360	0.03900		
Total	29	1.3545			

Appendix 1B
Limonene accumulation versus linalool concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
Linalool Conc.	5	1.6439	0.32878	13.42	0.000
Error	24	0.5878	0.02449		
Total	29	2.2317			

Appendix 1C
Tissues wet weight versus MVA concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
MVA conc.	5	0.05539	0.01108	0.48	0.787
Error	24	0.55224	0.02301		
Total	29	0.60763			

Appendix 1D
Limonene accumulation versus MVA concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
MVA conc.	5	3.648	0.72967	14.19	0.000
Error 0	24	1.234	0.05140		
Total	29	4.882			

Appendix 1E
Linalool accumulation versus MVA concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
MVA conc.	5	6.354	1.2709	8.80	0.000
Error	24	3.466	0.1444		
Total	29	9.820			

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Characterization of Fungi from Palm Kernel Cake (PKC) and the Effect of Storage Temperature on Fungi Growth

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ABSTRACT

The widespread contamination of animal feed with mycotoxin is not a new issue worldwide. Apart from economic loss, mycotoxin can have adverse health effects on humans due to the carcinogenicity, teratogenicity and mutagenicity potential of the toxins. Palm kernel cake (PKC) is the largest animal feed production in Malaysia. PKC is a by-product of palm kernel oil processing and it has been exported as animal feed. The purpose of this study was to isolate and characterise toxigenic fungi cultured in three different media, Dichloran Rose Bengal Chloramphenicol (DRBC) Agar, Dichloran 18% Glycerol (DG18) Agar and Malt Extract Agar (MEA), derived from PKC that is stored under three different temperatures, 4°C, 25°C and 60°C. Identification of fungi was carried out based on macroscopy and microscopy as well as molecular identification. Four mycotoxigenic fungi were found: *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus* and *Penicilium citrinum*. In order to characterise polymorphism of the isolates, RAPD assay was performed using OPA 3 as the primer. The software resulted in a constructed dendrogram that revealed the percentage of similarities between the typable isolates (*A. fumigatus, A. niger* and *P. citrinum*) within range from 20% to 80%. The effect of storage temperature on the strains' enumeration is reported in this work. The distributing strains are influenced by the storage temperature of the PKC matrices. The findings clearly show that Aspergillus species profused at 25°C PKC storage, while it was restricted at low and high temperature.

Keywords: Mycotoxin, PKC, fungi, media, RAPD assay, temperature

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INTRODUCTION

Mycotoxins are secondary metabolites produced by mycotoxigenic fungi in conditions that are favourable for fungal growth. Mycotoxin contamination of agricultural crops and animal feed is a serious issue since it has undesirable consequences on human and animal health. *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. are among the common types of mycotoxigenic fungi. Some of the common mycotoxins found in animal feed are aflatoxins and fumonisins.

Agriculture commodities are easily contaminated by fungi, in particular *Aspergillus* spp, when their storage conditions are favourable for fungi growth. This is a serious concern since *Aspergillus* spp. are capable of producing aflatoxins. Aflatoxins are produced by a number of strains of *A. flavus* and *A. parasiticus* in a variety of commodities that include cereals, figs, oilseeds, nuts and tobacco (Diener et al., 1987). Mycotoxin contamination of animal feed results in a reduction of its nutritional value; this is highly undesirable as it has unfavourable consequences on human and animal health.

The literature pertaining to the evaluation of protocols and methods used to define the relationship between isolates has increased significantly over the years. Randomly amplified polymorphic DNA (RAPD) has been highlighted by many researchers as the most convenient method for this purpose (Williams et al., 1990; Meyer et al., 1991). RAPD involves the application of short primers followed by PCR analysis using a large template of genomic DNA. These short primers will or will not amplify the DNA template. This process is dependent on the positions that are complementary to the sequence of the primers.

Malaysia is one of the major producers of palm oil and palm oil products in the world. The increasing demand for palm oil over the years has led to the increase in the production of palm kernel cake (PKC). In 2009, Malaysia produced 17.56 million tonnes of palm oil, from which 2.31 million tonnes of PKC were produced on 4.69 million hectares of planted land, and it is projected that these values will increase over the years (MPOB, 2009). In 2002, almost 4 million metric tonnes of PKC were generated all over the world (Atasie & Akinhanmi, 2009) and in 2006, Malaysia contributed 2.20 million tonnes of PKC and exported about 2.12 million tonnes of it (Ong et al., 2004). As little is known about fungi profiles isolated from PKC, the aim of this study was to isolate and identify fungi isolated from PKC and to determine the effect of media and temperature on fungal growth.

MATERIALS AND METHOD

Sample Collection

Samples of PKC (2 kg per pack) were collected from four different manufacturers in Selangor, Malaysia. The samples were kept at 4°C prior to being transported to the lab for further analysis.

Sample Preparation

A mass of 2 kg of samples were milled and divided into three parts. They were incubated at three temperatures, 4°C, 25°C and 60°C, for two weeks.

Isolation and Quantification of Fungi

Frequency of isolation of fungal species and total fungal count in palm kernel cake (PKC). Isolation and quantification of fungi were carried out in accordance with the procedure described by West (2009). The DRBC, DG18 and MEA culture media were used for comparative study of viable fungi-collecting media. A mass of 10 g of a PKC sample was weighed, after which sterile peptone water 0.1% (w/v) was added to it. The solution was homogenised using Stomacher® for 5 min and then put through serial dilution (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). It should be kept in mind that the elapsed time between the cessation of mixing in the dilution tube and the withdrawal of the sample using a pipette should not be more than a few seconds. Following this, 100 µl of each dilution was spread onto the surface of the DRBC, DG18 and MEA solid culture media using the spread plate method. The DRBC, DG18 and MEA plates were then incubated at 28°C. Each plate was prepared in triplicate. After five days of incubation, the fungal colonies were counted using a colony counter and expressed as the number of colonies forming units per gram (CFU/g). The isolates of *Aspergillus* spp. and *Penicillium* spp. were further sub-cultured onto potato dextrose agar (PDA).

Statistical analysis. The results were analysed using Analysis of Variance (ANOVA) by Minitab Statistical Software v.14 (Minitab Inc., State College, Pa., U.S.A.). One-way ANOVA was used to evaluate the mean differences in a) the culture medium of DRBC at the temperatures of 4°C, 25°C and 60°C ($p=0.794$); b) the culture medium of DG18 at the temperatures of 4°C, 25°C and 60°C, and; c) the culture medium of MEA at the temperatures of 4°C, 25°C and 60°C. The significance between the differences were determined using Tukey's multiple comparisons test.

Macroscopic and Microscopic Analysis

Macroscopic description. The morphological description of fungi was conducted according to the procedure given by Gao et al. (2007) with some modification. This procedure was carried out after five days of incubation at 28°C. The characteristics measured and observed by the naked eye were the diameter, colour, texture and underside (reverse) colour of the colonies. The micromorphological and structural characteristics of the colonies were measured using a dissecting microscope, namely the size of the metulae, shape of the conidial heads, the length, width and colour of the stipes, the shape, size and seriation of the vesicles and foot cell colour as well as the size of the phialides.

Microscopic observation. Taxonomic identification of the genera and species was performed based on microscopic

characteristics of the fungi by referring to the standard key (Pitt & Hocking, 1997). The mass of hydrophobic conidia from harvested mycelia were removed using ethanol 90% (v/v) and stained with lactophenol blue solution and observed microscopically.

Molecular Analysis

DNA extraction. Genomic DNA of fungi was extracted according to the procedure described by Gonzalez-Mendoza et al. (2010) with some modification. Mycelia along with their agars were harvested and each mixed with 200 µl of Tris-EDTA (TE) buffer solution in a microcentrifuge tube. Following this, purified water was added into the microcentrifuge tube until the volume of the solution was 100 mL. The homogenised mixture was centrifuged at 13,200 rpm for 5 min until two layers formed in the microcentrifuge tube. The first layer was collected and discarded. The suspension left was slowly mixed with 0.2 mL phenol:chloroform (1:1) and incubated at 65°C for 5 min. The remaining suspension in the microcentrifuge tube was slowly mixed with 0.2 mL of phenol:chloroform (1:1) and incubated at 65°C for 5 min. Following this, 0.4 mL of isopropanol was added and mixed, and the mixture was then incubated at 20°C for 20 min. The mixture was then centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed twice with 75% ethanol. The suspension was centrifuged at 13,200 rpm for 5 min and the pellet was suspended with 50 µl of TE buffer consisting of 100 mL of Tris-HCl, 20 mL of 0.5 M EDTA and 880 mL of purified water. The prepared DNA of the fungi was kept in a freezer at the temperature -20°C until further use.

Randomly amplified polymorphic DNA polymerase chain reaction (PCR) amplification. Polymerase chain reaction (PCR) assays were carried out in a DNA thermal cycler (Applied Biosystems 2720 thermal cycler, USA). The PCR amplification protocol was programmed as follows: (1) initial denaturation at 94°C for 5 min; (2) 45 cycles of denaturation at 94°C for 1 min; (3) annealing at 36°C for 1 min; (4) extension at 72°C for 1 min; and (5) final extension at 72°C for 10 min. The 25 µl mixture of PCR amplification consisted of 5 µl of PCR buffer, 3 µl of 2 mM MgCl2, 0.5 µl of dNTP, 0.3 µl of *Taq* polymerase, 1.0 µl of primers, 4 µl of DNA template and 11.2 µl of sterile double distilled water. PCR kits were purchased from Promega (Madison, WI, USA) and the primers were synthesised by 1st BASE Laboratories Sdn. Bhd., Selangor.

The PCR products were analysed on 1 % agarose gel in 0.5 X TBE buffer (54 g of Tris base, 27.5 g of Boric acid, 2.92 g of EDTA). The DNA ladder was used as the molecular size marker. DNA bands were stained with ethidium bromide and viewed under UV light. Thirteen primers were randomly screened as shown in Table 1.

Table 1
Screened primers for rapd-pcr characterisation of fungi isolates

Code	Sequence
OPA3	AGT CAG CCAC
OPA8	GTG ATC GCAG
OPA10	CAG CAC CCAC
OPA17	GAC CGC TTGT
OPA18	AGG TGA CCGT
OPA20	GTT GCG ATCC
OPB3	CAT CCC CCTG
OPB8	GTC CAC ACGG
OPB10	CTG CTG GGAC
OPB13	TTC CCC CGCT
OPB17	AGG GAA CGAG
OPB18	CCA CAG CAGT
OPB20	GGA CCC TTAC

RAPD analysis software. The DNA product was analysed by means of computer generated software, GelCompare 4.2 (Applied Maths, Belgium). A dendrogram was created using GelCompare 4.2 to observe the relatedness and similarities between the fungi isolates. A similarity coefficient curve was drawn based on Pearson correlation and dice band based with 1 % position tolerance. The Unweighted Pair Group Linkage Analysis method (UPGMA) was chosen for dendrogram construction.

RESULTS AND DISCUSSION

Isolation of Fungi

The growth of fungi in the PKC samples was analysed in the different temperatures used for PKC storage of 4°C, 25°C and 60°C using three different types of culture media, which were DG18, DRBC and MEA. In order to see the range of suitable storage temperatures for PKC in the industry, the stated temperatures were selected. The occurrence and quantification of fungi by mean in various selected temperatures and media are summarised in Table 1.

Table 2
Effect of culture medium and storage temperature on the frequency of fungi in the pkc samples (log cfu/g)s

Palm Oil Mill	Culture Medium	Temperature		
		4°C	25°C	60°C
1	DG18	3.79	3.68	2.64
	DRBC	2.10	3.17	3.83
	MEA	2.87	2.93	2.62
2	DG18	1.28	2.77	1.53
	DRBC	1.53	3.92	3.69
	MEA	1.20	3.00	1.62
3	DG18	0.00	0.00	0.00
	DRBC	1.43	1.43	0.00
	MEA	1.16	1.49	0.00
4	DG18	0.00	4.00	2.00
	DRBC	1.00	1.00	1.00
	MEA	2.10	0.00	0.00

Note: 1-4 values are means of the four different types of palm oil mills located in Klang. DRBC is Rose Bengal Chloramphenicol, DG18 is Dichloran 18 % glycerol and MEA is Malt Extract Agar

In the present study, total filamentous fungi counts were in line with the value reported in cereal and mixed feed (Dalcero et al., 1997). In general, the total fungi counts obtained in this study did not exceed the log 5 CFU/g level proposed as a feed quality limit (Chelkowski, 1991). It can be seen that a storage temperature of 25°C resulted in the highest frequency of fungi in the PKC samples, whereas the lowest frequency of fungi was obtained for the storage temperature of 60°C. In addition, the DG18 culture medium gave the highest occurrence of fungi compared to the DRBC and MEA culture media.

The one-way ANOVA statistical analysis showed that there were no significant differences ($p>0.05$) between a) the culture medium of DRBC at the temperatures of 4°C, 25°C and 60°C ($p=0.794$); b) the culture medium of DG18 at the temperatures of 4°C, 25°C and 60°C, and; c) the culture medium of MEA at the temperatures of 4°C, 25°C and 60°C where the p-values were equal to 0.794, 0.785 and 0.612, respectively. In addition, the statistical analysis also proved that there were no significant differences between a) the temperatures of 4°C, 25°C and 60°C at which the DRBC culture medium was stored; b) the temperatures of 4°C, 25°C and 60°C at which the DG18 culture medium was stored, and; c) the temperatures of 4°C, 25°C and 60°C at which the MEA culture media was stored where the p-values were equal at 0.678, 0.487 and 0.587, respectively.

The distribution of fungal species differred from one palm oil mill to another, which may have been due to the fact that each palm oil mill had its own method of storing PKC. Some palm oil mills may not have dedicated storage facilities for PKC and unfavourable storage conditions might have promoted the growth of fungi in the PKC, particularly that of the *Aspergillus* species.

The findings demonstrated that the fungi were readily isolated using the three media of DRBC, DG18 and MEA. However, it can be observed that the use of DRBC and DG18 culture media maximised the collection of fungi from the PKC samples. Moreover, the fungal colonies on DG18 and DRBC were clearer and the growth of the fungal colonies were slower in these media compared with their growth in the MEA culture medium. This finding was similar to the results of Al-Gabr et al. (2013); the outcome can be attributed to the ingredients in the culture media i.e. dichloran and chloramphenicol. Dichloran functions as an anti-fungal agent that prevents fungi from overgrowing, whereas the chloramphenicol in the DRBC and DG18 culture media restricted the growth of bacteria from the environment from contaminating the PKC samples. According to King et al. (1979), the DRBC culture medium is recommended for enumeration of yeasts and moulds. In addition, photodegradation of the DRBC culture medium resulted in a reactive oxygen species that inhibited the growth of *Saccharomyces* (Chilvers et al., 1999) and possibly other yeasts. The result, however, contradicted what had been defined previously, as the study represented

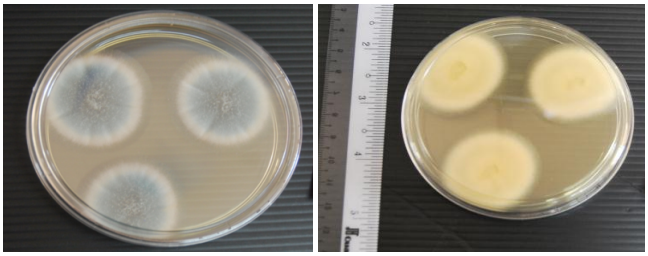
the optimum growth of fungi appearring in DG18, perhaps due to the development of yeast colonies inhibited in the medium (Deak et al., 2001). Based on the results obtained in this study, it can be deduced that DG18 is an excellent culture medium for the isolation of fungi from PKC samples and the storage temperature i.e. incubation temperature that results in the highest frequency of fungi is 25°C.

Macroscopic and Microscopic Characteristics of Fungi

Mycological analysis of PKC genotypes reported the existence of four principal genera filamentous fungi considered as the most important from a toxicological perspective. Mycological examination of PKC indicated three species of *Aspergillus* and one species of *Penicillium*. A total of 30 strains were isolated from the PKC matrices. Each of these species is discussed in the following sub-sections.

Aspergillus fumigatus.

Macromorphology of A. fumigatus. The diameter of the *A. fumigatus* isolates inoculated at three points on the PDA culture medium was within a range of 20-55 mm after five days of incubation. It could be observed that the fungal colonies did not overlap each other, as shown in Figure 1. Referring to the conidia of the isolates in Figure 1(a), it could be seen that the colour of the fungal colonies varied from bluish green to turquoise, whereas the texture of the fungal colonies appeared dense and smooth. From the underside, it could be seen that the colour of the fungal colonies varied from white to pale yellow, as shown in Figure 1(b).



(a) (b)

Figure 1. Image of *A. fumigatus* on PDA: (a) top view; (b) underside view

Micromorphology of A. fumigatus. Figure 2 shows an image of *A. fumigatus* as viewed under a microscope with 40× magnification. It could be observed that the conidial heads ranged from spatulate to pyriform. The length and width of the stipes were found to be within the range of 10-25 µm and 0.5-1.0 µm, respectively. The stipes and foot cells of the *A. fumigatus* isolates appeared colourless. The shape of the vesicles was subglobose. The seriation of the vesicles varied from radiate to columnar for most of

the isolates, while some were biseriate and uniseriate, as shown in Figure 2.

Figure 2. Image of *A. fumigatus* as viewed under a microscope with 40× magnification

Aspergillus niger

Macromorphology of A. niger. The diameter of *A. niger* isolates was within a range of 30-55 mm. The colour of the outer area of the fungal colonies was white and the fungal colonies did not overlap one another. Referring to the conidia of the isolates as seen in Figure 3(a), the colour of the fungal colonies was dark brown while their texture was rough, fluffy and less dense. In contrast, the colour of the fungal colonies on the reverse side varied from pale yellow to yellow at the centre and the fungal colonies were colourless near the edges, as shown in Figure 3(b).

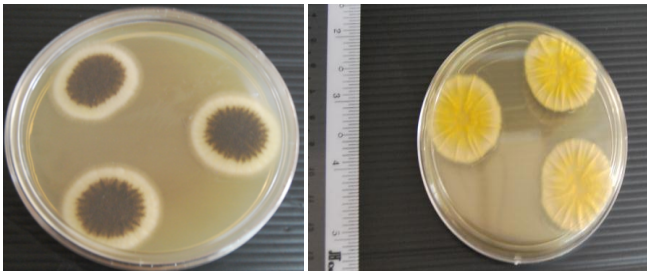


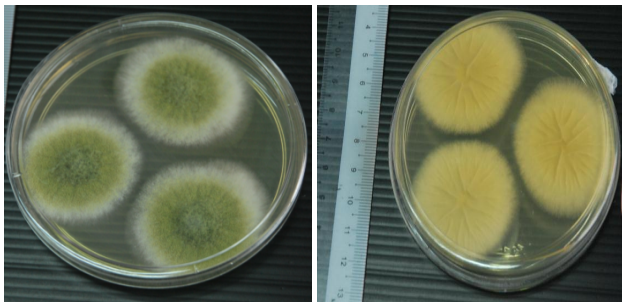
Figure 3. Image of *A. niger* on PDA: (a) top view, (b) underside view

Micromorphology of A. niger. Figure 4 shows an image of *A. niger*, as viewed under a microscope with 40× magnification. It could be observed that the shape of the conidial heads of *A. niger* varied from radiate to columnar. The length and width of the stipes were found to be within a range of 15-35 µm and 0.5-1.0 µm, respectively. The stipes were colourless, whereas the colour of the foot cells was pale brown. The shape of the vesicles was spherical, with a diameter ranging from 1.0 to 2.5 µm. The seriation of the vesicles varied from radiate to columnar for the *A. niger* isolates, while some were biseriate.

Figure 4. Image of *A. niger* as viewed under a microscope with 40× magnification

Aspergillus flavus

Macromorphology of A. flavus. The diameter of the *A. flavus* isolates inoculated at three points on the PDA culture medium was within a range of 25-35 mm after five days of incubation. The fungal colonies did not overlap one another, as shown in Figure 5. As seen in Figure 5(a), the colour of the fungal colonies in the conidia varied from olive green to yellowish green at the centre, while the colour at the edges was white. The texture of the fungal colonies was rough, less dense and somewhat fluffy. The colony reverse colour was generally colourless, but may appear brownish yellow in some areas, as shown in Figure 5(b). Dark yellow sclerotia were also present in some colonies, with various shapes and sizes.



(a) (b)

Figure 5. Image of *A. flavus* on PDA: (a) top view, (b) underside view

Micromorphology of A. flavus. Figure 6 shows an image of *A. flavus* as viewed under a microscope with 40× magnification. It could be observed that the shape of the conidial heads of *A. flavus* ranged from radiate to columnar. The length and width of the stipes for *A. flavus* were within a range of 10-30 µm and 0.5-1.0 µm, respectively. The colour of the stipes was clear brown, whereas the foot cells were colourless. The shape of the vesicle varied from spherical to pyriform, with a diameter within the range of 1.0 to 1.5 µm. The seriation of the vesicles varied from radiate to columnar for most of the *A. flavus* isolates, while some were biseriate and uniseriate, as shown in Figure 6.

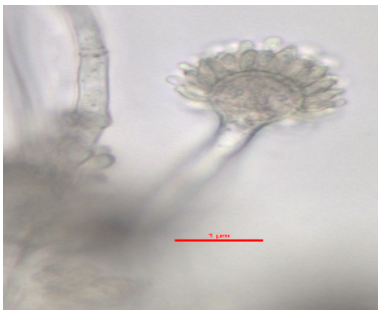
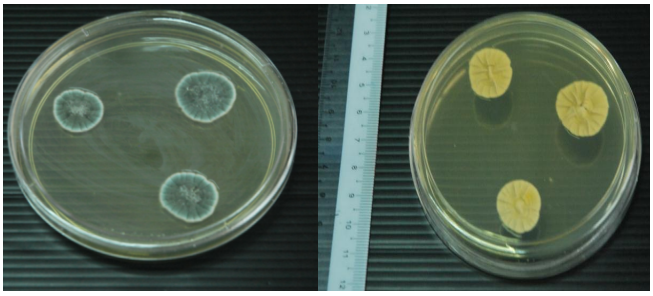


Figure 6. Image of *A. flavus* as viewed under a microscope with 40× magnification

Penicillium citrinum

Macromorphology of P. citrinum. The top view and underside view of *P. citrinum* on PDA culture media at 25°C after five days of incubation is shown in Figure 7(a) and Figure 7(b), respectively. The diameter of

the fungal colonies was found to be within a range of 12-15 mm. The fungal colonies had compact, dense conidiophores that were bluish green in colour. From the underside, it could be seen that the colour of the fungal colonies was yellowish orange, as shown in Figure 7(b).



(a) (b)

Figure 7. Image of *P. citrinum* on PDA: (a) top view, (b) underside view

Micromorphology of P. citrinum. Figure 8 shows an image of *P. citrinum* as viewed under a microscope with 40× magnification. Unlike *Aspergillus* spp., the conidia heads of *P. citrinum* were present in columns and the shape of the conidia heads varied from globose to subglobose, with a fine wall within a range of 2.5-3.0 µm. The growth of *P. citrinum* was rather fast on PDA compared to its growth on the DG18 culture medium. In addition, the fungal colonies appeared to be less dense.

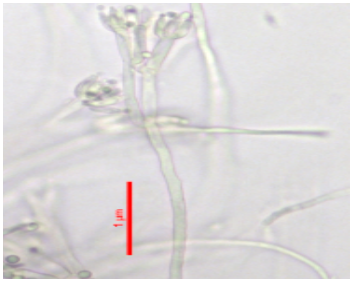


Figure 8. Image of *P. citrinum* as viewed under a microscope with 40× magnification

Molecular Characteristics

Randomly amplified polymorphic DNA cluster analysis. In this study, it was found that three out of four species, *A. fumigatus, A. niger* and *P. citrinum*, were typable on the RAPD assay when OPA 3 was used as the primer. Only *A. flavus* was not amplified by the primer. Thirteen primers were preliminarily assessed for RAPD PCR and only one primer was chosen based on its ability to produce consistent, distinguishable fragment patterns. The bands produced from the OPA 3 primer had a band size within a range of 1500-200 bp. The gel obtained from gel electrophoresis was analysed using the GelCompare 4.2 software to construct the dendrogram, as shown in Figure 9. The dendrogram was constructed based on the RAPD profiles generated using the OPA 3 primer.

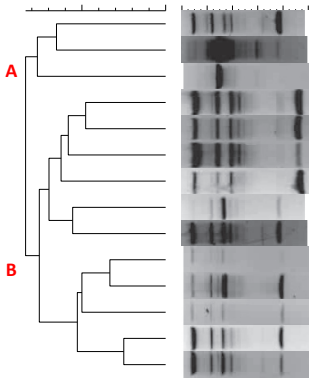


Figure 9. Dendrogram of typable fungi using OPA 3

The dendrogram in Figure 9 showed that the cluster was divided into two main groups, Group A and Group B, which consisted of 14 RAPD profiles. Only three strains were present in Group A: one strain of A. *fumigatus* and two strains of *P. citrinum*. In this segment, the strain of *A. fumigatu*s was isolated from the MEA culture medium stored at the temperature of 25°C. The two strains of *P. citrinum* were isolated from the DRBC culture medium at the storage temperature of 4°C.

Group B consisted of two main types of fungal species, with a total of 11 RAPD profiles. The first type consisted of six strains of *A. fumigatus*, making this group the largest among all the groups. In general, the origins of the fungal isolates in this segment were different. Two strains of *A. fumigatus* were isolated from the MEA culture medium at a storage temperature of 4°C, whereas one strain of *A. fumigatus* was isolated from the same medium at a storage temperature of 25°C. In addition, two strains of *A. fumigatus* from this group were isolated from the DRBC culture medium at 25°C and 60°C. One strain of *A. fumigatus* was also isolated from the DG18 culture medium at 4°C.

The second type of fungal species from Group B consisted of four strains of *A. fumigatus* and one strain of *A. niger*. Three strains of *A. fumigatus* in this group were isolated at the same storage temperature of 4°C; however, two strains were isolated from the DRBC culture medium, whereas one strain was isolated from the MEA culture medium. One strain of *A. fumigatus* was also isolated from the MEA culture medium at a storage temperature of 25°C. Finally, one strain of *A. niger* was isolated from the DG18 culture medium, which was stored at the temperature of 4°C. It should be noted that only one fungal species, *A. flavus*, was not typable using the OPA 3 primer in addition to some strains of A. niger.

RAPD was slightly different from conventional PCR analysis as it was not necessary for the analyst to have specialist knowledge regarding DNA sequence of the target organism. RAPD has been proven to be one of the quickest and easiest techniques for the detection of DNA polymorphism (Williams et al., 1990). RAPD is an alternative technique that can be used to distinguish genotypic variants (Meyer et al., 1991). RAPD basically involves the application of short primers followed by PCR analysis using a large genomic DNA template. These short primers will or will not amplify the DNA template. The process is generally dependent on the positions that are complementary to the sequence of primers.

In this study, only one primer was chosen, OPA 3, since it was able to construct genetic polymorphism between most of the fungal isolates. The remaining 12 primers were excluded from further analysis since they were not capable of producing consistent, distinguishable fragment patterns during gel electrophoresis.

Based on the dendrogram shown in Figure 9, it could be seen that the similarity values between the isolates varied from 20% to 80% for the primer, OPA 3. The total number of RAPD profiles was 14, and Group B was dominant among all the isolates, consisting of 11 RAPD profiles. This corresponded to 79% of the isolates and they all originated from the *A. fumigatus* species. Five strains of *A. fumigatus* were present in the MEA and DRBC culture media, and most of these strains were isolated at the storage temperature of 4°C. Both of these culture media had low similarity values at the same temperature due to various factors such as differences in the composition of the culture media (Gorski et al., 2006). The fitness between strains was influenced by the composition of the culture media; for instance, enrichment of the culture media with antibiotics would facilitate the isolation process. In this study, the DRBC culture medium was enriched with chloramphenicol that was addded to the medium; chloramphenicol suppresses bacterial growth and enables the growth of fungi compared to unselective culture media such as MEA.

Based on the results, Group A consisted of two fungal species, namely *A. fumigatus* and *P. citrinum*. There were differences between the two strains of *P. citrinum* even though they were isolated from the same culture medium (DRBC) at the same storage temperature (4°C). The differences between the *P. citrinum* isolates may have been due to genetic differences in the DNA (Buncic et al., 2001) such as differences in the serotypes or strains as well as the type of cell surface antigens. Furthermore, physiological differences may have led to low similarity values between the isolates.

CONCLUSION

In general, the frequency of fungi was at the highest at the storage temperature of 25°C, with a value of log 4.00 CFU/g. The DG18 culture medium was found to be the best medium for the collection of mycotoxigenic fungi from the PKC samples. In addition, the fungal colonies were more visible in the DRBC and DG18 culture media and the growth of the fungal colonies was slower in these media compared to in the MEA culture medium. A total of 30 strains were isolated successfully from the PKC samples. Four fungal species were identified, *A. fumigatus, A. niger, A. flavus* and *P. citrinum*, based on their macromorphological and micromorphological characteristics. These species are potential producers of mycotoxins, particularly *A. flavus*, which is well-known as a potent aflatoxin producer. A dendrogram was constructed from the RAPD profiles of 30 isolates in order to determine the genetic relatedness and similarities between the isolates. The isolates were identified based on the band

patterns on the dendrogram. The similarity values between the typable isolates, *A. fumigatus, A. niger* and *P. citrinum*, were within a range of 20-80%. In general, the temperature at which the PKC samples were stored had a significant effect on the growth of mycotoxigenic fungi.

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Biochemical and Nutritional Composition of Giant African Land Snail (*Archachatina marginata*) from Southwest Nigeria

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ABSTRACT

The survival of snails has been associated with the microclimate variables of their environment. Therefore, a comparative analysis of the biochemical composition of the haemolymph, mineral and proximate analysis of the flesh of the giant African land snail (*Archachatina marginata*) obtained from five southwestern states of Nigeria was conducted. Mature snails purchased from notable markets in Ogun, Oyo, Lagos, Osun and Ondo states were dissected. An analysis of the biochemical composition of the haemolymph and proximate composition of the flesh was done using standard methods, while a mineral composition analysis of the flesh and haemolymph was done using a Atomic Absorption Spectrophotometer and Flame Photometer. Snails from Oyo state had significantly higher ($p<0.05$) concentrations of glucose (35.00±0.20), protein (54.15±0.02) and lipid (22.90±0.05) in their haemolymph than those from the other locations. Concentrations of Na^{+}, Ca^{2+}, Cl- and PO_4^{2-} were observed to be significantly higher ($p<0.05$) in the haemolymph of the snails than in the flesh. Protein was observed to be higher than other metabolites in both the haemolymph and the flesh of *A. marginata* collected from the five states. The flesh of snails obtained from Ogun state recorded significantly higher ($p<0.05$) values of ash (1.73±0.02), crude fibre (1.01±0.01), crude protein (20.22±0.02) and carbohydrate (1.09±0.01) content than those from the other states examined. No significant difference ($p>0.05$) was observed in the values of Mg^{2+}, PO_4^{2-} and Cl^{-} across the five states. Although climatic conditions could have influenced some biochemical composition of the snails, the snails collected from the five southwestern states of Nigeria examined were equally nutritious.

Keywords: Haemolymph, proximate, mineral, environment, climate, snail biochemistry

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INTRODUCTION

Snails are invertebrates with a soft body that is covered by a hard calcerous shell. They belong to the phylum Mollusca and the class Gastropoda. They are bilaterally symmetrical with over 100,000 species known worldwide (Segun, 1995). They spend most of the daytime under stones, soil litter or decaying organic matter (Ajayi et al., 1978). According to Yoloye (1994), snails are the largest group among the molluscs after arthropods. Land snails' habitat ranges from the dense tropical high forest in southern Nigeria to the fringing riparian forests of the Guinea savannah (Ajayi et al., 1980; Odaibo, 1997).

In West Africa, snail meat has traditionally been a major source of protein in the diet of people living in the forest belt (Cobbinah, 1993). According to Fagbuaro et al. (2006), snail meat is now becoming a highly relished delicacy in Nigeria that is also an important source of animal protein for coastal communities. The edible portion of the snail is reported to be rich in protein (Imevbore & Ademosun, 1988), calcium, magnesium, zinc and iron and it has very low fat content (Babalola & Akinsoyinu, 2009; Uboh et al., 2010; Adeola et al., 2010). Its protein is also reported to contain all the essential amino acids such as lysine, leucine, isoleucine and phenylalanine that are needed by the body for metabolic activities (Aboua, 1995; Ademolu et al., 2004). Reports also have shown that snail meat has medicinal value and can be used to treat ailments such as whooping cough, anaemia, asthma and high blood pressure due to its relatively low cholesterol level but high mineral content (Ebenebe, 2000; Akinnusi, 1998).

Haemolymph is regarded as a blood analogue found in all arthropods and most molluscs (Abdussamad et al., 2010). It is composed of water, inorganic salts (mostly Na, Cl, K, Mg and Ca) and organic compounds (mostly carbohydrates, proteins and lipids) (Ademolu et al., 2011; Ademolu et al., 2006; Ademolu et al., 2009; Akinloye & Olorode, 2000). Since the haemolymph directly bathe snail organs in an open circulation (Miller & Harley, 1996), it has been associated with snail growth performance and susceptibility to infection and aggression (Ademolu et al., 2011). Ejidike et al. (2004) also reported that microclimatic variables like relative humidity, rainfall, photoperiod and temperature are important determinants that allow the snail species to thrive and that its survival depends greatly on these variables.

Alternative protein derived from the consumption of snails, especially in the southwestern region of Nigeria (Fagbuaro et al., 2006) has been drawing attention lately. Although there has been advocacy for snail rearing over the years, a substantial amount consumed is still sourced from the wild (Ademolu et al., 2004). Similarly, there has been speculation among the local people that the snails collected from some of the southwestern states of Nigeria are of better quality than those from other states. This however, has not received any scientific attention. In order to justify this claim and identify the land areas that support the success of these snails in the wild, there

is a need to assess the biochemical and nutritional composition of the commonly eaten giant land snail from the different states of southwestern Nigeria.

This study was therefore aimed at assessing the variation in the biochemical composition of the giant African land snail, *Archachatina marginata*, obtained from five southwestern states of Nigeria through biochemical evaluation of minerals found in the snail as well as through nutritional analysis of its haemolymph and flesh.

MATERIALS AND METHOD

Snail Sample Collection

Five southwestern states of Nigeria, Ogun, Oyo, Lagos, Osun and Ondo, were chosen for this study. The environmental description of each state is presented in Table 1. Twenty-five mature snails were purchased from notable snail markets from each state: Itoku market, Abeokuta (Ogun), Oje market (Oyo), Oyingbo market (Lagos), Oja Oba market, Osogbo (Osun) and Oja Oba market, Akure (Ondo). Snails were randomly

Table 1
Environmental description of the five southwestern states of Nigeria chosen for this study

	Coordinates	Climate type	Average daily temperature	Topography	Vegetation	Average rainfall
Oyo	8°00'N 4°00'E	Equatorial climate	25°C to 35°C	Gentle rolling low land	Rain forest and Guinea savannah	800 mm to 1800 mm
Lagos	6°27'11''N 3°23'45''E	Tropical climate	25°C to 29°C	Island, sandbars and lagoon	Tropical swamp forest consisting of fresh water and mangrove swamp forests	1270 mm to 2540 mm
Osun	7°30'N 4°30'E	Tropical climate	21.1°C to 31.1°C	Undulating land formation with isolated hills	Derived savannah, secondary forest regrowth	800 mm to 1500 mm
Ondo	7°10'N 5o05'E	Tropical climate	21°C to 29°C	Lowland with rugged hills	Mangrove swamps, rainforest, derived savannah	2,000 mm to 1150 mm
Ogun	7°00'N 3°35'E	Tropical climate	24°C to 30°C	Undulating lowlands of coastal secondary rocks with scattered hills and river valleys	Tropical rain forest and Guinea savannah	1000 mm to 2000 mm

Sources: OYSG (2015); LSG (2015); OSG (2015); TFN (2015); Online Nigeria (2015a, b, c); OSSG (2004)

purchased from various local snail traders who assured that the snails were sourced from the wild in the respective states. The snails were transferred to the laboratory of the Biological Sciences Department, Federal University of Agriculture, Abeokuta for biochemical analyses.

Biochemical Analysis of the Haemolymph

The apex of the snails' shell was broken and the haemolymph was collected using the method described by Ademolu et al. (2004). The protein concentration of each sample was determined immediately using the biuret method described by Henry et al. (1974). The glucose content was determined by the colorimetric method of Baumniger (1974). The lipids assay was done following the method of Grant et al. (1987). The haemolymph sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), phosphate (PO^{4-}) and chloride (Cl^-) were determined by the method described in the Association of Official Analytical Chemists (AOAC) publication (1990).

Proximate and Mineral Analyses of the Flesh

Dissected samples of snail flesh were dried at 60°C for 48 h in an oven. The dried samples were powdered in a Moulinex blender and sieved through a 450 µm sieve. The final powdered sample was stored in a desiccator and used for proximate and mineral content determination.

The powdered samples were digested with a mixture of per chloric acid and nitric acid (1:2 v/v) and cooled to room temperature. Na^+ and K^+ were determined by flame photometer while Ca^{2+}, and Mg^{2+} were determined using an atomic absorption spectrophotometer (Model AA.403). Methods in Henry et al. (1974) were used to assess the PO_4^{2-} and Cl^- content of the tissue.

Chemical analysis to determine the proximate composition of the flesh (crude protein, crude fibre, ash, moisture and fat content) was carried out by the method described by the AOAC (1990).

Data Analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS) version 20.0 (IBM Corp, 2011). The Analysis of Variance test (ANOVA) was used to test for significant differences in the measured variables of the snail flesh obtained from the different states in southwest Nigeria. A post-hoc test was conducted utilising the Duncan Multiple Range test, with the p value set at 0.05.

RESULTS

Biochemical Composition of Haemolymph

The concentrations of glucose, protein and lipid were significantly different ($p<0.05$) in the haemolymph of the snails obtained from the five states (Table 2). Snails obtained from Oyo state had significantly higher ($p<0.05$) concentrations of glucose

(35.00±0.20 mg/dl), protein (54.15±0.02) and lipid (22.90±0.05) in their haemolymph than those from other locations. Similarly, snails obtained from Oyo state recorded significantly higher ($p<0.05$) haemolymph concentrations of Ca^{2+} and Cl- (10.15±0.02 mg/dl and 99.50±0.20 mm/l, respectively). The lowest concentration of Ca^{2+} and Cl^- was recorded in the haemolymph of snails obtained from Lagos state (Table 2).

Table 2
Biochemical composition of the haemolymph of archachatina marginata obtained from five southwestern states of Nigeria

	Glucose (mg/dl)	Protein (g/l)	Lipid (mg/dl)	Na^+ (mm/l)	Ca^{2+} (mg/dl)	Cl^- (mm/l)	PO_4^{2-} (mg/l)
OGUN	15.70±0.20^{d}	27.35±0.02^{d}	15.50±0.20^{d}	134.00±0.20^{bc}	9.95±0.02^{a}	94.50±0.20^{c}	1.70±0.10^{c}
ONDO	20.00±0.20^{b}	32.20±0.20^{b}	15.20±0.10^{d}	134.00±0.20^{bc}	9.95±0.03^{a}	97.50±0.20^{b}	2.30±0.20^{b}
LAGOS	16.35±.02^{c}	28.25±0.02^{c}	16.75±0.02^{c}	136.50±0.20^{a}	9.50±0.20^{b}	94.00±0.20^{c}	2.85±0.02^{a}
OYO	35.00±0.20^{a}	54.15±0.02^{a}	22.90±0.05^{a}	133.50±0.20^{c}	10.15±0.02^{a}	99.50±0.20^{a}	2.65±0.02^{ab}
OSUN	15.75±0.02^{d}	21.15±0.01^{e}	18.20±0.10^{b}	134.50±0.10^{b}	9.85±0.01^{a}	98.00±0.20^{b}	2.85±0.02^{a}

^{abcd}Mean values (± Standard Error) in the same column having the same superscript are not significantly different ($p>0.05$)

Proximate Composition of Snail Flesh

Protein content of the snail flesh from all the states ranged from 12.54±0.02 to 20.22±0.02 g/100g. Snail flesh obtained from Ogun state recorded significantly higher ($p<0.05$) values of ash (1.73±0.02), crude fibre (1.01±0.01), crude protein (20.22±0.02), carbohydrate (1.09±0.01) and fat (2.09±0.11) content than the values recorded for snail flesh from the other southwestern states of Nigeria (Table 3).

Table 3
Proximate composition (g/100g) of the flesh of archachatina marginata obtained from five southwestern states of Nigeria

	Moisture Content	Fat Content	Ash Content	Crude Fibre	Crude Protein	Carbohydrate
OGUN	73.95±0.02^{e}	2.09±0.11^{a}	1.73±0.02^{a}	1.01±0.01^{a}	20.22±0.02^{a}	1.09±0.01^{a}
ONDO	77.00±0.20^{d}	1.77±0.02^{b}	1.53±0.01^{b}	0.89±0.02^{b}	17.88±0.01^{b}	0.94±0.01^{b}
LAGOS	78.85±0.02^{b}	1.60±0.10^{bc}	1.40±0.05^{c}	0.82±0.01^{c}	16.42±0.02^{c}	0.92±0.02^{bc}
OYO	79.67±0.02^{a}	1.46±0.02^{c}	1.35±0.01^{c}	0.79±0.01^{c}	15.77±0.01^{d}	0.86±0.02^{c}
OSUN	77.41±0.01^{c}	1.73±0.02^{b}	1.51±0.01^{b}	0.88±0.01^{b}	12.54±0.02^{e}	0.95±0.02^{b}

^{abcd}Mean values (± Standard Error) in the same column having the same superscript are not significantly different ($p>0.05$)

Mineral Composition of Snail Flesh

Calcium (Ca^{2+}) was significantly lower ($p<0.05$) in the flesh of snails from Oyo and Osun states than in the flesh of snails from Ogun Ondo and Lagos (Table 4). There was no significant difference ($p>0.05$) in the values of Mg^{2+}, PO_4^{2-} and Cl^- in snail flesh from the different states. However, K^+ was higher in the flesh of the snails compared to other minerals, followed by Na^+, PO_4^{2-}, Ca^{2+}, Mg^{2+} and Cl^-.

Table 4
Mineral composition of the flesh of archachatina marginata obtained from five southwestern states of Nigeria

	Na^+ (mm/l)	K^+ (mm/l)	Ca^{2+} (mg/dl)	Mg^{2+} (mg/dl)	PO_4^{2-} (mg/l)	C^{l-} (mm/l)
OGUN	1.05±0.02^b	2.97±0.01^c	0.42±0.02^{ab}	0.27±0.02^a	0.53±0.01^a	0.02±0.00^a
ONDO	1.27±0.02^a	3.43±0.02^a	0.43±0.02^a	0.27±0.02^a	0.54±0.02^a	0.02±0.00^b
LAGOS	1.29±0.01^a	2.90±0.05^c	0.41±0.01^{ab}	0.29±0.01^a	0.56±0.02^a	0.02±0.00^{ab}
OYO	1.09±0.02^b	3.19±0.01^b	0.32±0.01^c	0.28±0.01^a	0.52±0.02^a	0.02±0.00^{ab}
OSUN	1.23±0.02^a	3.11±0.01^b	0.36±0.02^{bc}	0.27±0.02^a	0.55±0.02^a	0.02±0.00^{ab}

^{abc}Mean values (± Standard Error) in the same column having the same superscript are not significantly different ($p>0.05$)

DISCUSSION

The results of this study showed that location was a factor affecting the chemical constituency of the haemolymph of the giant African land snail found in five southwestern states of Nigeria. Ejidike et al. (2004) reported that dietary variables have influence on the physiology of giant African land snails. It was therefore not unexpected that environmental and dietary variables could have contributed to the variations observed in the composition of snail flesh in snails obtained from the different locations.

The concentration of glucose, protein and lipid was significantly higher in the haemolymph of snails obtained from Oyo state than those obtained from the other states of Southwest Nigeria. Of the five states examined, Oyo state has the highest average daily temperature (25°C-35°C). Since haemolymph is the fluid that bathes the flesh of snails, any physiological process that takes place in the body of the snails must be reflected in the haemolymph (Akinloye & Olorode, 2000). Ademolu et al. (2006) recorded a strong relationship between the haemolymph concentration of glucose, lipid and protein and the modified environment. Snails are ectothermic animals; their physiological processes are affected by environmental factors like temperature (Odiete, 1999). It is therefore not unexpected that higher concentrations of glucose, protein and lipid in the haemolymph of the snails from Oyo state could have been aimed at overcoming the challenges that arose from the higher average daily temperature of the state.

However, concentrations of glucose, protein and lipid recorded in snails from the five states compared well with those of normal and albino *A. marginata* (Ademolu et al., 2006; Ademolu et al., 2011).

The concentration of sodium, calcium, chloride and phosphate was observed to be higher in the haemolymph of the snails than in their flesh. The function of the haemolymph in the open circulatory system of snails is to serve as transport of nutrients to the various body parts (Miller & Harley, 1996). Hence, it is expected that the haemolymph should contain higher concentrations of minerals for absorption into body tissue (as recorded by the current study). Haemolymph biochemical properties of *A. marginata* have been reported to influence physiological processes (Akinloye & Olorode, 2000) and growth performance (South, 1990; Ademolu et al., 2006) of the land snail.

South (1990) and Ademolu et al. (2009) also reported that Na^+ and Cl^- were the most abundant ions in the haemolymph of the slug *Arion ater* and the land snail, *A. marginata*, respectively. The role of Na^+ in nervous communication is significant. Therefore, higher concentrations of Na^+ recorded in the haemolymph of snails in this study could have been responsible for the control of the contraction of their muscular foot (Miller & Harley, 1996).

The concentration of protein was higher than that of other metabolites in both the haemolymph and the flesh of *A. marginata* from the five states. This is in agreement with South (1992), who reported that protein was the most abundant solute in snail haemolymph. Similarly, protein was also reported to be higher than other metabolites in *A. marginata* reared under various environments (Ademolu et al., 2006; Ademolu et al., 201). Snails are thus a good source of protein (Akinnusi, 2002; Amusan & Omidiji, 1988; Imeuevbove & Ademosun, 1988) regardless of the location from which they were collected.

A. marginata collected from the five southwestern states of Nigeria was high in nutritional composition such as crude protein, carbohydrate, fat, ash and fibre. Values of crude protein, carbohydrate, fat, ash and fibre obtained compared well with those of *A. marginata, A. achatina* and *Limicolaria* spp. (Fagbuaro et al., 2006) and *Achatina achatina* (Babalola & Akinsoyinu, 2009). However, the values were higher than those of *Limicolaria* spp. and *Achatina fulica* (Babalola & Akinsoyinu, 2009) and the fresh water snail, *Pila ampullacea* (Obande et al., 2013).

This study showed that higher concentrations of lipid, protein and glucose recorded in snails from Oyo state could be a means for the snails of surviving high average daily temperature. Also, concentrations of minerals were higher in the haemolymph than in the flesh of the snails. *Archachatina marginata* collected from the five southwestern states of Nigeria examined was equally nutritious.

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Optimisation of Soaking Conditions to Improve the Quality of Frozen Fillets of Bocourti's Catfish (*Pangasius bocourti* Sauvage) using Response Surface Methodology (RSM)

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ABSTRACT

Bocourti's catfish (*Pangasius bocourti* Sauvage) is one of Asia's economic freshwater fish. The fish has high demand in Asian markets particularly as a frozen product due to its tasty meat and a size that makes it suitable for cooking as fish steak. However, there are few studies about its production and quality. For frozen products, soaking in appropriate chemicals and conditions is the key procedure that food producers use to improve the quality of the frozen fillet. Therefore, this research aims to establish the optimum conditions for soaking. Several phosphate compounds, sodium acid pyrophosphate (SAPP), tetra sodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP) and sodium hexametaphosphate (SHMP), were investigated. It was found that the best phosphate compound was STPP that provided high L*, a*, gained weight, cooking yield and sensory acceptability with less drip loss and cooking loss. Consequently, the optimisation of the soaking condition was conducted. The three main factors, STPP (0.13-4.38% w/v), soaking time (3-37 min) and salt concentration (0.30-3.70% w/v), were optimised by response surface methodology (RSM). The results showed that the optimised condition was STPP of 2.56%, soaking time of 37 min and salt concentration of 3.70%. At this condition, the obtained frozen fillets showed a good result with gained weight of 11.45%, cooking yield of 73.11%, a* of 10.97 and ^{o}h of 9.94, respectively.

Keywords: phosphate, *Pangasius bocourti* Sauvage, frozen fillets, response surface methodology, soaking conditions

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INTRODUCTION

Bocourti's catfish (*Pangasius bocourti* Sauvage) is an important fish for aquaculture in Asian countries. Bocourti's catfish is a hybrid from the female striped catfish (Pangasiidae) and the male Snail eater Pangasius (Wiwat & Chaisiri, 1995). Nowadays, it has been promoted as a new commercial fish and supported for cage aquaculture in many countries along the Mekong River, such as Thailand, Cambodia and Vietnam (Cacot et al., 2002). Its estimated monetary value is 1.2 million tons in 2014 and it is exported to more than 151 countries (FAO, 2014). The striped catfish is produced as a fillet product for export to European, USA, Russia and Asian markets (Doungporn & Kriangsak, 2010; Chatchai et al., 2011).

Although frozen fillets of Bocourti's catfish has high demand in markets, there are few studies on its production and quality. The freshness, colour and weight of fillets are significant factors for customer's consideration. To improve the frozen products, soaking in appropriate chemicals and conditions is the key procedure that food producers use to improve the quality of the frozen fillet. Phosphates or related compounds and sodium chloride (NaCl) have been used to improve meat properties during frozen food processing and storage. Phosphates could improve water retention and reduce weight loss in the thawing process (Masniyom et al., 2005). The synergistic application of NaCl and phosphates provide good water holding capacity (WHC) and cooking yield (Rattanasatheirn et al., 2008). Therefore, several types and amounts of phosphates with addition of NaCl are used in the soaking process to improve the quality of fillets (Thorarinsdottir et al., 2004; Margrethe et al., 2005; Rattanasatheirn et al., 2008; Kin et al., 2010).

Response surface methodology (RSM) was introduced in 1951 (Box & Wilson, 1951). The concept of RSM is to use an experimental design with a sequence level such as the Mixture design, Box-Behnken design and Central composite design to obtain the optimal response. A second-degree polynomial model is recommended to create a mathematical model for response prediction. At present, RSM is recognised as an effective tool to improve the qualities and processes and is widely used in food industries (Wangtueai & Noomhorm, 2009; Koli et al., 2011; Bai et al., 2015; Murthy et al., 2015).

While the production of frozen Bocourti's catfish fillets is increasing due to market demand, proper research-based information about the product and its quality is lacking. Thus, the aim of this study was to determine the effect of soaking conditions on some key quality parameters of frozen Bocourti's catfish fillets.

MATERIALS AND METHOD

Raw Materials

Bocourti's catfish (*Pangasius bocourti* Sauvage) was collected from cage aquaculture in the Mekong River at Nakhon Phanom Province, Thailand (17.4108 in latitude and 104.779 in longitude of decimal degrees). The samples used were from

the same crop (nine-month-old fish) with average body weight of 1 kg. The fish were caught and packed in iced and transported in plastic boxes to the Food Science Laboratory, Rajamangala University, Sakon Nakhon campus, within 2 h. The fish were slaughtered, manually eviscerated, filleted and de-skinned by hand. The fillets had an individual average weight of 150 g/piece.

Chemicals

Food grade sodium tripolyphosphate (STPP), tetrasodium pyrophosphate (TSPP), sodium hexametaphosphate (SHMP) and sodium acid pyrophosphate (SAPP) were purchased from Haifa Chemicals Ltd. (Bangkok, Thailand). Refined NaCl (99.99%) was obtained from Thai Refined Salt Co., Ltd. (Bangkok, Thailand).

Investigation of Suitable Phosphates

Four phosphate-related solutions (STPP, TSPP, SHMP and SAPP) were each prepared in tap water at 2.0% (w/v) with NaCl (2.5% w/v). Then, fish fillets were dipped in the phosphate solution at 4°C for 10 min and drained of excess water in a strainer for 1 min. The obtained fillets were individually packed into polyethylene bags and placed in a freezer (NFT-4258, Cleo Natural Group co. Ltd., Nonthaburi, Thailand) at -20°C for 24 h.

Physical Properties Determination

The physical parameters, gained weight, cooking loss and cooking yield, were analysed following the method of Rattanasatheirn et al. (2008). The gained weight was calculated using the following equation:

Gained weight (%) = [(weight after soaking – weight before soaking)/weight before soaking] × 100

Cooking loss and yield of the frozen fillets were thawed in the refrigerator at 4 ºC for 24 h. The weight of fillets were measured before and after cooking by steaming at 95±2ºC for 15 min to determine cooking loss and cooking yield as following:

Cooking loss (%) = [(weight after thawing – weight after steaming)/ weight after thawing] × 100

Cooking yield (%) = (weight after steaming/ weight after thawing) × 100

For drip loss, the frozen fillets were weighted and thawed in a refrigerator at 4ºC for 24 h and excessive water was removed from the fish surface using filter paper. Then, the weight of each fillet was measured and the drip loss calculated as per the following (Gonçalves & Ribeiro, 2009).

Drip loss (%) = [(weight before thawing – weight after thawing)/weight before thawing] × 100

Chemical Properties

The moisture content of frozen fillets were investigated according to the standard

method of 934.01AOAC (2000). The colour of the fillets was determined using a colourimeter (Hunter Lab, Colorflex, USA).

Sensory Analysis

A frozen fillet was thawed in the refrigerator at 4ºC for 24 h. The sample was then cut into square cubes of 30×30×20 mm, wrapped in aluminum foil and cooked in a steaming pot at 95±2ºC for 15 min. A sensory test was conducted by 40 non-trained panelists for the fillet's appearance and texture using a 9-point hedonic scale.

Optimisation of Soaking Conditions

The phosphates giving the best results from the previous experiment were used for soaking optimisation. Three main factors, phosphate concentration (1-3.5% w/v), time (10-30 min) and NaCl concentration (1-3% w/v), were investigated for optimum conditions. The experimental design was created by central composite design (CCD) by Minitab (Trial version 16, Minitab Inc., State College, PA, USA). Twenty treatments could be observed. The code and real value of each factor is shown in Table 1.

Table 1
Experimental design range and values of the independent variables in the central composite design (CCD) of fish fillets in optimisation step

Independent Variables	Symbols	Levels				
		Code Value				
		-1.0	0.0	+1.0	+1.7	
		Real Value				
Phosphate Concentration (%)	X_1	0.13	1.00	2.25	3.50	4.38
Soaking Time (min)	X_2	3	10	20	30	37
NaCl Concentration (%)	X_3	0.3	1.0	2.0	3.0	3.7

Statistical Analysis

The response model was calculated by Minitab. The model proposed was $Y = \beta_0 + \Sigma \beta_i x_i + \Sigma \beta_{ii} x_2 + \Sigma\Sigma \beta_{ij} x_i x_j$, where x_i and x_j are the code independent variables, whereas β_0, β_i, β_{ii}, and β_{ij} are the coefficients for intercept, linear, quadratic and interaction. One-way ANOVA was performed for significant difference using SPSS statistical package 16.0 software (SPSS Inc., USA). Duncan's new multiple range test (DMRT) was used to test for the differences between means with a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

Investigation of Suitable Phosphates

The physical property results of the fillet dip in different phosphates (SAPP, TSPP, STPP and SHMP) is shown in Table 2. It was found that moisture content was at a similar level for all the treatments (80-82%). The highest moisture content was observed in STPP, implying that the least water loss occurred in the soaking step. The control (no phosphate) had a moisture content of 80%, which matched the results obtained in a similar study of lingcod fillet

(Duan et al., 2010). The gained weight ranged between 17 and 40%. STPP showed a highly significant difference compared to TSPP, SHMP, SAPP and the control. Xiong et al. (2000) studied the effects of different phosphate compounds on chicken meat and reported that triphosphate and pyrophosphate compounds showed similar levels but at higher levels than hexametaphosphate compounds. The mechanism of phosphate forming in the meat could be explained as follows: Phosphate attacks inside structures of myofribrilla protein, leading to pH change and increasing ionic bonds, resulting in higher water holding in muscle protein and increasing binding of water and protein (Thorarinsdottir et al., 2004).

Table 2
Gained weight and moisture content of fish fillets after soaking and drip loss, cooking loss and cooking yield of frozen fish fillets treated with different phosphates

Phosphates	Moisture (%)	Gained Weight (%)	Drip Loss (%)	Cooking Loss (%)	Cooking Yield (%)
SAPP	81.28±0.12^{b}	17.00±0.71^{c}	3.50±0.32^{b}	19.36±1.09^{b}	71.64±0.76^{b}
TSPP	82.19±0.53^{ab}	32.00±0.73^{b}	2.50±0.34^{b}	20.97±0.40^{b}	75.85±1.05^{a}
STPP	82.33±0.24^{a}	40.00± 0.32^{a}	1.18±0.10^{c}	18.28±0.11^{c}	75.85±0.39^{a}
SHMP	81.97±0.61^{b}	24.00±0.20^{c}	5.59±0.20^{a}	24.53±1.89^{a}	71.23±2.58^{b}
Control	80.16±0.26^{c}	13.00±0.44^{d}	5.60±0.32^{a}	25.26±1.09^{a}	67.64±0.76^{c}

Note: SAPP; Sodium acid pyrophosphate, TSPP; Tetra sodium pyrophosphate, STPP; Sodium tripolyphosphate, SHMP; Sodium hexametaphosphate, Control; no phosphate/-mean±SD and different letters (a, b, c) in the same column indicate significant ($p \leq 0.05$) differences among different phosphates

Drip loss is the weight loss owing to water leaking from frozen food products during the thawing process, causing cell membrane damage and distortion resulting in water leakage from cells (Xiong, 1997). Phosphates could increase binding between water and protein during freezing storage (Woyewoda & Bligh, 1986). In this study, drip loss ranged between 1.8 and 5.6%. STPP showed the lowest drip loss value with a significant difference comparing with other phosphates ($p \leq 0.05$). This implied that STPP could maintain the highest water holding capacity of protein in the freezing step. STPP showed the least cooking loss with significant difference ($p \leq 0.05$) at 18.28%. For cooking yield, TSPP and STPP provided the highest value of 75.85% ($p \leq 0.05$).

Colour

The results of fillet colour are shown in Table 3. A high L* value for STPP and SAPP was observed. The L* value showed the lightness of the sample due to associate expansion capacity (Altan et al., 2008) and lighter colour tended to customer preference (Huda et al., 2010). Fillets dipped in STPP had high a* value ($p \leq 0.05$) compared with those that used other treatments while c*

(colour intensity) had the highest value for SAPP and the control. There was no significant difference of °h in all the treatments.

Table 3
Colour of frozen fish fillets treated with different phosphates

Treatment	Colour				
	L*	a*	b*^{ns}	c*	°h^{ns}
SAPP	66.28±1.17^{a}	4.01±0.85^{ab}	9.25±0.35	56.28±1.17^{b}	9.84±33.74
TSPP	62.57±5.20^{b}	3.67±2.34^{ab}	8.33±1.86	5.20±3.00^{b}	9.18±2.63
STPP	67.63±3.64^{a}	5.22±1.42^{a}	8.74±0.71	3.64±2.10^{b}	10.22±1.14
SHMP	63.37±2.71^{b}	2.32±178^{ab}	8.72±0.92	2.71±1.56^{a}	9.07±1.31
Control	52.77±1.60^{c}	1.14±0.54^{b}	7.75±0.48	56.77±1.60^{b}	7.90±0.56

Note: SAPP; Sodium acid pyrophosphate, TSPP; Tetra sodium pyrophosphate, STPP; Sodium tripolyphosphate, SHMP; Sodium hexametaphosphate, Control; no phosphate/-mean±SD and different letters (a, b, c) in the same column indicate significant (p≤0.05) differences among different phosphates, ns; non-significantly difference (p>0.05)

Sensory Analysis

The sensory test (cooked fillets) was conducted by 40 non-trained panelists using a 9-point hedonic scale, where 1 = extremely dislike; 5 = neither like nor dislike; 9 = extremely like. The results of the sensory score are shown in Table 4. Fillets dipped in SHMP and STPP had high a appearance score at 7.16 and 7.05 (p>0.05). There were no significant differences for colour and taste scores. STPP had the highest odour, texture and overall consistency score with 6.75, 7.20 and 7.75, respectively. Similarly, Goncalves and Riberio (2009) found that STPP showed high sensory scores for frozen shrimp, caused from increasing water holding capacity resulting in higher sensory evaluation scores (Goncalves et al., 2008).

Table 4
Sensory evaluation of cooked fillet using 9-point hedonic scale

	Sensory Analysis					
Treatment	Appearance	Colour	Odour	Taste^{ns}	Texture	Overall
SAPP	6.33±1.04^{b}	6.37±1.37	5.87±1.36^{b}	6.62±1.20	6.70±1.16^{ab}	6.86±1.12^{b}
TSPP	6.33±1.04^{b}	6.50±1.14	6.75±1.56^{a}	6.95±1.36	6.79±1.18^{ab}	7.04±0.95^{ab}
STPP	7.05±1.06^{a}	6.55±1.28	6.75±1.35^{a}	6.83±1.08	7.20±1.12^{a}	7.75±0.94^{a}
SHMP	7.16±1.04^{a}	6.40±1.07	6.37±1.31^{ab}	6.83±1.09	7.16±09.1^{a}	7.12±0.99^{ab}
Control	6.07±1.12^{ab}	6.59±1.44	6.26±1.34^{ab}	6.12±0.85	6.54±1.35^{b}	7.04±0.99^{b}

Note: SAPP; Sodium acid pyrophosphate, TSPP; Tetra sodium pyrophosphate, STPP; Sodium tripolyphosphate, SHMP; Sodium hexametaphosphate, Control; no phosphate/-mean±SD and different letters (a, b, c) in the same column indicate significant (p≤0.05) differences among different phosphates, ns; non-significantly difference (p>0.05)

Compared with four phosphate compounds, STPP showed the best results, such as gained weight, drip loss, cooking loss and cooking yield and provided a high L* and a*; these significantly related to the customer's preference. Moreover, STPP is available in the market at low cost, is considered safe for consumption and handling, and is widely used in the food industry. Therefore, STPP was utilised and studied in the soaking process in the further optimisation step.

Optimisation of Soaking Conditions

The central composite design (CCD) was used to create treatments. The main factor was phosphate concentration (X_1), soaking time (X_2) and NaCl concentration (X_3). Twenty treatments were found and the results are shown in Table 5 and Table 6. Each response value was calculated using a regression model in terms of linear, intercept and quadratic model, which is expressed as the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta 2_3 X_2 X_3$$

Table 5
Treatments created by central composite design (CCD) and physical properties

Treatment	Code factor			Response									
	X_1	X_2	X_3	Moisture (%)	Gained weight	Cook loss	Cook yield	Drip loss	L*	a*	b*	C*	°h
1	0	0	1.7	79.50	19.99	19.99	69.68	2.40	60.81	11.00	76.64	2.49	10.53
2	1	1	1	80.90	19.01	32.80	81.08	4.67	58.12	11.87	74.21	3.25	11.39
3	-1	-1	1	78.00	20.41	19.41	78.57	1.20	64.05	13.35	75.73	3.35	12.87
4	0	1.7	0	80.60	21.25	36.30	80.4	3.95	50.23	15.28	61.00	7.36	13.38
5	0	0	-1.7	80.80	18.87	18.25	72.17	2.52	58.76	14.74	75.79	3.62	14.27
6	-1	1	1	78.20	15.80	18.63	75.56	5.44	55.22	15.64	63.75	6.91	14.02
7	0	0	0	80.70	22.68	22.90	72.21	4.28	57.14	15.01	68.27	5.57	13.94
8	0	0	0	78.05	23.91	22.90	83.45	4.28	59.70	15.89	73.09	4.63	15.20
9	0	-1.7	0	78.55	17.89	18.05	72.67	2.76	61.95	14.18	72.02	4.38	13.48
10	0	0	0	76.46	21.28	28.73	71.23	4.52	61.64	14.88	79.08	2.88	14.57
11	-1	1	-1	77.45	18.31	27.37	77.18	3.28	58.80	13.72	68.98	4.74	12.87
12	1	-1	1	80.45	21.95	13.20	86.9	2.40	62.28	12.57	78.08	2.61	12.26
13	1	1	-1	80.65	14.96	25.82	78.83	3.43	62.72	14.23	78.13	2.94	13.92
14	0	0	0	78.70	22.68	22.90	78.74	3.28	60.84	14.64	77.09	3.33	14.23
15	0	0	0	77.70	22.68	22.90	78.38	4.08	65.85	12.82	81.40	1.91	12.63
16	-1.7	0	0	79.65	22.79	19.41	82.4	1.20	64.08	14.36	72.48	4.34	13.69
17	0	0	0	80.10	19.88	26.79	87.9	3.68	56.35	15.23	67.19	5.94	14.01
18	1.7	0	0	75.40	20.13	29.12	72.17	4.58	54.98	14.74	62.37	6.89	12.97
19	1	-1	-1	80.90	11.32	18.63	78.94	1.20	60.82	14.43	71.34	4.63	13.67
20	-1	-1	-1	78.20	13.84	19.41	78.38	2.40	59.90	11.08	58.30	3.09	14.22

Note: X_1: Phosphate concentration, X_2: Soaking time, X_3: NaCl concentration

Table 6
Treatments created by central composite design (CCD) and sensory evaluation

Treatment	Code factor			Response					
	X_1	X_2	X_3	Appearance	Colour	Odour	Taste	Texture	Overall
1	0	0	1.7	79.50	19.99	19.99	69.68	2.40	60.81
2	1	1	1	80.90	19.01	32.80	81.08	4.67	58.12
3	-1	-1	1	78.00	20.41	19.41	78.57	1.20	64.05
4	0	1.7	0	80.60	21.25	36.30	80.4	3.95	50.23
5	0	0	-1.7	80.80	18.87	18.25	72.17	2.52	58.76
6	-1	1	1	78.20	15.80	18.63	75.56	5.44	55.22
7	0	0	0	80.70	22.68	22.90	72.21	4.28	57.14
8	0	0	0	78.05	23.91	22.90	83.45	4.28	59.70
9	0	-1.7	0	78.55	17.89	18.05	72.67	2.76	61.95
10	0	0	0	76.46	21.28	28.73	71.23	4.52	61.64
11	-1	1	-1	77.45	18.31	27.37	77.18	3.28	58.80
12	1	-1	1	80.45	21.95	13.20	86.9	2.40	62.28
13	1	1	-1	80.65	14.96	25.82	78.83	3.43	62.72
14	0	0	0	78.70	22.68	22.90	78.74	3.28	60.84
15	0	0	0	77.70	22.68	22.90	78.38	4.08	65.85
16	-1.7	0	0	79.65	22.79	19.41	82.4	1.20	64.08
17	0	0	0	80.10	19.88	26.79	87.9	3.68	56.35
18	1.7	0	0	75.40	20.13	29.12	72.17	4.58	54.98
19	1	-1	-1	80.90	11.32	18.63	78.94	1.20	60.82
20	-1	-1	-1	78.20	13.84	19.41	78.38	2.40	59.90

Note: X_1: Phosphate concentration, X_2: Soaking time, X_3: NaCl concentration

In this study, the significant models ($p \leq 0.05$) were cooking yield, gained weight and ^{o}h. Moreover, a* also showed a fine model with high R^2 as well as this factor was the important response for consumer acceptability (Huda et al., 2010). In addition, the a* value cloud showed accumulation of brown-coloured methemoglobin from the indication of hemoglobin oxidation in flesh fish (Kachele et al., 2017). Therefore, the models of cooking yield, °h, a* and gained weight were applied in the process of response optimisation step. The parameters, regression coefficient and full models are shown in Table 7 and Table 8. Gained weight, cooking yield, a* and ^{o}h showed good performances with R^2 of 0.7545, 0.6851, 0.7192 and 0.7138, respectively. These responses were used to create the response surface plots as shown in Figure 1. To find the optimum condition for the soaking process, the multi-response optimiser function in Minitab software was used for calculation. The response of gained

weight and cooking yield were set at the maximum while the a* and °h values were set at the minimum together with weight and important of all response equal one. It was found the optimum point was 2.56% of phosphate concentration (code value = 0.25), soaking time was 37 min (code value = 1.68) and 3.7% of NaCl concentration (code value = 1.68). The fillets soaking in this condition had 11.45% of gained weight, 73.11% of cooking yield, 10.97 of a* and 9.94 of °h, respectively. Thorarinsdottir et al. (2001) reported that addition of NaCl (0.5-5%) could increase space in myofibrillar protein and reduce repulsive charges, resulting in increasing water holding capacity. However, the limitation of the current work for model development and optimisation was that important parameters could not be developed to predict the models. Further optimisation should be focused on some parameters involving economic factors and consumer preference in fish fillet processing such as gained weight and drip loss.

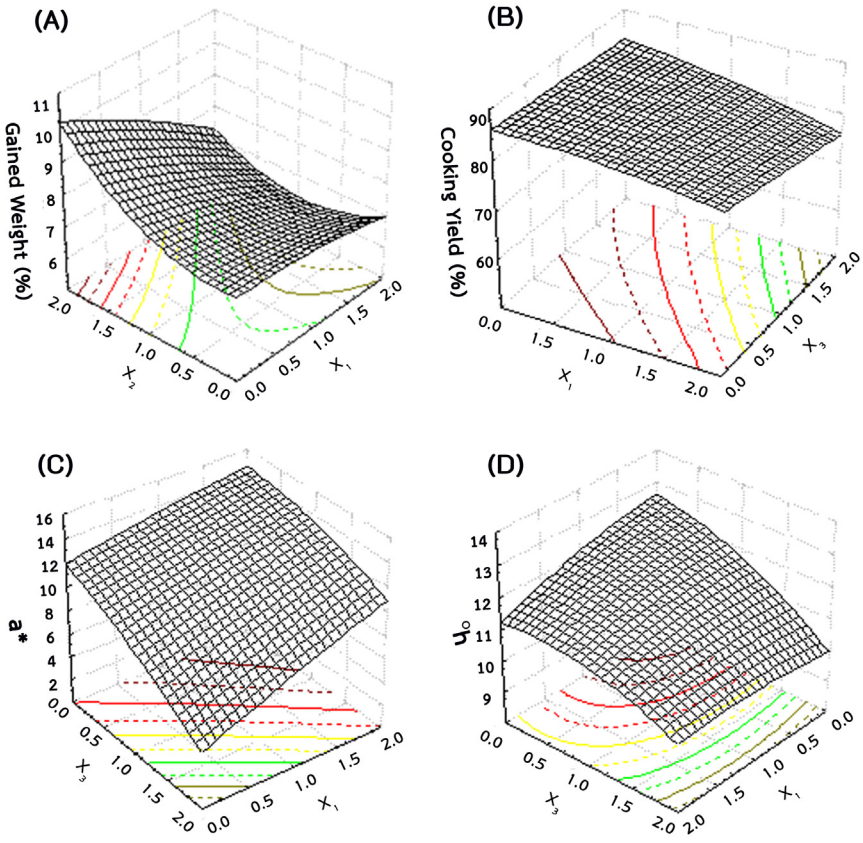

Figure 1. Response graphs of gained weight (A), cooking yield (B), a* (C) and °h (D)

Table 7
Parameters, regression coefficient and p-value of used responses

Parameter	Term	Gained weight		Cooking yield		a*		^{o}h	
		Coef.	p-Value	Coef.	p-Value	Coef.	p-Value	Coef.	p-Value
β_0	Intercept	6.717	0.000	80.042	0.000	14.766	0.000	14.093	0.000
β_1	X_1	0.352	0.377	3.299	0.031	-0.003	0.000	-0.288	0.219
β_2	X_2	0.769	0.071	0.800	0.556	0.430	0.16	-0.724	0.749
β_3	X_3	0.030	0.938	-0.741	0.570	-0.463	0.133	-0.764	0.006
β_{11}	X_{12}	-0.181	0.635	-1.064	0.424	-0.205	0.473	-0.236	0.296
β_{22}	X_{22}	0.976	0.078	1.385	0.483	-0.141	0.621	-0.203	0.366
β_{33}	X_{32}	0.083	0.870	-0.045	0.980	-0.799	0.016	-0.566	0.025
β_{12}	X_1X_2	-0.708	0.085	-1.899	0.168	-0.729	0.078	-0.054	0.854
β_{13}	X_1X_3	-0.213	0.677	-1.415	0.980	-1.051	0.018	0.466	0.136
β_{23}	X_2X_3	-0.672	0.100	-0.258	0.844	-0.105	0.781	0.173	0.56

Table 8
The full quadratic polynomial models of each response

Response	Quadratic Polynomial Model	R^2	p-Value
Gained weight	$Y_1 = 6.71+0.35X_1+0.76X_2+0.03X_3 -0.18X_1^2+0.97 X_2^2+ 0.08 X_3^2 - 0.7X_1X_2 - 0.21 X_1X_3 - 0.67X_2X_3$	0.7545	0.051
Cooking yield	$Y_2 = 80.04+3.29X_1 +0.8X_2-0.74X_3 -1.06X_1^2+1.38 X_2^2 – 0.04 X_3^2 -1.89X_1X_2 -1.41 X_1X_3 - 0.25X_2X_3$	0.6851	0.015
a*	$Y_3 = 14.76-0.003X_1 +0.43X_2-0.46X_3 -0.2X_1^2-0.41X_2^2 – 0.79X_3^2 -0.72X_1X_2 -1.05X_1X_3-0.1X_2X_3$	0.7192	0.059
^{o}h	$Y_4 = 14.09-0.28X_1 -0.72X_2-0.76X_3 -0.23X_1^2-0.2X_2^2 – 0.56X_3^2 -0.05X_1X_2 +0.46X_1X_3+0.17X_2X_3$	0.7138	0.050

Note: X_1: Phosphate concentration, X_2: Soaking time, X_3: NaCl concentration

CONCLUSION

Four phosphate compounds were compared on their effect on physical appearance, colour analysis and sensory analysis of frozen fillets of Bocourti's catfish. STPP showed the best results in gained weight, drip loss, cooking loss and cooking yield and provided a high L* and a* colour. Soaking conditions were optimised using the response surface methodology. The optimum conditions were 2.56% of STPP concentration, 3.7% of NaCl concentration and a soaking time of 37 min for improvement of the quality of frozen fillets, showing gained weight of 11.45%, cooking yield of 73.11%, a* of 10.97 and ^{o}h of 9.94.

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Effects of Short- and Long-Term Temperature on Seed Germination, Oxidative Stress and Membrane Stability of Three Rice Cultivars (Dular, KDML105 and Riceberry)

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ABSTRACT

The reduction in rice productivity as a result of elevated daily temperature due to climate change is a major concern for Thailand. This study aimed to investigate hydrogen peroxide and malondialdehyde (MDA) content and electrolyte leakage of rice seedlings grown from seeds exposed to different temperature (25°C, 35°C and 40°C) treatments over a short (one week) and long (two weeks) period before germination. Three rice cultivars were investigated, Dular, KDML105 and Riceberry. The experiment was designed in RCBD with six replications. The results indicated that Riceberry seeds produced a greater percentage of normal seedlings after both short- and long-term heat treatment (40°C). By contrast, KDML105 seeds exposed to 40°C for one and two weeks gave rise to the highest percentage of abnormal seedlings. The highest oxidative stress indicated by the accumulation of hydrogen peroxide was found in abnormal seedlings of cvs. KDML105 and Riceberry after short- and long-term heat (40°C) exposure, respectively. The effect of heat stress on membrane stability was indicated by MDA content and electrolyte leakage. MDA content was the highest in abnormal seedlings of cv. Riceberry after heat exposure for two weeks. High electrolyte leakage due to both short- and long-term high temperature treatment was found in abnormal seedlings of all the rice cultivars. Heat exposure to rice seeds at 40°C for one week induced the highest percentage of abnormal seedlings in KDML 105 coinciding with the highest hydrogen

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peroxide content and membrane damage. These results provide crucial information for consideration in breeding programmes for heat-tolerant rice cultivars.

Keywords: Temperature stress, seed germination, oxidative stress, membrane stability

INTRODUCTION

Rice (*Oryza sativa*) is one of the most important crops in Thailand. The main rice production area is found in Northeastern Thailand. One of the most common rice cultivating methods in this region is the sowing of seeds before the rainy season, a practice that often exposes seeds to high temperatures. The current maximum temperature of the warmest month in Thailand and Northeastern Thailand are approximately 41°C and 40°C, respectively (Thai Meteorological Department, 2017). In addition, the maximum temperature of the warmest month in Thailand is predicted to rise by approximately 1.5-2°C (Trisuart et al., 2011). Peng et al. (2004) reported that rice becomes heat sensitive and this reduces grain yield by 10% during the reproductive stage for every 1°C rise in daily temperature, i.e. every increase by 1°C leads to a reduction of 10 % yield. High temperatures also affect seed germination and the physiology of seedlings. Essemine et al. (2010) reported that ungerminated seeds and damaged embryo were found in wheat seeds exposed to 45°C. Piramila et al. (2012) found that *Vigna mungo* seeds showed a significantly decreased germination percentage and seed vigour index after heat exposure. Similarly, the germination percentage of *Cassia tora* seeds exposed to 40°C, 50°C and 60°C for 10 days decreased by 85%, 63% and 32%, respectively compared to seeds incubated at room temperature (Pant et al., 2012).

High temperatures lead to oxidative burst, indicated by the production of reactive oxygen species (ROS), namely, superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•) (Hammond-Kosack & Jones, 1996). These ROS exert a toxic effect in cells via lipid peroxidation, protein degradation, DNA damage, plasma membrane damage (Fridovich, 1986; Halliwell & Gutteridge, 1989), electrolyte leakage (Abass & Rajashekar, 1991), cell structure damage (Mittler et al., 2004) and other physiological changes such as declining photosynthetic rate, increasing respiration rate (Paulsen, 1994) and finally, cell death (Maxwell et al., 1999). The autocatalytic peroxidation of membrane lipids and pigments and modification of membrane permeability and function are the main effects of ROS (Hasanuzzaman et al., 2012). Lipid peroxidation is indicated by malondialdehyde (MDA) content. A high temperature of 40/45°C (day/night temperature) leads to MDA content increases in rice and maize by 1.8-fold and 1.2- to 1.3-fold, respectively. Similarly, the H_2O_2 levels at 40/35°C showed a 1.9- to 2.0-fold elevation in rice and 1.4- to 1.6-fold elevation in maize relative to their control (Kumar et al., 2012). Membrane stability indicated by electrolyte leakage was used in one study for screening thermal tolerance in wheat (Saadalla et al., 1990), and MDA

content was also found to be an indicator of membrane stability (Fan et al., 2015).

In Thailand, Khao Dawk Mali 105 (KDML105) is a Thai rice variety commonly known as jasmine rice. KDML105 is well-known in the global market because of its distinctive characteristic of a white colour, long grains and slender shape. When it is cooked, it is soft and releases a natural aromatic fragrance (Ranna et al., 2016; Bureau of Rice Research and Development, 2017). Riceberry is the most famous Thai brown rice. It is a dark violet grain and contains a high amount of antioxidants. It is soft and releases an aroma after cooking (Rice Science Center and Rice Gene Discovery, 2017). Finally, Dular is a traditional rice variety from India (Wang et al., 1998) and is classified as highly heat tolerance using as donor parents in a breeding programme (Magnibas et al., 2014). This research focused on determining the effects of seed pretreatment with different temperatures on seed germination, oxidative stress and membrane stability in seedlings of these three rice cultivars i.e. Dular (Magnibas et al., 2014), KDML 105 and Riceberry, all of which have different levels of heat tolerance.

MATERIALS AND METHOD

Seed Germination and Stress Conditions

Dry seeds of three rice cultivars, that is, Dular (obtained by Biotechnology Research and Development Office, Thailand), KDML105 and Riceberry (obtained by Assist. Prof. Dr. Jirawat Sanitchon, KKU), were used as the experimental materials. This experiment was carried out in the Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Thailand during the period April-August 2015. For heat treatment, 20 sterilised seeds were placed in a Petri dish each and incubated at 25°C (optimum temperature for germinating seeds and growing worldwide plants), 35°C (optimum temperature for growing rice plants) and 40°C (maximum temperature of the warmest month in Northeastern Thailand) for one week (minimum duration for which dry rice seeds can be exposed to high temperatures under dry ungerminated seed broadcasting cultivation) and two weeks (moderate duration for which dry rice seeds can be exposed to high temperatures under dry ungerminated seed broadcasting cultivation). After heat exposure, the seeds were placed on germination paper and put in a plastic box under humidity control by spraying with water. The germination boxes were placed in a growth chamber (25°C and 80% of RH) under normal light. Seeds were considered as germinated after the radicles had emerged to a length of 2 mm. Germination was recorded at 14 days according to AOSA (1990) and then germination percentage (% GP) was calculated according to ISTA (1985) (Equation 1) and Pirasteh-Anosheh et al. (2011). Normal and abnormal seedlings

were collected for measurement of hydrogen peroxide and malondialdehyde content and electrolyte leakage.

$$\text{Germination percentage (\% GP)} = \frac{\text{No. of normal seedlings x 100}}{\text{No. of total seeds}} \quad (1)$$

Measurement of Hydrogen Peroxide Content

Hydrogen peroxide content was determined according to Velikova et al. (2000). Sample tissues (0.5 g) were ground in an ice bath with 5 ml of 0.1% w/v trichloroacetic acid (TCA) and centrifuged at 12,000×g for 15 min. The supernatant (0.5 ml) was transferred to a 15-ml test tube and 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide were added to the supernatant and mixed throughly. The mixture was measured spectrophotometrically at 390 nm.

Measurement of MDA Content

The assay of lipid peroxidation measured the amount of MDA formation according to Heath and Packer (1968). Sample tissues (0.1 g) were transferred to a 15-ml test tube to which 1.4 ml of distilled water was added; this was then mixed using a vortex mixer. Thiobarbituric acid (TBA) reagent (1.5 ml of 0.5% (w/v) TBA in 20% (w/v) TCA) was then added and mixed using a vortex mixer. The mixture was boiled in a water bath for 25 min. The reaction was stopped by placing the tube on ice for 5 min and then centrifuged at 1000×g for 10 min to remove cell debris. The absorbance of the supernatant was measured spectrophotometrically at 532 nm and 600 nm.

Measurement of Electrolyte Leakage

Electrolyte leakage (EL) measurement (in %) was determined according to Bajji et al. (2001). Fresh seedling samples (0.1 g) were placed in a 15-ml test tube containing 10 ml of deionised water and incubated at room temperature for 24 h. The electrical conductivity (EC_1) of the suspension was measured, and the tube was then heated to 100°C for 15 min. The samples were then cooled to 25°C and the final electrical conductivity (EC_2) was measured.

Experimental Designs and Statistical Analysis

The experiment was carried out in a randomised completely block design (RCBD) with six replications. Data were analysed using analysis of variance (ANOVA) at the significant level of p=0.05 and means comparison or means separation among various treatments was determined through Duncan's multiple range tests (DMRT) at p=0.05 using the Statistical Package for the Social Sciences software (SPSS) for windows version 17.0.

RESULTS AND DISCUSSION

Effects of High Temperatures on Characteristics of Seed Germination and Seed Germination Percentage

Characteristics of seed germination in three rice varieties, Dular, KDML105 and

Riceberry are shown in Figure 1. Normal seedlings (n) in all rice varieties showed white and long roots. Root length ranged from 3 to 6 cm. The leaf blade was green and approximately 5-6 cm long. Leaf numbers per plant was approximately 2-3. Abnormal seedlings (ab) showed white roots and their root length was 0.5-3 cm. The leaf blade was yellow or brown, folded and curved and approximately 3-5 cm long. The leaf number per plant was in the range of 1-2. In ungerminated seeds (un), the seed coats were dark brown and black. A milky liquid was expressed when the seeds were crushed. The ungerminated seeds were classified as dead seeds according to ISTA (1985).

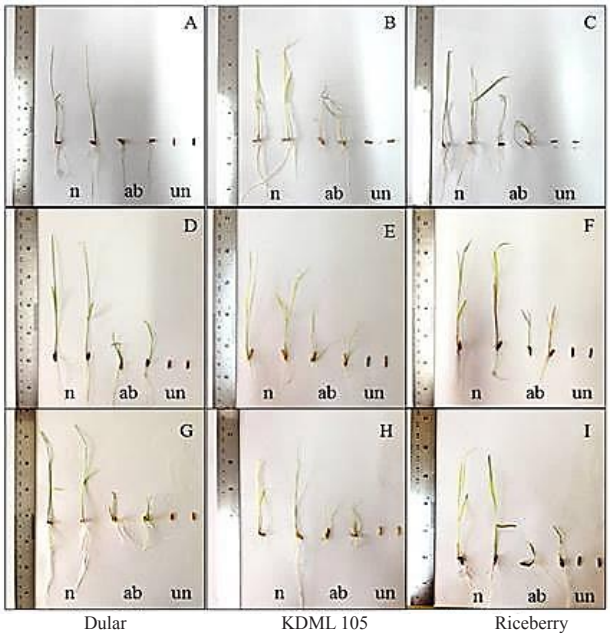

Figure 1. The characteristics of normal seedlings (n), abnormal seedlings (ab) and ungerminated seeds (un) of three rice cultivars: Dular (A, D and G), KDML105 (B, E and H) and Riceberry (C, F and I) grown from seeds treated at 25°C, 35°C and 40 C for one week (left seedlings) and two weeks (right seedlings)

Many physiological changes under high temperature stress may be reflected in the abnormality of seed germination. The interaction between the cultivars and temperature (cv x Temp) and between the cultivars and exposure time (cv x Time) was significant for all instances of seed germination and the interaction of cv x Temp x Time was also significant (Table 1). The significant differences were mainly due to high temperature adversely affecting seed germination of all the rice cultivars (Figure 2 and Table 1). Riceberry seeds produced the highest percentage of normal seedlings after the seeds were exposed to 40°C for one week (62.5 ± 1.44%). However, when the seeds were treated at 40°C for two weeks, the Dular seeds showed the highest

percentage of normal seedlings (61.67 ± 1.67%). The exposure temperature of 40°C for one and two weeks (36 ± 6% and 27.5 ±1.44%) induced the lowest percentage of normal seedlings in cv. KDML105. This result was supported by the work of Abernethy (1989), who reported reduction of seed germination percentage in rice due to high temperature (51°C) exposure, and Ali et al. (2013), who reported that the high temperature of 42 ± 3°C resulted in slow seed germination and decreased the percentage of seed germination. Moreover, Akman (2009) showed that temperature exposure at 35, 38 and 41°C reduced the germination of rice and sorghum. The results of abnormal seedlings are shown in Figure 2C and D. The results show that KDML105 was the highest percentage of abnormal seedlings after temperature exposure at 40°C for one week (70 ± 2.88%) and two weeks (70%) compared to other rice cultivars. Spears et al. (1997) found that high temperature at 38/33°C (day/night) resulted in a low percentage of normal seedlings but exhibited a higher percentage of abnormal seedlings. This study found that Dular seeds were the most tolerant to high temperature, showing the lowest percentage of abnormal seedlings after the seeds were exposed to 40°C for one and two weeks (20 ± 2.8% and 18.33 ± 1.67%). No instances of temperature treatment affected the percentage of ungerminated seeds in all the rice cultivars.

Table 1
Analysis of variance of the effects of rice seed cultivars, temperatures and temperature-exposure duration on the percentage of normal seedling germination, abnormal seedling germination and ungerminated seeds under Thailand experimental conditions

Sources	% Normal Seedlings	% Abnormal Seedlings	% Ungerminated Seeds
	Mean square		
Cultivars (cv)	1766.90**	3680.57**	844.51**
Temperature (Temp)	323.35**	36.96	29.79
Temperature-Exposure Duration (Time)	1622.51**	1340.01**	8.96
cv * Temp	545.57**	1004.06**	117.29**
cv * Time	440.35**	471.13**	37.85**
Temp * Time	1043.97**	810.29**	8.68
cv * Temp * Time	241.57**	212.65**	68.40**
Error	35.70	35.07	10.70

** Indicates significant at $p \leq 0.05$ probability levels

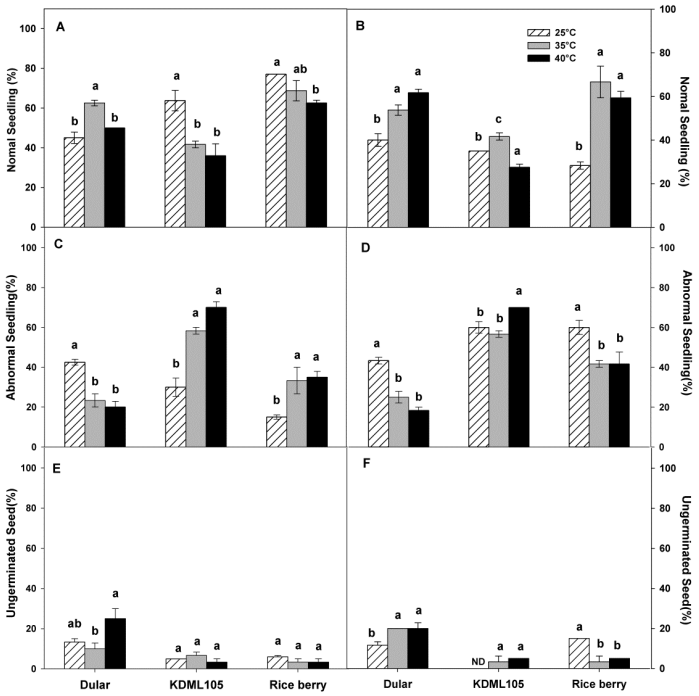

Figure 2. Effect of exposing seeds to different temperatures (25°C, 35°C and 40°C) for one (A, C, E) and two (B, D, F) weeks before germination on percentages of normal seedlings (A and B), abnormal seedlings (C and D) and ungerminated seeds (E and F) in rice cvs. Dular, KDML105 and Riceberry. The values are means ± SE (n=3 to 4). ND=not determined. Different letters in each cultivar indicate significant differences among temperature treatments tested by DMRT at $p \leq 0.05$

Effect of High Temperature on Oxidative Stress and Membrane Stability of Rice Seeds

Hydrogen peroxide accumulation in the abnormal seedlings grown from seeds treated at 40°C for one week was higher (0.5-3-fold) than in those treated at 35°C for one week. The increasing trend of hydrogen peroxide accumulation was found in the abnormal seedlings germinated from Dular and KDML105 seeds treated at 35 and 40°C for two weeks (Figure 3B). This result related to the interaction of cv x Time, cv x Seedling x Temp and cv x Time x Seedling x Temp, which showed significant differences (Table 2). From this study, the increasing content of hydrogen peroxide in the abnormal seedlings grown from seeds treated at 35 and 40°C suggested that abnormal seedlings suffered from oxidative stress induced by ROS, leading to growth inhibition, similar to the results reported by Schöffl et al. (1999). The reactive oxygen species can oxidise membrane lipid resulting in cell membrane damage (Bowler et al., 1992), leading to cell death (Abernethy et al., 1989). Ali et al. (2013) reported that at the germination stage, rice showed high H_2O_2 content due to heat exposure. Timabud (2015) also indicated that rice var. IR64 showed high

H_2O_2 content in high temperature treatment. Normal and abnormal seedlings of rice cv. Riceberry grown from seeds treated at 25°C for two weeks showed the highest level of hydrogen peroxide accumulation (2- and 3-fold higher than seedlings of rice cvs. Dular and KDML105, respectively).

Table 2
Analysis of variance of the effects of rice cultivars, temperatures, temperature-exposure duration and seedling normal status on H_2O_2, MDA and EL of rice seedlings under Thailand experimental conditions

Sources	H_2O_2 mmol/gFW	MDA mmol/gFW	EL (%)
	Mean square		
Cultivars (cv)	5.30 E-8**	0.001**	1376.69**
Temperature-Exposure Duration (Time)	1.05 E-8**	4.90 E-5	26.98**
Temperature (Temp)	5.51 E-8**	0.001**	4644.58**
Seedling Normal Status (Seedling)	1.12 E-7**	0.002**	11078.41**
Seedling * Temp	0.20 E-8**	0.001**	772.50**
cv * Time	1.53 E-8**	0.001**	73.71**
cv * Seedling * Temp	0.27 E-8**	0.007**	51.39**
Time * Seedling * Temp	0.13 E-8**	5.74 E-5^{ns}	107.26**
cv * Time * Seedling * Temp	0.37 E-8**	0.45 E-5^{ns}	78.95**
Error	1.80E-10	2.15E-5	6.46

ns and ** Indicates non-significant and significant at $p \leq 0.05$ probability levels, respectively

The decline in membrane stability can be determined by MDA content and electrolyte leakage (EL) (Fan et al., 2015). A trend of higher MDA content in all the rice cultivars treated with different temperatures for one and two weeks was found in abnormal seedlings rather than in normal seedlings as shown in Figure 3C-D and Table 2 (the interaction of cv x Time and cv x Seedling x Temp was significant for MDA content). The highest MDA content was found in abnormal seedlings of Riceberry (0.13 ± 0.008 µmol/g FW) compared with other rice cultivars (Table 2); MDA content was significant in the different cultivars. Furthermore, EL in the abnormal seedlings grown from seeds treated at 25 and 40°C for one and two weeks was higher than in those grown from seeds treated at 35°C. In addition, the EL percentage in normal seedlings germinated from all the seeds of the rice cultivars treated at 25°C and 40°C for two weeks showed a higher trend than for temperature exposure at 35°C. Ali et al. (2013) reported that MDA content and EL in rice seedlings treated at 42°C for 72 h was higher than for seedlings treated at 42°C for 24 and 48 h. Moreover, Zhang et al. (2005) suggested that heat stress severely affected mesophyll cell damage and induced

increased membrane permeability. Either denaturation of proteins or an increase in unsaturated fatty acids caused the higher fluidity in the lipid bilayer of biological membranes. The lipids were then destroyed by the lipid peroxidation process, which produces MDA as a final product (Savchenko et al., 2002). The integrity and functions of biological membranes are sensitive to high temperature; for example, heat stress alters the tertiary and quaternary structures of membrane proteins. Such alterations

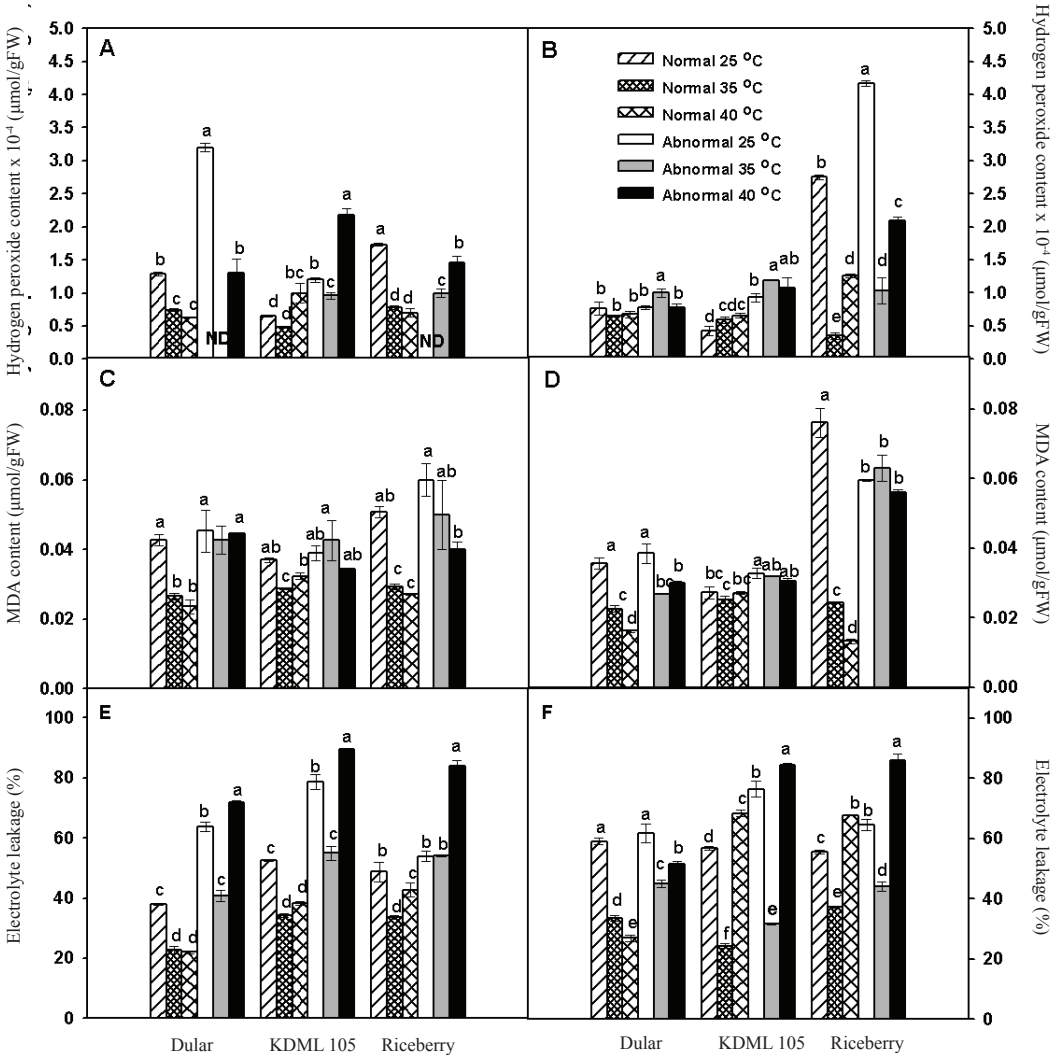

Figure 3. Effect of exposing seeds to different temperatures (25°C, 35°C and 40°C) for one and two weeks before germination on hydrogen peroxide content (A and B), MDA content (C and D) and electrolyte leakage (E and F) in seedlings of rice cvs. Dular, KDML105 and Riceberry. The values are means ± SE (n=3 to 4). ND=not determined. Different letters in each cultivar indicate significant differences among temperature treatments tested by DMRT at $p \leq 0.05$

enhance the permeability of membranes (Savchenko et al., 2002), as is evident from the greater loss of electrolytes after heat exposure (Figure 3E-F and Table 2). Even though Riceberry exhibited high oxidative stress and membrane stability after long high temperature exposure in this study, it may have been recovered by ROS scavenging or membrane repair immediately after attack with ROS. Consequently, ROS was unaffected by heat temperature and ROS levels were higher in the normal seedlings.

CONCLUSIONS

From the results, it was concluded that the dry rice seeds exposed to a high temperature (40°C) resulted in a higher percentage of abnormal seedlings, particularly in KDML105. A higher potential for heat tolerance was found in seeds of rice cvs. Dular and Riceberry. Short-term heat exposure (40°C) induced an increasing trend of oxidation stress in all the abnormal seedlings. Seeds of rice cvs. Dular and Riceberry were susceptible to both short- and long-term exposure to low temperature at 25°C, as shown by the increased oxidative stress and decreased membrane stability. Both short- and long-term heat exposure at 40°C induced increased membrane damage in the abnormal seedlings, indicated by the high EL percentage.

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Kenaf-Based Composite Posts as Alternative Supports for Black Pepper (*Piper nigrum* L.)

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ABSTRACT

Black pepper is a perennial climber and the provision of a supporting post is important for the successful establishment of black pepper planting in the producing countries. With the high cost of establishing black pepper planting using the traditional Belian post, there is a need to develop alternative posts for its sustainability. The study was carried out to evaluate the use of Kenaf composite posts for black pepper planting compared to the commonly used Belian post as supporting material. Three types of Kenaf composite posts, namely, Kenaf Extrusion, Kenaf Pultrusion and Kenafkrete posts, together with mechanically controlled fibre-glass posts, were investigated for black pepper planting. The epiphytical response of black pepper plant, accelerated laboratory decay test, leaf temperature, leaf-to-air vapour pressure deficit and the leaf gas exchange rate were the parameters assessed in this study to indicate the suitability of the composite post for black pepper growing. The trial plot was established at thoroughly exposed conditions to determine the sustainability of Kenaf composite posts at open field conditions. The study demonstrated that adventitious roots of black pepper plants were able to bind on all types of support except the Kenafkrete post, which showed a low number of cling roots. On durability observation, the Kenaf Extrusion post showed severe bending and was intolerant to field weather conditions, whereas the Kenafkrete post showed moderate level of cracks on the post. The laboratory decay test indicated that Kenaf composite posts were highly resistant to wood decay fungi

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and performed better than Belian post. It was discovered that Kenaf Extrusion contributed to an adverse microclimate environment for the growth of black pepper by showing significantly ($p<0.05$) higher leaf temperature and leaf-to-air VPD. Leaf photosynthesis rates (*A*) and leaf stomatal conductance (*gs*) of black pepper supported by Belian, Kenaf Pultrusion, Kenafkrete and fibre-glass were comparatively ($p<0.05$) higher than recorded for Kenaf Extrusion. The results of this study implied that among the supports studied, Kenaf Pultrusion and fibre-glass posts showed positive results as good alternative supports to the currently used Belian post for black pepper planting.

Keywords: Black pepper (*Piper nigrum* L.), Kenaf (*Hibiscus cannabinus* L.), supporting post, composite post

INTRODUCTION

Black pepper (*Piper nigrum* L.) is the most important spice traded internationally and it is cultivated in many tropical regions of the world like India, Brazil, Vietnam, Indonesia, Malaysia and Sri Lanka. As black pepper is a climber, it requires physical post support for proper growth, development and yield. Providing ideal support is important for successful establishment of black pepper holdings. Both deadwood support and living support can be used for black pepper cultivation. In Malaysia and parts of Indonesia, the preferred support material is the wooden pole of the Belian tree (*Eusideroxylon zwageri* Teijsm. & Binn.), also known as Borneo Ironwood, which is high density, heavy and construction-timber resistant to termite attack (Dinesh et al., 2005). The Ironwood poles are durable, lasting for more than 20 years, well beyond the lifespan of pepper plants (Paulus et al., 2011). However, Belian posts are expensive and their acquisition would be the most expensive item in the establishment of a farm. Other hardwood such as Selangan Batu (*Shorea* sp.) and semi-hardwood, mainly Somah (*Ploiarium alternifolium* [Vahl] Melchior) have been used by farmers. Most of them do not last beyond five years after planting. Therefore, farmers sink a new post to reinforce the original post when the latter is at the initial stage of rotting.

As an alternative to Belian posts, farmers have been using living supports for pepper cultivation. In 1992, MARDI had introduced Dedap (*Erythrina indica*) as an alternative live support system for pepper cultivation in Johor for its low establishment cost (Abd. Rahman Azmil & Yau, 1992). However, it was found that the living supports were severely infested by the insect pest, Erythrina gall wasp (*Quadrastichus erythrinae* Kim) (Paulus & Megir, 2006) and therefore, no longer recommended for black pepper planting. Currently in Sarawak, two living supports, Gliricidia (*Gliricidia sepium* [Jacq.] Kunth ex Walp) and Simpuh (*Dillenia suffruticosa* [Griff ex Hook. F. & Thomson] Martelli) are commonly used for the planting of black pepper (Paulus & Megir, 2006). For Gliricidia, a terminal shoot arising from the upper portion of the stake has to be pruned regularly to maintain a height for black pepper growing, and this

task is labour intensive. As for Simpuh, the post can only be obtained from trees found in young secondary forests in Sarawak.

With the high cost of deadwood support and unavailable ideal living support, there is a need to develop alternative posts for black pepper planting. Other hardwood and semi-hardwood timber species have been tried as substitutes for Belian, but most of them were found to be unsatisfactory due to lack of durability. The use of cement posts in pepper planting has been practised in most farms in Peninsular Malaysia with satisfactory outcome. However, growth performance at the early stage has been reported to be less promising. Researchers revealed that the burning surfaces of cement posts once exposed to sunshine may discourage attachment and growing of adventitious roots on the post. The nature of this climber shows more preferable cling on hardwood species like *Eusideroxylon zwageri*, probably due to less desiccated surfaces compared to cement posts. Therefore, the key success of developing a viable post for pepper planting is the durability of the post and at the same time, the post surfaces must be desirable for the pepper root to cling on.

Kenaf fibre composite presents as a potential material to develop as an alternative post for black pepper planting support with its good mechanical strength. Previous studies have shown that Kenaf fibre composite has properties superior to unreinforced matrices such as polypropylene, which has poor impact strength (Rowell et al., 1999; Anuar et al., 2008). Other studies also showed that natural fibres such as Kenaf have been used with biodegradable polymer as fibre reinforced biocomposites due to their good mechanical properties, lower cost and light weight (Yussuf et al., 2010). In the field of technical utilisation of natural fibres, Kenaf reinforced composites are one of the most important areas for further exploration (Huda et al., 2008). Therefore, Kenaf fibre composites incorporated with long lasting binders like PVC, cement and fibre-glass are potential solutions in developing the ideal post for black pepper growing.

The objective of this study was to evaluate the effect of different types of alternative Kenaf composite posts for the sustainable growth of the black pepper plant. The aim was to examine field performance including epiphytical response of adventitious roots, black pepper's photosynthetic rate, stomatal conductance rate, leaf vapour pressure deficit (VPD) and leaf temperature and to run laboratory tests to determine the appropriateness of Kenaf-based composite as an alternative to currently recommended posts in black pepper cultivation. This is the first report on using Kenaf composite posts as an alternative support for black pepper cultivation in Malaysia.

MATERIALS AND METHOD

Plant Materials

In this study, the observation plot was established at Serikin Farm, Bau, Sarawak (East Malaysia) in September 2014. The black-pepper cuttings of the variety Semongok Aman were obtained from the nursery of Serikin farm. The black-pepper cuttings were placed in rooting beds covered

with river sand to the level of the fourth node of a five-node cutting. Watering of cuttings was done manually once a day. After five weeks, the rooted cuttings were transplanted to the observation plots.

Kenaf Composite Materials

Kenaf-based composite and fibre-glass posts were provided by the National Kenaf and Tobacco Board and certified by SIRIM. Three types of Kenaf-based composite posts were used in this study, Kenaf Extrusion (60% Kenaf fibre, 40% PVC), Kenaf Pultrusion (80% glass fibre yarn, 10% glass fibre mat, 10% Kenaf yarn) and Kenafkrete (10% Kenaf, 30% pilling rode, 60% cement). The fibre-glass post that was composited with synthetic fibres was used as a comparison for mechanical strength with the natural-fibre Kenaf composite post. Belian (Ironwood) was used as a control for examining plant growth of the black pepper.

Experimental Design

The experimental design for this study was a completely randomised design (CRD) with five types of different support poles as treatment. The treatments were: (i) Control – black pepper vines supported on Belian posts, (ii) Kenaf Extrusion – black pepper vines supported on Kenaf-based composite extrusion posts, (iii) Kenaf Pultrusion – black pepper vines supported on Kenaf-based composite pultrusion posts, (iv) Kenafcrete – black pepper vines supported on Kenaf-based concrete posts, and; (v) Fibre-glass – black pepper vines supported on fibreglass posts. Each treatment had five replicates, with each replicate represented by one pole.

The Epiphytical Response of Adventitious Roots of Black Pepper Plant

The percentage of support cling roots was determined in this study by counting the number of posts with cling roots.

$$\text{Support cling roots} = \frac{\text{number of posts with positive cling roots}}{\text{number of posts tested}} \times 100\%$$

Sustainability of Kenaf Composite Posts

A trial plot was established at thoroughly exposed conditions to determine the response of composite posts under open-weather conditions. The sustainability of Kenaf composite posts was evaluated based on the physical observation of the posts.

The Accelerated Laboratory Decay Test

The accelerated laboratory decay test was employed using the agar block culture method, a modified harmonised procedure adopted from Tan et al. (2010). Composite samples and Belian samples were processed to test specimen size as indicated in Table 1. White rot fungus *Coriolus versicolour* (L. ex. Fr.) and brown rot fungus *Gloeophylum trabeum* (Pers. Ex. Fr.) were used in the test. Test specimens were exposed to healthy cultured fungus in agar-based media in culture bottles and subjected to a 16-week trial before being assessed for their weight loss based on the difference in oven-dry

weight between before and after fungus exposure, and expressed in percentage. In this study, the decay test on Kenafkrete was omitted due to the required test specimen size as stipulated in the testing standards.

Leaf Temperature and Leaf-to-Air Vapour Pressure Deficit (VPD) Measurement

Using the method employed by Day (2000), leaf temperature and VPD were computed by calculation based on air temperature. It was measured by a fine wire thermocouple using a LICOR LI-6400 XT infrared gas analyser (IRGA) (Lincoln, Nebraska, USA).

Measurement of Gas Exchange of *Piper nigrum*

Gas exchange measurement was determined according to the method by DiCristina and Germino (2006). It was carried out on young fully expanded leaves with the same orientation and layer in the vine crown (middle bottom). Measurements of net photosynthesis on an area basis (*A*) (µmol CO_2 $m^{-2}s^{-1}$) and leaf stomatal conductance (*gs*) (mol H_2O $m^{-2}s^{-1}$) of 25 different leaves per treatment were monitored using a LICOR LI-6400 XT infrared gas analyser (IRGA) (Lincoln, Nebraska, USA). Light intensity (Photosynthetically active radiation, PAR) within the sampling chamber was set to PAR at 900 µmol $m^{-2}s^{-1}$, which was presumed to be the intensity at which photosynthetic rates for black pepper would be maximal (Mathai, 1983; Vijayakumar et al., 1984). The CO_2 flow into the chamber was maintained at a concentration of 400 µmol mol^{-1}. The humidity flow into the chamber was fixed at 500 µmol s^{-1}. Measurement was done on gas exchange parameters at between 1100 and 1200 h.

Data Analysis

One-way Analysis of Variance (ANOVA) at p=0.05 was carried out to determine differences of effects among different types of post. If then required, means separation was analysed using Duncan's Multiple Range Test (DMRT) (Duncan, 1955) at p=0.05 level. All statistical analyses were performed using IBM SPSS statistics 21 (IBM Corp, 2012).

Table 1
Test specimen size and number of replicates per fungus for each type of post

Type of Post	Test Specimen Size (mm)	No. of Replicates per Fungus
Belian	20 x 20 x 6	10
Fibre-glass	22 x 25 x 2	10
Kenaf Extrusion	25 x 25 x 5	10
Kenaf Pultrusion	25 x 25 x 3	10

RESULTS AND DISCUSSION

Plant Growth and the Epiphytical Response of Adventitious Roots of Black Pepper Plant on Kenaf Composite Posts

Table 2 shows the results of the ANOVA analysis and Duncan's mean separation tests for black pepper plant growth and adventitious root response on Kenaf composite posts. There were significant differences ($p<0.05$) in the number of leaves

and number of lateral branches among the plants trained on different types of support. The results of the present work showed that plants on Belian post and fibre-glass post grew vigorously, with both supports recording the highest number of leaves. Kenafkrete recorded the lowest growth of black pepper plant compared to the Kenaf Extrusion post and Kenaf Pultrusion post. Since the black pepper plant is a climber, the attachment of adventitious roots on the support posts is essential. This study demonstrated that the adventitious roots of black pepper plants were capable of binding on different types of support (Figure 1A-E). From observation, Kenaf Extrusion, the Pultrusion posts and the Fibre-glass posts had similar binding strength for black pepper adventitious roots (Table 2). Although fibre-glass is a synthetic fibre with no organic materials, the response of black pepper growth and attachment of adventitious roots had no significant difference compared to when the Belian post was used. Kenafkrete showed low inclination for black pepper plant attachment as the concrete post got heated up in the daytime, resulting in drying of clinging roots. The binding feature of adventitious roots is important for enhancing the growth of young shoots upward and allowing them to continue to grow to form black pepper plant canopies.

Sustainability of Kenaf Composite Posts

In this study, the sustainability of Kenaf composite posts was evaluated against the field condition. From observation, the Kenaf Extrusion posts showed significant bending and cracking on the post after eight months' exposure in the open field (Figure 1F). It is believed this was due to the high percentage of PVC composition in the Kenaf Extrusion post with low heat stability under field weather conditions. Kenafkrete is also not recommended for black pepper planting as the post tends to crack easily after long periods of planting. However, it is interesting that fibre-glass was found to be suitable for growing black pepper plants. The fibre-glass posts and Kenaf Pultrusion posts that incorporated fibre-glass performed well in field conditions, causing the black pepper plants to grow vigorously on the posts. The present study also implied that the Kenaf composite posts and fibre-glass did not harbour pests and diseases that could hinder the growth of black pepper plants.

Table 2
Plant growth and response of adventitious roots of black pepper plants on kenaf composite posts

Support	No. of Leaves/Plant	No. of Lateral Branches/Plant	Support Cling Roots (%)
Belian	9.6 ± 1.52[a]	3.4 ± 2.70[b]	100
Kenaf Extrusion	5.6 ± 2.30[ab]	5.6 ± 2.30[ab]	60
Kenaf Pultrusion	7.6 ± 5.27[ab]	7.6 ± 5.27[ab]	60
Kenafkrete	2.8 ± 2.77[b]	5.0 ± 3.16[ab]	40
Fibre-glass	9.2 ± 4.44[a]	9.2 ± 4.4[a]	60
ANOVA (F value) _Support	3.140*	3.620*	-

* indicates significant at p=0.05. Means followed by the same letter within a column are not significantly different by DMRT at p=0.05. The percentage of support cling roots is only based on the positive response observation; no statistical analysis was done. Mean ± standard error

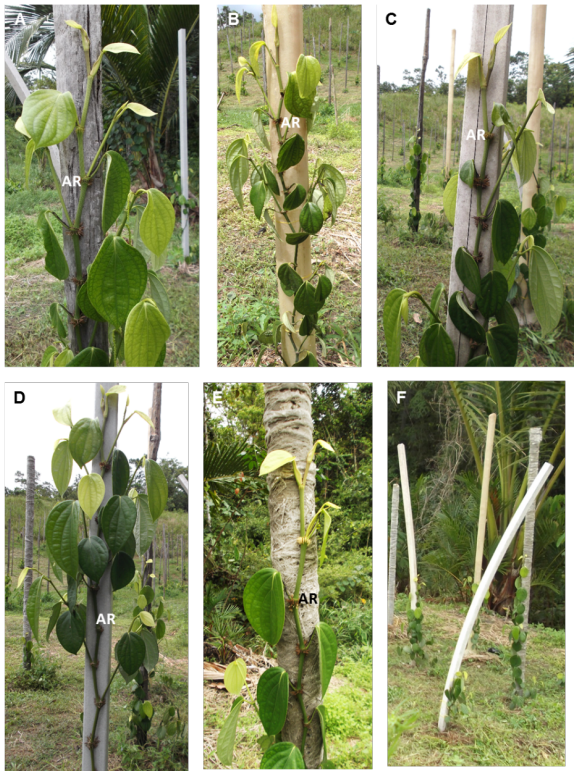

Figure 1. Plant growth and attachment of adventitious roots of black pepper plant on different supports. (A) Belian. (B) Fibre-glass (C) Kenaf Extrusion (D) Kenaf Pultrusion (E) Kenafkrete (F) Bending of the Kenaf Extrusion posts after eight months of planting. AR, Adventitious Roots

The Accelerated Laboratory Decay Test

In this study, the accelerated laboratory decay test focused on durability of the posts against white rot fungi (*Coriolus versicolour*) and brown rot fungi (*Gloeophyllum trabeum*). These fungi are known for their highwood degradation capacity and therefore, were used as an efficient fungus reference for wood degradation in standard tests (Kerem et al., 1999; van Acker et al., 1999; Zabel et al., 1992). Table 3 presents the results of the analysis of the average weight loss of the support posts (Belian, fibre-glass, Kenaf Extrusion and Kenaf Pultrusion) after 16 weeks of exposure to *C. versicolour* and *G. trabeum*. The average weight loss using Belian was the highest, 3.85% and 3.23% in *C. versicolour* and *G. trabeum*, respectively. The Kenaf composite produced through pultrusion method recorded the lowest average weight loss, with 0.03% in *C. versicolour*. However, there was slight increase of weight in Kenaf pultrusion treated with *G. trabeum* (-0.01%), which was due to the variation among the replicates tested. This was followed by the fibre-glass posts, with average weight losses of 0.3% in *C. versicolour* and 0.31% in *G. trabeum*. The Kenaf-composite posts produced through Extrusion method recorded 0.98% average weight loss in *C. versicolour* and 0.08% in *G. trabeum*. In general, the percentage of average weight loss in Belian was significantly higher than when the fibre-glass and Kenaf composite posts were used. According to the standard, an average weight loss of 0%-10% is considered to be highly resistant and weight loss higher than 45% is considered to be slightly resistant or non-resistant (Kartal & Avrilmis, 2005). Thus, all the sample posts tested in this study fell in the category of highly resistant against both white rot and brown rot fungi. The moisture content after exposure to the fungi in the Belian post was also significantly higher compared to the that in the rest of the samples, with 62.62% in *C. versicolour* and 43.44% in *G. trabeum*. The fibre-glass and Kenaf Pultrusion posts showed moisture content in the range of 1.8%-2% and Kenaf Extrusion recorded moisture content in the range of 11%-13%. The high resistance against these two aggressive fungi by fibre-glass and Kenaf composite posts might have been due to the composition of the posts. Fibre-glass is a synthetic fibre consisting of numerous extremely fine fibres of glass that are not affected by the cellulases and lignin-degrading enzymes produced by the decaying fungi (Aziz & Ansell, 2004; Tanaka et al., 1999). The Kenaf Pultrusion composite was more resistant to fungus decay compared to the Kenaf Extrusion composite because Kenaf Pultrusion consisted mainly of fibre-glass compared to the Kenaf Extrusion with its higher percentage of Kenaf fibre. Evidently proved in this study, the Kenaf composite posts were highly resistant against the wood decay fungi and performed better than Belian, which had higher weight loss albeit being in the same resistance class.

Table 3
Mean weight loss % of materials exposed to white rot fungi (Coriolus Versicolour) and brown rot fungi (Gloeophyllum Trabeum) in 16-week trial test

Test Fungi	Post Materials	Weight Loss %	Initial MC* %	MC* % After Exposure	Resistance Classification
C. versicolour	Belian	3.85 ± 0.53^{a}	7.97 ± 0.12^{a}	62.62 ± 2.73^{a}	Highly resistant
	Fibre-glass	0.30 ± 0.07^{c}	0.61 ± 0.05^{b}	1.96 ± 0.39^{c}	Highly resistant
	Kenaf (Extrusion)	0.98 ± 0.31^{b}	0.86 ± 0.08^{b}	12.73 ± 0.55^{b}	Highly resistant
	Kenaf (Pultrusion)	0.03 ± 0.04^{c}	0.34 ± 0.01^{b}	1.80 ± 0.30^{c}	Highly resistant
G. trabeum	Belian	3.23 ± 0.91^{a}	7.87 ± 0.25^{a}	43.44 ± 6.32^{a}	Highly resistant
	Fibre-glass	0.31 ± 0.13^{b}	0.58 ± 0.09^{b}	1.94 ± 0.98^{c}	Highly resistant
	Kenaf (Extrusion)	0.08 ± 0.18^{b}	0.62 ± 0.03^{b}	11.30 ± 0.46^{b}	Highly resistant
	Kenaf (Pultrusion)	-0.01 ± 0.08^{b}	0.34 ± 0.01^{b}	1.83 ± 0.30^{c}	Highly resistant
ANOVA (F value)					
_Support with *G. versicolour*		319.661*	5507.667*	4116.520*	
_Support with *G. trabeum*		109.727*	2680.667*	374.121*	

* indicate significant, at p=0.05. Means followed by the same letter within a column in each fungus are not significantly different by DMRT at p=0.05. The negative mean value indicates relatively small increase in weight loss of Kenaf Pultrusion post. * MC: Moisture content. Mean ± standard error

Piper nigrum Gas Exchange Characteristics

The photosynthetic rates (*A*) of plants supported by Kenaf Pultrusion, Kenafkrete and fibre-glass posts recorded higher values, which indicated better growth performance for black pepper plants supported by these types of post (Table 4). The favourable outcome from the result of net photosynthetic rate was that Kenaf Pultrusion, Kenafkrete and fibre-glass posts were able to support the growth of black pepper plants fairly well and in a similar manner as Belian hardwood. The *A* rate was reduced by 17% in black pepper plants subjected to Kenaf Extrusion compared to that of the Belian posts. The result for net photosynthetic rate of black pepper might well be associated with changes in its leaf temperature and leaf-to-air VPD (Table 4). Day (2000) and Aspinwall et al. (2016) reported that increase in leaf-to-air VPD and temperature, acting singly or interactively, reduced photosynthetic carbon gain of a plant. The negative response of *A* rate to increasing VPD in plants has been described in several studies as well (Warkentin et al., 1992; Darlington et al., 1997, Ambrose et al., 2016).

The results in Table 4 showed that the leaf stomatal conductance (*gs*) of black pepper supported by Kenaf Extrusion post declined by 39% of that of Belian. With regard to external factors, stomata responded to many environmental factors including leaf temperature and leaf-to-air VPD (Jones, 1992; Kagotani et al., 2015). Vann et al. (1994) and Marias et al. (2017) discovered significant inhibition of both *A* and *gs* rates in most plants at

air temperatures >33°C, thus relating the response to the current range limits and changes that might be linked to a warming climate. Table 4 also shows that there was no significant difference ($p<0.05$) in *gs* between the Belian and other posts including Kenaf Pultrusion, Kenafkrete and fiberglass. This suggesting that leaf stomatal conductance of black pepper plants responded positively when supported by these posts.

Leaf Temperature and Leaf-to-Air Vapour Pressure Deficit (VPD)

The black pepper plants supported by Kenaf Extrusion posts demonstrated significantly higher leaf temperature compared to the plants supported by Belian, Kenaf Pultrusion, Kenafkrete and fiber-glass support posts. Kenaf Extrusion consists of mainly heat sink thermoplastic material; this might have contributed to the increase in the leaf temperature. The finding was almost similar to a study done by Sulok et al. (2015), who reported that thermoplastic materials tended to heat up during summer under heat-exposed conditions. Leaf temperatures of plants supported by Belian, Kenaf Pultrusion, Kenafkrete and fibre-glass were not significantly different, indicating that in terms of temperature microclimatic effect on the leaves, these support posts were on par with the Belian posts.

Table 4 also shows leaf-to-air vapour pressure deficit (VPD) subjected to different support posts. Leaf-to-air VPD with Kenaf Extrusion support posts recorded the highest value at 1.89 kPa while that of the Belian support posts exhibited the lowest value at 1.64 kPa. This result might be due to the surface characteristics of the Kenaf Extrusion thermoplastic material affects heat transmission into the environment. Agarwal and Gupta (2011) and Sulok et al. (2015) reported that a coarse and dull coloured thermoplastic surface is more likely to absorb and emit heat and therefore, increases the surrounding temperature compared to a lighter coloured and smooth surface. In other crops such as corn and wheat, a small increase in temperature can increase leaf-to-air VPD during exposure to extreme heat radiation (Day, 2000; Will et al., 2013; Sulok et al., 2015).

Table 4
Effect of kenaf-based composite posts on leaf photosynthetic rate, leaf stomatal conductance, leaf temperature and leaf-to-air vapour pressure deficit (vpd) of Piper nigrum

Support	Photo (μmol CO_2 $m^{-2}s^{-1}$)	Stomatal cond (mol H_2O $m^{-2}s^{-1}$)	Leaf temp (°C)	Leaf VPD (kPa)
Belian	$12.06 + 0.56^{a}$	$0.39 + 0.98^{a}$	$30.99 + 0.81^{b}$	$1.64 + 0.94^{b}$
Kenaf Extrusion	$10.02 + 0.73^{b}$	$0.24 + 0.72^{b}$	$33.97 + 0.42^{a}$	$1.89 + 0.78^{a}$
Kenaf Pultrusion	$12.49 + 0.43^{a}$	$0.36 + 0.39^{a}$	$30.83 + 0.34^{b}$	$1.68 + 0.46^{b}$
Kenafkrete	$12.58 + 0.61^{a}$	$0.36 + 0.55^{a}$	$31.31 + 0.75^{b}$	$1.72 + 0.32^{b}$
Fibre-glass	$11.91 + 0.59^{a}$	$0.38 + 0.87^{a}$	$31.60 + 0.68^{b}$	$1.70 + 0.55^{b}$
ANOVA (F value)	16.353*	8.324*	11.333*	5.668*

* indicate significant, at p=0.05. Means followed by the same letter down the column are not significantly different by DMRT at p=0.05. Mean ± standard error

CONCLUSION

In this study, the adventitious roots of black pepper plant demonstrated positive response to all types of support except the Kenafkrete posts. The laboratory decay test showed that Kenaf composite posts were highly resistant to wood decay fungi and performed better than Belian posts. For the measurement of leaf temperature and leaf-to-air VPD, Kenaf Extrusion contributed to an adverse microclimate environment for the growth of black pepper plants. The gas exchange rate of black pepper plants supported by Belian, Kenaf Pultrusion, Kenafkrete and fibre-glass were comparatively higher than that of Kenaf Extrusion. Therefore, the above findings have demonstrated that Kenaf Pultrusion and fibre-glass posts are suitable as an alternative support to Belian posts compared to those of Kenafkrete and Kenaf Extrusion. However, follow-up research is needed for further verification of the usage of Kenaf composite posts, which include aspects such as the efficiency of fertiliser use and the productivity of the plant.

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Genetic Diversity and Relationship of Sabah Traditional Rice Varieties as Revealed by RAPD Markers

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ABSTRACT

Sabah, also known as North Borneo, is one of the states in Malaysia. It is home to many local varieties of rice, but the self-sufficiency quotient for rice production is only about 30%. Knowledge of the genetic diversity of crops has been utilised to increase crop yields including rice in different countries, but the information regarding the genetic diversity of Sabah traditional rice varieties is very limited. Therefore, we report a comprehensive genetic diversity and relationship study of 22 Sabah traditional rice varieties in three main divisions of Sabah including the West Coast Division (WCD), Sandakan Division (SD), and Interior Division (ID) using 11 random amplified polymorphic DNA (RAPD) markers. Our results showed that more than half of the collected rice seeds were medium in size and shape, with moderately high head rice recovery and low moisture content. In addition, about half of them were categorised with high to very high amylose content. Genetic analysis revealed a total of 75 bands were produced using all RAPD markers with 100% polymorphism, and a high degree of genetic variation among all Sabah traditional rice varieties was obtained. The genetic differentiation of Sabah's traditional rice varieties was more likely to occur within divisions rather than among divisions. Furthermore, Sabah traditional rice varieties in ID showed the greatest genetic diversity and polymorphic loci, and were closely related to rice varieties in SD but genetically dissimilar to those in WCD as revealed in both phylogenetic tree and principal component analysis. In conclusion, this study provides breeders with reliable information regarding diversity

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of Sabah's traditional rice varieties; the data could also be beneficial for local rice yield enhancement.

Keywords: Genetic diversity, Sabah's traditional rice, RAPD, phylogenetic tree, principal component analysis

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's populations (Malik et al., 2008). About 163.1 million hectares of rice are cultivated, and the worldwide annual rice production was around 748 million tons in 2016 (FAO, 2017). In Malaysia, the production of rice has only been able to meet approximately 65% of domestic needs, and the remaining 35% are imported from other countries (Vengedasalam et al., 2011). Sabah, also known as North Borneo, is one of the states in Malaysia. It is home to many local rice varieties but the self-sufficiency level is only about 30% (Mohamad Shokur et al., 2015). Hence, the National Food Security Policy launched in 2008 has identified Sabah as one of the regions in which local rice plantations and production need to be increased in order to sustain domestic demand.

Genetic diversity of crops is mainly influenced by several factors such as genetic drift, mating system, evolutionary history and life history (Loveless & Hamrick, 1984). Knowledge of genetic diversity of crops such as rice, including their wild relatives and traditional varieties, is essential for crop management, crop improvement by selection, usage of crop germplasm, genetic mapping and detection of genome structures to ensure crops with superior characteristics are planted (Sasaki, 2005; Sabu et al., 2006; Varshney et al., 2008; Pooja & Katoch, 2014). Besides, rice is an ideal crop for genetic diversity assessment due to the significant level of genetic polymorphisms present in the genome (Wang et al., 1995; Latif et al., 2011).

Recently, one study reported that rice varieties from Sabah and Sarawak (East Malaysia) were clustered together when compared to rice varieties from Peninsular Malaysia (Razak et al., 2016). However, the genetic diversity of rice in different divisions of Sabah was not investigated. Therefore, this study emphasises the genetic diversity and relationship of 22 Sabah's traditional rice varieties from three main divisions of Sabah including the West Coast Division (WCD), Sandakan Division (SD) and the Interior Division (ID) using 11 random amplified polymorphic DNA (RAPD) markers.

MATERIALS AND METHOD

Plant Materials and DNA Isolation

A total of 22 germplasms of Sabah's traditional rice varieties were collected from different divisions of Sabah including WCD (n=9), SD (n=5) and ID (n=8). The length, width, thickness and colour of the rice seeds were determined. The size of the rice seeds were grouped into short (<6 mm), medium (between 6-7 mm) and long (>7 mm) based on their length. The shape of the rice seeds was determined based

on the length/width ratio and categorised into bold (ratio of 1.1-2.0), medium (ratio of 2.1-3.0) and slender (ratio>3.0) (JICA, 2013). The head rice recovery, moisture content and amylose content were determined according to previous approaches (Jindal & Siebenmorgan, 1987; Avaro et al., 2009; Kamruzzaman et al., 2012). Table 1 shows the characteristics of all Sabah's traditional rice varieties in this study. Genomic DNA from seeds was isolated using the cetyl trimethylammonium bromide (CTAB) method with slight modifications from previously described (Sharma et al., 2013) where CTAB was used instead of polyvinylpyrrolidone (PVP) and β-mercaptoethanol.

Table 1
Characteristics of Sabah's traditional rice varieties presented in this study

Rice Varieties	Length (mm)	Width (mm)	Thickness (mm)	Size[a]	Shape[b]	Colour[c]	Head Rice Recovery	Moisture Content	Amylose Content[d]
PBT02	7.40	1.84	1.30	LG	S	RB	78.57%	7.99%	IM
PBT06	6.30	2.79	1.59	MD	M	PB	74.29%	8.45%	H
PBT07	6.50	2.84	1.85	MD	M	PB	85.42%	8.15%	H
PBT08	6.00	3.00	1.90	MD	B	RB	85.42%	8.30%	L
PBT09	7.60	3.20	1.94	LG	M	B	81.82%	8.11%	IM
PBT10	6.70	2.72	1.45	MD	M	PB	80.00%	6.39%	VH
PBT11	6.20	2.79	1.59	MD	M	RB	96.00%	7.10%	IM
PBT12	6.30	3.07	1.82	MD	M	CW	87.10%	8.59%	IM
PBT13	5.30	1.88	1.32	ST	M	CW	75.56%	8.22%	VH
PBT14	7.20	2.52	1.82	LG	M	CW	84.85%	8.32%	H
PBT16	6.50	2.34	1.68	MD	M	PB	68.75%	6.28%	L
PBT17	6.80	2.12	1.35	MD	S	RO	91.30%	9.53%	IM
PBT18	6.20	1.91	1.44	MD	S	CW	86.36%	13.13%	IM
PBT19	6.80	2.98	1.64	MD	M	PB	91.67%	7.96%	L
PBT20	7.50	2.60	1.49	LG	M	CW	80.49%	8.67%	L
PBT21	7.20	2.46	1.70	LG	M	CW	88.00%	12.32%	H
PBT22	6.30	2.47	1.68	MD	M	CW	79.55%	8.68%	H
PBT23	5.70	2.09	1.26	ST	M	CW	90.91%	8.20%	VH
PBT24	7.20	2.18	1.52	LG	S	RO	86.84%	8.79%	H
PBT25	6.80	2.98	1.64	MD	M	RB	84.62%	8.54%	IM
PBT26	5.50	2.90	1.88	ST	B	CW	89.13%	6.61%	H
PBT27	6.90	2.12	1.74	MD	S	CW	82.93%	8.01%	L

[a]ST, short (length<6 mm); MD, medium (length between 6-7 mm); LG, long (length >7 mm).
[b]Based on length/width ratio. B, bold (ratio between 1.1-2.0); M, medium (ratio between 2.1-3.0); S, slender (ratio >3.0).
[c]RB, reddish brown; PB, purplish black; B, black; CW, creamy white; RO, reddish orange.
[d]L, Low (15-22% amylose content); IM, intermediate (23-26% amylose content); H, high (27-30% amylose content); VH, very high (>30% amylose content).

PCR Amplification Using RAPD Markers

A total of 22 RAPD primers were initially screened for the presence of bands. Out of them, only 11 were with reproducible amplification and were selected to perform genetic diversity analysis for all rice varieties (Table 2). PCR was carried out in a final 25 µL reaction volume containing 50 ng of template DNA, 1x PCR Buffer, 2.0 mM of $MgCl_2$, 0.2 mM of dNTPs mixture, 0.2 µM of each primer and 0.2 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif). The conditions of PCR were set at 1 cycle of initial activation for 4 min at 95°C, 40 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 40°C, extension of 2 min at 72°C, and 1 cycle of final extension for 2 min at 72°C in SpeedCycler^2 thermal cycler (Analytik Jena, Jena, Germany). Amplified PCR products were analysed using Fragment Analyzer™ (Advance Analytical Technologies, Ames, IA).

Table 2
List of 11 RAPD primers and genetic variation in 22 of Sabah's traditional rice varieties

Primer Name	Sequence (5'-3')	Range of Size (bp)	Total no. of Bands	No. of Polymorphic Bands	Percentage of Polymorphism (%)
OPA-01	CAGGCCCTTC	698-3000	9	9	100
OPA-02	TGCCGAGCTG	298-2951	8	8	100
OPA-03	AGTCAGCCAC	372-2131	8	8	100
OPA-04	AATCGGGCTG	431-3144	7	7	100
OPA-10	GTGATCGCAG	406-2738	9	9	100
OPA-12	TCGGCGATAG	1491-1848	1	1	100
OPA-13	CAGCACCCAC	447-2875	8	8	100
OPB-07	GGTGACGCAG	444-6104	9	9	100
OPB-10	CTGCTGGGAC	341-1870	7	7	100
OPB-12	CCTTGACGCA	278-2525	4	4	100
OPC-15	GACGGATCAG	305-3000	5	5	100
	Total		75	75	100

Genetic Diversity and Relationship Analysis

Each polymorphic band was scored as a binary code of 1 (presence) or 0 (absence). Jaccard's similarity matrix was calculated using DendroUPGMA online software (http://genomes.urv.cat/UPGMA/). GenAlEx ver.6.41 software (Peakall & Smouse, 2006) was used to calculate the genetic parameters including number of alleles (N_a), number of effective alleles (N_e), expected heterozygosity (H_e) and Shannon's information index (I). The same software was used to determine molecular variance (AMOVA) and perform the principal component analysis (PCA). Molecular Evolutionary Genetic Analysis 6 (MEGA6)

Software (Tamura et al., 2013) was utilised to construct a phylogenetic tree using the Neighbour Joining method with bootstrap replicates of 1000. In addition, the genetic differentiation index of PhiPT (ϕ_{st}) among divisions, Nei's genetic distance and Nei's genetic identity were also calculated using GenAlEx ver.6.41 software. The gene flow level (Nm) was determined based on the formula previously described (Slatkin & Barton, 1989).

RESULTS AND DISCUSSION

DNA-based molecular markers were reported as useful tools in the assessment of genetic diversity and elucidation of the relationship between different rice varieties (Ragunathanchari et al., 2000; Shivapriya & Hittalmani, 2006). Various molecular techniques to study genetic diversity are available including amplified fragment length polymorphism (AFLP) (Zabeau & Vos, 1993), restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), simple sequence repeats (SSRs) (Tautz, 1989) and RAPD (Williams et al., 1990). Among them all, the RAPD approach is the most inexpensive and rapid as it requires no information regarding the genome of the plant. In addition, it has been widely applied in rice genetic diversity studies (Rahman et al., 2007; Rajani et al., 2013; Abdul-razzak Tahir, 2014; Alam et al., 2014; Hasan & Raihan, 2015).

The characteristics of Sabah's traditional rice varieties in this study showed that more than half of the rice seeds were medium in size (59.09%) and shape (68.18%) (Table 1). The head rice recovery of the rice seeds was considered moderately high (ranging from 60.75% to 96.00%) but all had low moisture content (<14.00%). It is recommended to harvest rice at 18-24% moisture content to avoid fissuring of the seeds. The optimum milling potential for rice is at the moisture content of 14% wet weight basis (JICA, 2013). In addition, about half of the collected rice seeds (45.45%) were categorised with high to very high amylose content, making them less tender, dry when cooked and hard upon cooling (JICA, 2013).

In this study, all the amplified bands generated by 11 RAPD markers were analysed using Fragment Analyzer™ (Figure 1). A total of 75 bands were scored (Table 2). The number of bands produced ranged from 1 to 9 bands with the minimum number of one band produced by OPA-12 primer and the maximum number of 9 bands were produced by OPA-01, OPA-10 and OPB-07 primers. The size of bands produced ranged from 278 to 6104 bp. All 11 RAPD primers produced 75 polymorphic bands with 100% polymorphism. The high level of polymorphism generated by all RAPD markers in this study was dramatically higher compared to the results of previous studies conducted in Thailand (Kanawapee et al., 2011), Bangladesh (Hasan & Raihan, 2015), Iraq (Abdul-razzak Tahir, 2014) and India (Rajani et al., 2013). Guo et al. (2007) reported that geographic isolation may play an important role during the process of genetic diversification and variation. As Sabah is geographically isolated from the mainland of Asia, the significant high level

of polymorphism could be due to great intra-specific variation among the traditional rice varieties in different divisions of Sabah for better adaptation to environment changes and survival rate.

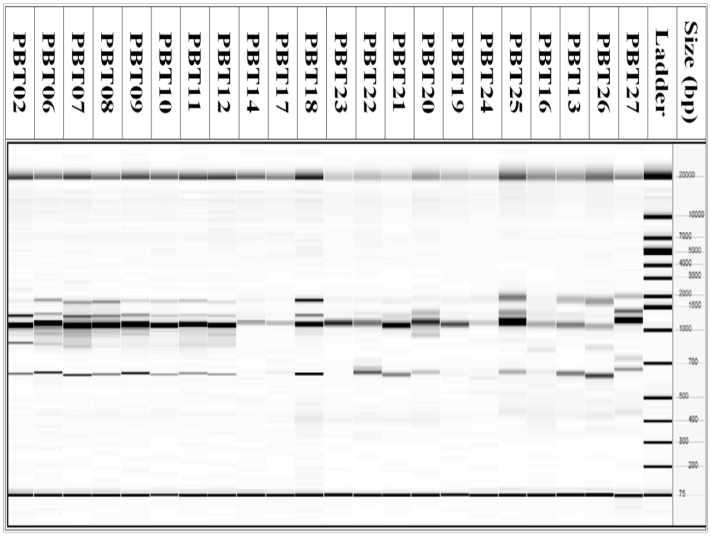

Figure 1. A representative banding profile of 22 different traditional rice varieties of Sabah using OPB-07 RAPD primer generated from Fragment Analyzer™

Our study showed that PBT10 and PBT11 rice varieties from WCD had the closest genetic relationship (Jaccard's similarity coefficient=0.864) (Table 3). On the other hand, PBT22 and PBT24 rice varieties from ID had the most distant genetic relationship (Jaccard's similarity coefficient=0.095). Jaccard's coefficient of similarity in this study ranged from 0.095 to 0.864, representing a high level of genetic variation among all Sabah traditional rice varieties. A high level of genetic variation of rice was also reported in different countries including in Thailand, with genetic similarity ranging from 0.64 to 0.94 (Kanawapee et al., 2011), India, with genetic similarity ranging from 0.47 to 0.81 (Rajani et al., 2013) and Bangladesh, with genetic similarity ranging from 0.101 to 0.911 (Hasan & Raihan, 2015).

Table 3
Similarity matrix of Sabah traditional rice varieties using Jaccard's similarity coefficient

	PBT02	PBT06	PBT07	PBT08	PBT09	PBT10	PBT11	PBT12	PBT13	PBT14	PBT16	PBT17	PBT18	PBT19	PBT20	PBT21	PBT22	PBT23	PBT24	PBT25	PBT26	PBT27
PBT02	1.000	0.735	0.517	0.607	0.712	0.596	0.635	0.593	0.510	0.491	0.490	0.392	0.698	0.611	0.654	0.623	0.446	0.426	0.109	0.400	0.377	0.537
PBT06		1.000	0.579	0.712	0.796	0.673	0.714	0.667	0.617	0.560	0.469	0.458	0.816	0.623	0.700	0.667	0.509	0.462	0.163	0.409	0.469	0.608
PBT07			1.000	0.759	0.709	0.660	0.667	0.655	0.491	0.527	0.393	0.407	0.667	0.643	0.596	0.569	0.410	0.439	0.143	0.308	0.444	0.571
PBT08				1.000	0.755	0.851	0.816	0.765	0.620	0.482	0.453	0.500	0.741	0.685	0.636	0.636	0.491	0.500	0.170	0.396	0.426	0.706
PBT09					1.000	0.720	0.796	0.780	0.600	0.547	0.520	0.451	0.824	0.698	0.679	0.648	0.500	0.455	0.174	0.404	0.490	0.593
PBT10						1.000	0.864	0.804	0.644	0.434	0.429	0.478	0.673	0.680	0.596	0.596	0.472	0.480	0.200	0.395	0.458	0.702
PBT11							1.000	0.771	0.652	0.444	0.469	0.458	0.712	0.686	0.604	0.604	0.481	0.462	0.163	0.409	0.500	0.640
PBT12								1.000	0.638	0.463	0.460	0.511	0.698	0.673	0.593	0.593	0.473	0.453	0.186	0.370	0.460	0.660
PBT13									1.000	0.400	0.561	0.590	0.588	0.560	0.510	0.571	0.469	0.478	0.167	0.350	0.524	0.682
PBT14										1.000	0.404	0.524	0.566	0.538	0.519	0.549	0.480	0.400	0.100	0.217	0.294	0.407
PBT16											1.000	0.568	0.481	0.574	0.521	0.521	0.388	0.422	0.188	0.351	0.395	0.522
PBT17												1.000	0.500	0.500	0.511	0.511	0.571	0.512	0.161	0.231	0.349	0.511
PBT18													1.000	0.655	0.765	0.731	0.545	0.500	0.170	0.396	0.426	0.582
PBT19														1.000	0.673	0.776	0.491	0.529	0.182	0.362	0.423	0.615
PBT20															1.000	0.755	0.620	0.540	0.159	0.340	0.460	0.596
PBT21																1.000	0.620	0.571	0.186	0.340	0.431	0.596
PBT22																	1.000	0.532	0.095	0.184	0.333	0.472
PBT23																		1.000	0.200	0.317	0.422	0.480
PBT24																			1.000	0.217	0.152	0.143
PBT25																				1.000	0.282	0.429
PBT26																					1.000	0.429
PBT27																						1.000

When stratified to different divisions, the estimated allele frequency with number of different alleles (N_a) and effective alleles (N_e) was highest in ID (N_a=1.693±0.080, N_e=1.505±0.043), while the lowest in SD (N_a=1.413±0.093, N_e=1.366±0.044) (Table 4). Among all the three divisions, Sabah's traditional rice varieties in ID showed the highest genetic diversity (I=0.434±0.029, H_e=0.239±0.013) and percentage of polymorphic loci (82.67%), whereas the lowest genetic diversity (I=0.315±0.034, H_e=0.213±0.024) and percentage of polymorphic loci (58.67%) were in WCD, which further supported that PBT22 and PBT24 from ID had the lowest Jaccard's similarity coefficient while PBT10 and PBT11 from WCD had the highest Jaccard's similarity coefficient. The AMOVA analysis revealed that the total genetic differentiation coefficient of the three divisions was 0.145, indicating that the genetic differentiation among the divisions was relatively small (Table 5). The genetic differentiation of Sabah's traditional rice varieties was more likely to occur within divisions (85%) rather than among divisions (15%), and a similar finding was obtained by a study conducted in China (Hu et al., 2014).

Table 4
Genetic parameters of Sabah's traditional rice varieties in different divisions

Division	PPL (%)	N_a	N_e	I	H_e
WCD	58.67	1.427±0.087	1.372±0.045	0.315±0.034	0.213±0.024
SD	61.33	1.413±0.093	1.366±0.044	0.321±0.033	0.214±0.023
ID	82.67	1.693±0.080	1.505±0.043	0.434±0.029	0.291±0.021
Overall	100.00	1.511±0.051	1.414±0.026	0.357±0.019	0.239±0.013

WCD, West Coast Division; SD, Sandakan Division; ID, Interior Division; PPL, percentage of polymorphic loci; N_a, number of different alleles; N_e, number of effective alleles; I, Shannon's information index; H_e, expected heterozygosity

Table 5
AMOVA analysis of Sabah's traditional rice varieties in different divisions

Source	*df*	SS	MS	Variance Component	Variation (%)	PhiPT	*p*
Among Division	2	47.107	23.553	1.807	15.00	0.145	<0.001
Within Division	19	202.439	10.655	10.655	85.00		<0.001
Overall	21	249.545		12.462	100.00		

df, degree of freedom; SS, sum of squares; MS, mean of sum of squares

The phylogenetic analysis classified Sabah's traditional rice varieties into four groups, with close relationship found between SD and ID as revealed in Group 2 to 4 (Figure 2). Although some rice varieties in SD and ID were grouped together with WCD in

Group 1, the PCA revealed no evidence of genetic overlapping of rice varieties in WCD to either SD or ID (Figure 3). From the PCA analysis, rice varieties in SD and ID exhibited scattered distribution and presented overlapped rice varieties. This indicated that rice varieties in SD and ID contained the richest genetic information and were closely related, as supported by a low PhiPT genetic differentiation index (0.022) and high gene flow (N_m) value (11.114) (Table 6) as well as a low Nei's genetic distance (0.104) and high Nei's genetic identity (0.901) (Table 7) between SD and ID. The accuracy of the phylogenetic analysis and PCA in this study can be improved by including the environmental changes and human activity over time as these two factors may influence the genetic diversity of plants (Tilman & Lehman, 2001; Helm et al., 2009).

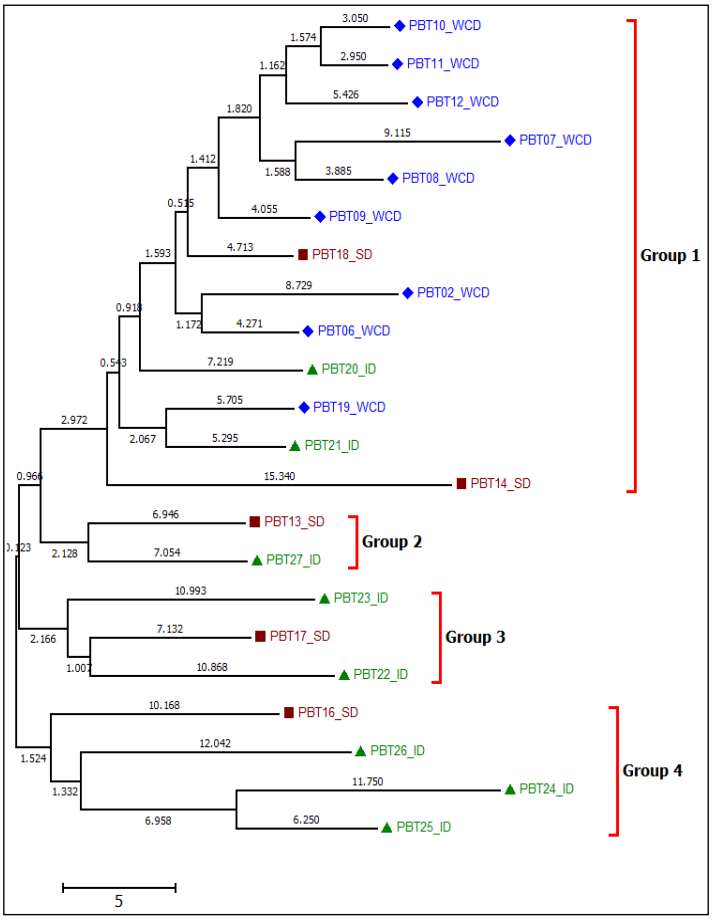

Figure 2. Phylogenetic tree of the traditional rice varieties of Sabah in different divisions constructed using the Neighbour Joining method with 1000 bootstrap replicates in MEGA

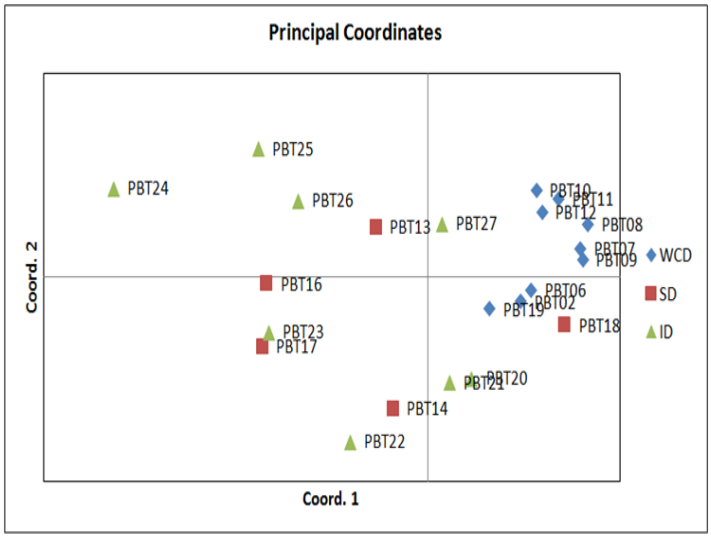

Figure 3. Principal component analysis (PCA) of genetic diversity among the traditional rice varieties of three divisions of Sabah including West Coast Division (WCD), Sandakan Division (SD) and Interior Division (ID)

Table 6
Genetic differentiation of PhiPT (lower left) and gene flow (N_m) (upper right) analysis among Sabah's traditional rice varieties in different divisions

Division	WCD	SD	ID
WCD	-	1.059	1.073
SD	0.191	-	11.114
ID	0.189	0.022	-

WCD, West Coast Division; SD, Sandakan Division; ID, Interior Division

Table 7
Nei's genetic distance (lower left) and Nei's genetic identity (upper right) of Sabah's traditional rice varieties in different divisions

Division	WCD	SD	ID
WCD	-	0.862	0.851
SD	0.149	-	0.901
ID	0.161	0.104	-

WCD, West Coast Division; SD, Sandakan Division; ID, Interior Division

CONCLUSION

In summary, genetic diversity assessed using 11 RAPD markers in this study revealed a high level of polymorphism and genetic variation among all Sabah's traditional rice varieties. The genetic differentiation of Sabah's traditional rice varieties was more likely to occur within divisions rather than among divisions. Sabah's rice varieties in ID had the highest genetic diversity and polymorphic loci, and were closely related to rice varieties in SD while distanced to WCD. Therefore, cross-breeding of Sabah's traditional rice varieties in WCD with those in either SD or ID is recommended to enhance the genetic diversity of rice varieties. As Sabah's traditional rice plantations are mainly grown by conventional farmers who do not have a proper recording system and comprehensive

information of genetic diversity in their rice varieties, information on genetic diversity and the relationship between Sabah's traditional rice varieties in this study may provide insight for local breeders into selective breeding and cross-breeding programmes for rice yield increment.

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Effect of Naphthalene Acetic Acid (NAA) on Oil Content and Quality of the Mustard Plant (*Brassica campestries* L.)

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ABSTRACT

An experiment was carried out to evaluate the biochemical response of mustard to naphthalene acetic acid (NAA). Six levels of NAA such as G0 (no NAA or control), G1 (30 ppm ha^{-1}), G2 (50 ppm ha^{-1}), G3 (70 ppm ha^{-1}), G4 (90 ppm ha^{-1}) and G5 (110 ppm ha^{-1}) were tested. The leaf chlorophyll content was recorded at 30, 40, 50 and 60 days after emergence (DAE). The NAA significantly influenced the biochemical property. The highest chlorophyll level was recorded in 70 ppm NAA ha^{-1} at 50 DAE. At 45 DAE, the highest nitrogen content was noted in 70 ppm NAA ha^{-1}. The 70 ppm NAA ha^{-1} also showed the maximum oil content. The minimum acid value, peroxide and saponification values were found in 70 ppm NAA ha^{-1}. The maximum iodine value was observed in 70 ppm NAA ha-1. Nonessential chemicals likes stearic, palmitic and erucic acid were augmented in the mustard with a decrease in the NAA level while necessary fatty acids were highest in 70 ppm NAA ha^{-1}. It is suggested that 70 ppm NAA ha^{-1} can be used to grow quality mustard plants.

Keywords: *Brassica campestries*, fatty acid, NAA, oil content, oil quality

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INTRODUCTION

Mustard is a vital oilseed crop in Bangladesh and its oil is generally used as a cooking oil. Generally, *Brassica campestris* and *B. juncea* are cultivated in Bangladesh for producing edible oils (Kaul & Das, 1986). Edible oils are essential for meeting calorie requirements due to its high-energy

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component. Each gram of oil/fat provides 9 kcal of energy, whereas each gram of carbohydrate/protein supplies only about 4 kilocalories of energy (Stryer, 1980). Edible oils also carry fat-solubles vitamin such as A, D, E and K. In nutrition, fat or oil is necessary for the absorption of these vitamins. Cooking oil that contains necessary fatty acids such as linolenic and linoleic acid is assumed to be of better quality (Egesel, 2009). Moreover, better quality cooking oil possesses minor peroxide, acid and non-saponification values and greater iodine value (Ahsanullah, 1994).

Bangladesh has been suffering from severe scarcity of edible oils for the last several decades. Bangladesh receives about 30% oil from local production (3.8 g/day/head) while the remaining 70% is imported (Wahhab et al., 2002).

Growth-stimulating chemicals are used to modify the growth and development of the mustard plant to increase its yield and also to improve the quality of products. Naphthalene acetic acid (NAA) is an important synthetic auxin (Yamamoto & Yamamoto, 1998). It is important to study the performance of NAA on the growth, yield and biochemical attributes of mustard plant varieties especially the ones that have been approved for cultivation in Bangladesh. Setia et al. (1993) reported that the effect of growth regulators on photosynthesis assimilate partitioning and yield of *B. juncia* and *B. campestris* was positive. However, no data are available on the effect of naphthalene acetic acid (NAA) on the biochemical response of rapeseed. Therefore, there is scope for work on the biochemical aspects of rapeseed, especially growth regulators and sulphur-based fertilisers. The influence of naphthalene acetic acid on the oil content and quality of rapeseed has not been stated. So, the present study was undertaken with the following objectives: To study the influence of naphthalene acetic acid on chlorophyll and nitrogen content in the leaf, oil and protein content in the seed and the nutritive merits of the oil of the mustard plant.

MATERIALS AND METHOD

Experimental Site and Soil Characteristics

An experiment was conducted at the Oil Seed Research Centre, BARI, Gazipur from July 2010 to April 2012. The tested plot was situated in grey terrace soil. The geographical location was at about N-24° 23′ and E-90° 08′. The elevation was 8.4 m above sea level. The texture of the tested plot was silty clay and the plot was located on high ground. The chemical properties of the soil were pH 6.4, OM content 0.87%, total nitrogen 0.09% and exchangeable K 59 ppm. The sulphur level was critical (Table 1). The soil was slightly acidic (pH 6.0) in nature and low in total N (0.088%) and the available S was at 9 ppm. Available P and exchangeable K were above the critical level (Table 1).

Table 1
Initial soil properties of the experimental field

Properties	Initial Value	Critical Level
pH	6.0	-
Total –N (%)	0.088	- 0.12
P (ppm)	14.00	10.00
S (ppm)	9.00	10.00
K (ppm)	63.0	46.9

Extraction method:
Total-N: Kjeldahl method
Available P: Olsen method
Available S: Calcium dihydrogen phosphate
Exchangeable K: N NH_4OA_c extraction method

Cropping Period

Rabi, Kharif-I and Kharif-II are the main cropping periods in Bangladesh. The Rabi season lasts from 15 October to 15 March, while the Kharif-I season is from 15 March to the end of June and the Kharif-II season is from 1 July to 15 October. Rapeseed is sown in November. The weather data related to the planting seasons are presented in Table 2.

Table 2
Weather data related to the growing season

Month and Year	Temperature (°C)			Precipitation (mm)	Relative Humidity (%)		
	Max.	Min.	Avg.		Max.	Min.	Avg.
Nov. 2010	30.6	19.3	25.0		93.4	68.4	80.9
Dec. 2010	26.2	13.5	19.9	53	91.0	68.8	79.9
Jan. 2011	23.3	10.5	16.9		93.1	66.7	79.9
Feb. 2011	28.0	14.3	21.2		88.2	52.1	70.2
Nov. 2011	29.2	16.7	23.0	2	90.2	67.9	79.1
Dec. 2011	25.2	13.5	19.4		91.4	67.6	79.5
Jan. 2012	23.9	12.3	18.1	12	88.3	61.5	74.9
Feb. 2012	30.0	13.5	21.8		87.4	46.2	66.8

Source: Meteorological Centre, Ministry of Defense, BARI, Gazipur, BD

The Test Crop

Rapeseed (*Brassica campestries* L.) cv. BARI Sarisha-15 was used as the test crop. It was collected from ORC, BARI, Gazipur. BARI Sarisha-15 was a semi dwarf, early-growing plant.

Experimental Design and Treatments

The trial was set up in RCBD design and replicated three times. The six rates of NAA were: G0 (nil NAA or control), G1 (30 ppm ha^{-1}), G2 (50 ppm ha^{-1}), G3 (70 ppm ha^{-1}), G4 (90 ppm ha^{-1}) and G5 (110 ppm ha^{-1}). After certain periods (30, 40, 50 and 60 DAE), the leaf samples were collected for analysis of their biochemical properties. The experimental unit plot size was 4 m × 3 m.

Fertilizer Rate, Use and Other Actions

N, P, K, S, Zn and B were used from urea, triple superphosphate (TSP), muriate of potash (MoP) and gypsum, zinc oxide and

boric acid, respectively. N, P, K, S, Zn and B were used at the rate of 120, 34, 45, 60, 1.8 and 1.8 kg ha^{-1}, respectively. Half amounts of nitrogen and whole amounts of P, K, S, Zn, B and half of N were applied as the base during the final land preparation. The remaining N was top-dressed at the flower initiation time. During the whole growing season, irrigation was provided three times. At first, light irrigation was provided 5 days before planting. At the vegetative stage, irrigation was done for the second time. The last irrigation was provided at the siliqua filling stage. Mulching and other plant protection measures were taken as per requirement.

Seed Sowing and Harvesting

The rapeseeds were sown on 14 and 16 November of 2010 and 2011, respectively with 30 cm × 5 cm spacing. The rapeseed plants were harvested on February 25, 2011 and April 2, 2012, respectively.

Sampling Techniques

Chlorophyll content and nitrogen in the leaf samples were detected at 30, 45, 60 DAE and 30, 40, 50, 60 DAE for N and chlorophyll, respectively. Some properties such as oil content and protein content of the rapeseeds and the biochemical properties (acid, peroxide, iodine and saponification values and fatty acid content) were assessed after harvesting.

Analysis of Soil Sample

Analysis for soil acidity, nitrogen, phosphorus, potassium and sulphur was done following the standard laboratory method. A glass electrode pH meter was used to determine soil pH. Total nitrogen content was determined using the micro-Kjeldahl method (Page et al., 1989); available phosphorus was detected using the Olsen method (Jackson, 1973), exchangeable potassium was measured following the NH_4OAC extraction method (Black, 1965) and sulphur was measured using a spectrophotometer at the wave length of 420 nm (Page et al., 1989).

Biochemical Properties of Oil

The Cocks and Van Rede (1966) and Mehlenbacher (1960) methods were used to determine the oil content of rapeseed and fatty acid composition was determined using the gas-liquid chromatography method (Jellum & Worthington, 1966). A total of 12 mg oil was taken and transesterified at the same time with 5 ml ethylate reagent and shaken. A salt solution (80 g NaCl and 3 g sodium hydrogen sulphate in 1 L water) of 10 ml was added and shaken. As soon as the two layers were separated, the benzene phase was transferred to small test tubes and the samples were then ready for gas chromatography. Peak areas were measured with an electronic digital integrator Thermo Fisher Scientific, Trace GC Ultra- with Tri-Plus autosample (Nagraj, 2009). The

following operating parameters were used: split injection mode, the injection rate was 0.2 µml and the injector temperature was 250°C, where the capillary column (30 m × 0.25 µ × 0.25 µ) was used. The method for esterification of fatty acids was developed by the Swedish Seed Association, Svalov, Sweden; by this method, the oil was used for transesterification. The procedure to determine the acid value was from Devine and Williams (1961). A volume of 25 ml diethyl ether was mixed with 25 ml alcohol and 1 ml of phenolphthalein solution and carefully neutralised with 0.1 M sodium hydroxide. An amount of 2 g oil was dissolved in the mixed neutral solvent and titrated with aqueous 0.1 M sodium hydroxide. The mixture was constantly shaken until a pink colour was seen (Chapman, 1979; IUPAC, 1979). Iodine value was determined using the Hanus method (AOAC, 1960). An amount of 1 g oil sample was placed in a 500 mL volumetric flask. A volume of 15mL of carbon tetrachloride was added to the sample and the mixture was swirled to ensure that the sample was completely dissolved. A volume of 25 mL of Wijs solution was then dispensed into the flask. The flask was stoppered and swirled to ensure complete mixing. The sample was then placed in the dark for 30 min at room temperature. The flask was removed from storage and 20 mL of 10% potassium iodide (KI) solution was added, followed by 150 mL of distilled water. The mixture was titrated with 0.1 N thiosulphate ($Na_2S_2O_3$) solution that was added gradually and with constant and vigorous shaking until its yellow colour had almost disappeared. Then, 1.5 mL of starch indicator solution was added and the titration was continued until the blue colour disappeared. The Cocks and Van Rede (1966) method was used to determine the peroxide value. An amount of 1 g oil was placed in a clean dry boiling tube and 1 g powdered potassium iodide was added together with 20 ml of solvent mixture 2 vol. glacial acetic acid + 1 vol. chloroform. The tube was placed in boiling water so that the liquid boiled within 30 s. It was allowed to boil vigorously (<30 s). The contents were quickly poured into a flask containing 20 ml of potassium iodide solution 5%. The tube was washed out twice with 25 ml water and titrated with 0.002 M sodium thiosulphate solution using starch (Mehlenbacher, 1960; IUPAC, 1979). The saponification value was estimated using the Pearson technique (1970). A volume of 2 g oil sample was placed in a volumetric flask. Then, 25 mL of 1.0 N alcoholic KOH was pipetted and allowed to drain for about 1 min into the mixture. A condenser was connected to the flask and the mixture sample allowed to boil gently but steadily for 45 min for complete saponification. The flask and the condenser were then cooled but not sufficiently to form a gel. The inside of the condenser was washed down with about 1 ml of distilled water. The condenser was disconnected and 1 mL of phenolphthalein indicator was added. The solution was titrated with 0.5 N hydrochloric acid (HCl) until the pink colour of the mixture disappeared.

Chlorophyll Content Determination

The specific absorption co-efficient of Mckinney (1940) and the formula of Maclachalan and Zalik (1963) were used to determine chlorophyll a and b.

The formulae used were:

$$\text{Chlo. a} = \{(12.3 \times D663 - 0.86 \times D645) \times V\} / \{d \times 1000 \times W\} \text{ mg/g fresh leaf}$$

$$\text{Chlo. b} = \{(19.3 \times D645 - 3.6 \times D663) \times V\} / \{d \times 1000 \times W\} \text{ mg/g fresh leaf}$$

where, Chlo. a = Chlorophyll a

Chlo. b = Chlorophyll b

D = Visual density (OD) at wave length

V = Final volume

W = Weight (Fresh leaf pigment materials)

d = Light path length in cm

Statistical Analysis

The analysis of variance for different properties of oil were performed following the ANOVA technique and the mean values were adjudged by DMRT (p=0.05) method (Steel & Torrie, 1960). SAS software (version 9.1) was used to analyse the data. Microsoft EXCEL 2003 was used to compute and prepare the graphs.

RESULTS AND DISCUSSION

Different levels of napthalene acetic (NAA) significantly influenced the chlorophyll content and nitrogen in the leaf, oil and protein content in the rapeseed and its nutritive potential.

Influence of NAA on Chlorophyll 'a' Content in Rapeseed Leaf

The different NAA levels at different days after emergence (DAE) significantly influenced the chlorophyll 'a' content. From 2010-2011, 50 DAE showed the significantly (p=0.05) higher chlorophyll 'a' (1. 61 mg g-1) (Figure 1).

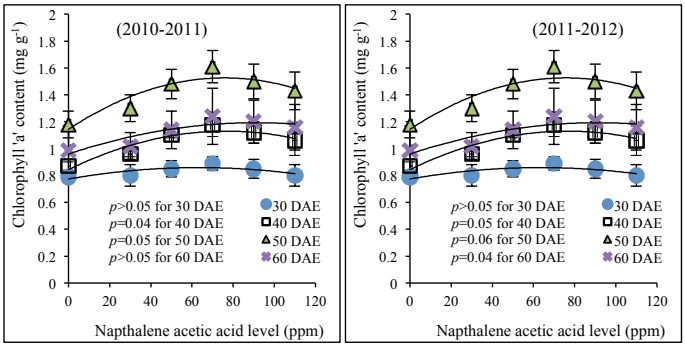

Figure 1. Chlorophyll 'a' content of rapeseed leaf as influenced by napthalene acetic acid at different sampling dates (DAE=days after emergence). The mean data (±SE) over replication are presented

The maximum chlorophyll 'a' content (1.61 mg g^{-1}) was found in G3 (70 ppm ha^{-1}) among the NAA levels. The response function showed the quadratic association among the chlorophyll 'a' content and NAA in regression analysis (Figure 1). For chlorophyll 'a' (1.51 mg g^{-1}), the calculated suitable dose of NAA was 72 ppm ha^{-1} at 50 DAE (Table 3). Therefore, applying 72 ppm ha^{-1}, the highest chlorophyll 'a' content (1.51 mg g^{-1}) of rapeseed leaf can be done. Chlorophyll 'a' content of rapeseed leaf was positively correlated (R^2=858^{NS}) with the applied NAA at 50 DAE. (Table 3).

Table 3
Response function of chlorophyll 'a' content (mg g^{-1}) in Rapeseed leaf to NAA

2010-2011	Regression Equation	R^2 Value	Optimum Dose (kg ha^{-1})	Maximum Chlorophyll 'a' content (mg g^{-1}) For Optimum Dose
30 DAE	$y = -2E\text{-}05x^2 + 0.0027x + 0.7742$	0.6458^{NS}	67	0.86
40 DAE	$y = -5E\text{-}05x^2 + 0.0075x + 0.844$	0.8802^{NS}	75	1.13
50 DAE	$y = -7E\text{-}05x^2 + 0.0101x + 1.1442$	0.8585	72	1.51
60 DAE	$y = -3E\text{-}05x^2 + 0.0051x + 0.9604$	0.8043^{NS}	85	1.18
2011-2012				
30 DAE	$y = -2E\text{-}05x^2 + 0.0027x + 1.0836$	0.4131^{NS}	74	1.17
40 DAE	$y = -2E\text{-}05x^2 + 0.0025x + 1.272$	0.8557^{NS}	63	1.35
50 DAE	$y = -5E\text{-}05x^2 + 0.0074x + 1.2119$	0.8531^{NS}	68	1.48
60 DAE	$y = -2E\text{-}05x^2 + 0.0026x + 1.0511$	0.975*	65	1.13

Note: NS, not significant; *, significant at 5% level

Apparently, chlorophyll 'a' at 40 and 60 DAE showed similar values but there is a significant difference in the values between the two dates. The control showed the minimum chlorophyll 'a' content in all the sampling dates (Figure 1). Chlorophyll 'a' content increased due to application of NAA in 2011-2012 that followed the same trend. At 30 and 60 DAE, the chlorophyll 'a' was significantly different in the rapeseed leaf samples (Figure 1b). At 30, 40, 50 and 60 DAE, the optimum doses of NAA were 74, 63, 68 and 65 ppm NAA ha^{-1} for maximising chlorophyll 'a' of 1.17, 1.35, 1.48 and 1.13 mg g^{-1}, respectively (Table 3). The chlorophyll 'a' content was increased with the increase in NAA level up to 70 ppm ha^{-1}; after that, chlorophyll 'a' decreased. Chlorophyll 'a' content also increased with the increase on different days after emergence but decreased after 50 DAE (Figure 1).

Chlorophyll 'b' Content in Rapeseed Leaf as Influenced by NAA

Different DAE significantly influenced the chlorophyll 'b' content. The chlorophyll 'b' content was increased with the increase in NAA level up to 70 ppm ha^{-1} in 2010-11

on different sampling dates. G3 (70 ppm NAA ha^{-1}) showed the highest chlorophyll 'b' content. A quadratic relationship was found between the chlorophyll 'b' and NAA level (Figure 2). At 40, 50 and 60 days after emergence the optimum doses were 71, 76 and 77 ppm NAA ha^{-1}, respectively (Table 4). The NAA level significantly influenced the chlorophyll 'b' of the mustard leaf at different sampling dates in 2011-12. The maximum chlorophyll 'b' (0.49 mg g^{-1}) was found at 50 DAE, which was significantly higher than at the other DAE. At 30 DAE, the lowest chlorophyll 'b' was recorded, while 70 ppm NAA ha^{-1} showed the maximum chlorophyll 'b' content. The response function showed a quadratic relationship between chlorophyll 'b' and the NAA level. At 50 DAE, the optimum dose of NAA was 72 ppm ha^{-1} (Table 4).

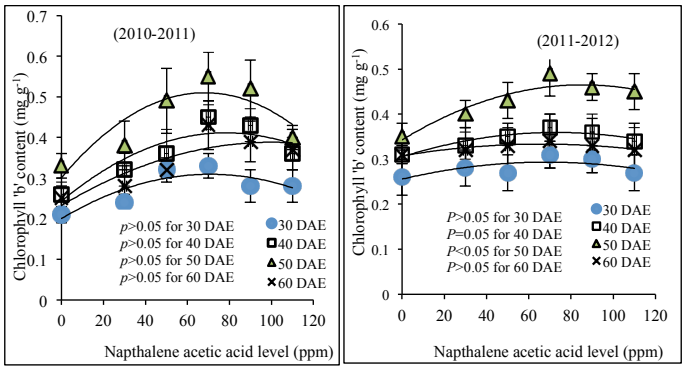

Figure 2. Chlorophyll 'b' of rapeseed leaf as influenced by NAA at different sampling dates (DAE=days after emergence). Data are presented as mean (±SE) over replications

Applying 72 ppm NAA ha^{-1}, the highest chlorophyll 'b' (0.50 mg g^{-1}) in rapeseed leaf can be predicted. Chlorophyll 'b' was positively associated with the applied NAA at 40 DAE (R^2=0.868), 50 DAE (R^2=0.892) and 60 DAE (R^2=0.835) (Table 4). At 40, 50 and 60 DAE, the optimum doses of NAA were 75, 72 and 66 NAA ppm ha^{-1} for maximising chlorophyll 'b' (0.38, 0.50 and 0.34 mg g^{-1} for 40, 50 and 60 DAE, respectively) (Table 4). The photosynthetic activities of green plants are directly influenced by the chlorophyll content of leaves. The NAA level significantly influenced the chlorophyll a and b content in leaf. This was attributed to the increased proportion of gana per plastid volume in the chloroplasts.

Table 4
Chlorophyll 'b' (mg g^{-1}) in Rapeseed leaf as influenced by NAA under response function

2010-2011	Regression Equation	R^2 Value	Optimum Dose (kg ha^{-1})	Maximum Chlorophyll 'b' Content (mg g^{-1}) for Optimum Dose
30 DAE	$y = -2E\text{-}05x^2 + 0.0031x + 0.2004$	0.7633^{NS}	77	0.32
40 DAE	$y = -3E\text{-}05x^2 + 0.0043x + 0.2429$	0.8123^{NS}	71	0.40
50 DAE	$y = -4E\text{-}05x^2 + 0.0061x + 0.3009$	0.7745^{NS}	76	0.50
60 DAE	$y = -2E\text{-}05x^2 + 0.0031x + 0.2322$	0.7634^{NS}	77	0.43
2011-2012				
30 DAE	$y = -8E\text{-}06x^2 + 0.0011x + 0.2556$	0.5109^{NS}	68	0.34
40 DAE	$y = -1E\text{-}05x^2 + 0.0015x + 0.3049$	0.868^{NS}	75	0.38
50 DAE	$y = -2E\text{-}05x^2 + 0.0029x + 0.3426$	0.8921^{NS}	72	0.50
60 DAE	$y = -6E\text{-}06x^2 + 0.0008x + 0.3074$	0.8353^{NS}	66	0.34

Note: NS, not significant

Lakshmamma and Rao (1996a) reported that spraying 5-20 ppm NAA at the flowering stage progressively increased the chlorophyll content in the leaves of black gram. The highest chlorophyll and RNA content were recorded with 20 ppm NAA in the presence of endogenous gibberellic acid (Brain & Hemming, 1958). Spraying NAA (0.04 % solution) at 35 and 75 DAE increased the total chlorophyll content in the soybean leaves by 2.08, 2.21 and 1.68% over the control (Kalarani & Jeyakumar, 1998). Sivakumar et al. (2002) reported that application of 20 ppm NAA increased the content of chlorophyll in the leaf of pearlmillet. Kumar et al. (2005) also found that application of NAA (20 ppm) increased the chlorophyll content of cotton.

Nitrogen Content in Rapeseed Leaf as Influenced by NAA

NAA levels significantly influenced the nitrogen content at different DAE (Figure 3). The highest nitrogen content (6.03 and 5.7% for 2010-11 and 2011-12, respectively) was recorded at 45 DAE, which was significantly higher than the other DAE. The maximum nitrogen content was found in 70 ppm NAA ha^{-1} at all sampling dates (Figure 3). A quadratic relationship was found between the nitrogen content and applied NAA (Figure 3). In 2010-11, the optimum doses of NAA were 75, 72 and 76 ppm NAA ha^{-1} for 30, 45 and 50 DAE, respectively (Figure 3). Applied NAA at 30, 45 and 50 DAE was positively correlated with the nitrogen content in rapeseed leaf (R^2=0.819 and 0.434 and 0.794 for 30, 45 and 50 DAE, respectively) (Table 5). At 30 and 45 DAE, the nitrogen content was apparently similar but there was a significant difference between 30 and 45 DAE. After application of NAA, the nitrogen content increased in the rapeseed leaf in 2011-12, which followed the same trend. In rapeseed leaf, the nitrogen content was positively

($p<0.01$ and 0.18 for 30 DAE and 45 DAE, respectively) correlated ($R^2=0.956^*$ and 0.685 for 30 and 45 DAE, respectively) with NAA at 30 and 45 DAE. In 2011-2012, the optimum doses of NAA were 67, 79 and 77 ppm ha^{-1} for 30, 45 and 50 days after emergence, respectively (Table 5). It may be mentioned that the nitrogen content of the rapeseed leaf samples increased with the increase in NAA but after 70 ppm NAA ha^{-1} it was decreased. Shende et al. (1987) reported that foliar application of different growth regulators increased the nitrogen content in the leaves. Forty-five days after emergence, the nitrogen content was gradually decreased; this might have been due to ageing.

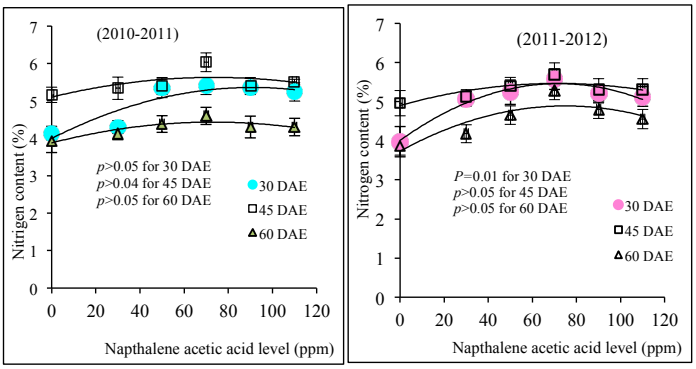

Figure 3. Nitrogen content (%) in rapeseed leaf as influenced by NAA at different DAE in 2010-11 and 2011-12
Note: DAE = days after emergence. Data are presented as mean (±SE) over replications

Table 5
Nitrogen content (%) in Rapeseed leaf as influenced by NAA under response function

2010-2011	Regression Equation	R^2 Value	Optimum Dose (kg ha^{-1})	Maximum Nitrogen Content (%) for Optimum Dose
30 DAE	$y = -0.0002x^2 + 0.03x + 3.995$	0.819^{NS}	75	5.43
45 DAE	$y = -1E\text{-}04x^2 + 0.0144x + 5.1004$	0.434^{NS}	72	5.68
60 DAE	$y = -0.0001x^2 + 0.0153x + 3.8757$	0.794^{NS}	76	4.41
2011-2012				
30 DAE	$y = -0.0003x^2 + 0.0404x + 4.0083$	0.956*	67	5.45
45 DAE	$y = -0.0001x^2 + 0.0158x + 4.8988$	0.685^{NS}	79	5.61
60 DAE	$y = -0.0002x^2 + 0.0308x + 3.7334$	0.765^{NS}	77	4.70

Note: NS, not significant; *, significant at 5% level

Protein Content in Rapeseed as Influenced by NAA

The NAA significantly influenced the protein content in rapeseed. G2 (50 ppm NAA ha^{-1}) showed the maximum protein content (23.7%), which was significantly higher than in the other treatments (Figure 4A). G2 was also 20.3% higher than the control. Kalarani and Jeyakumar (1998) reported that spraying 0.04 % NAA solution at 35 and 75 DAE caused the soluble protein content of soybean leaves to increase by 9.84, 17.58 and 9.13% over the control. Karim (2005) also reported that spraying 20 ppm NAA on the foliage produced the highest amount of protein in chickpeas but the protein content in the seeds decreased with the increase in NAA concentration. Sivakumar et al. (2002) observed that application of 20 ppm NAA increased the protein content in the leaf of pearl millet. Ullah et al. (2007) also reported that the highest protein content was found with 50 ppm NAA.

Oil Content in Rapeseed as Influenced by NAA

The NAA significantly influenced the oil content in rapeseed. In rapeseed, oil content varied from 42.45 to 43.05, having the maximum (43.05%) in G3 (70 ppm NAA ha^{-1}) followed by G4 (42.85%) (Figure 4B). The control showed the minimum oil content (42.0%) (Figure 4B). The similar oil content was found in G4 and G5. With the increase in NAA level, the oil content of rapeseed increased up to 70 ppm NAA ha^{-1} (Figure 4B). The protein content was reduced in the same treatment, while the oil content was the highest (Figure 4A). The best rate of the NAA might have enriched enzyme activities; this implicates that oil synthesis resulted in increased amounts of oil in the seed.

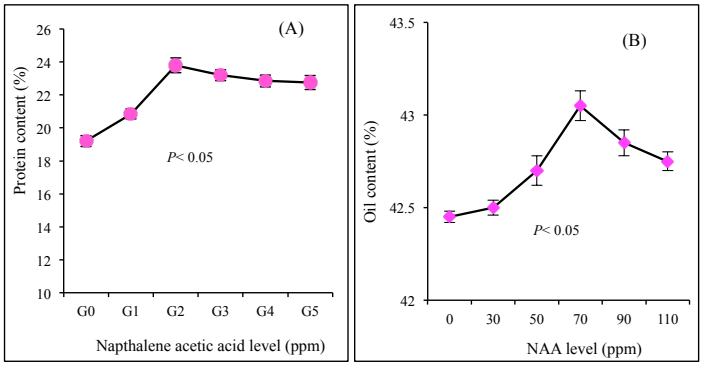

Figure 4. Effect of NAA on protein (%) (A) and oil content (B) in rapeseed. Data are presented as mean (±SE) over replications

Bhat et al. (2004) reported that application of 50 ppm NAA caused significant improvement in oil content of the seed of the mustard plant. Karim (2005) carried out an experiment with different concentrations of NAA on chickpea foliage and found that 20 ppm NAA produced the highest fat in the seeds. Oluwatosin (1997) reported positive correlation between protein and lipid content of cowpea.

Chemical Characteristics of Rapeseed Oil as Influenced by NAA

Acid, iodine, peroxide and saponification values determine the quality of oil. The NAA significantly influenced the acid value. The highest acid value (0.86) was found in G1 (30 ppm NAA ha^{-1}), which was statistically different form other treatments. G3 (70 ppm NAA ha^{-1}) showed the minimum acid value (0.37) (Figure 5A). Mondal (1999) reported that the growth regulators significantly influenced the acid value. G3 showed the maximum iodine value (86.7 and 95.6 for 2010-11 to 2011-12, respectively) (Figure 5B). In 2010-11, a quadratic relation was found between the NAA and iodine value. The optimum dose of NAA was found to be 70.7 ppm NAA ha^{-1}. A quadratic relation was also found between the iodine value and applied NAA in 2011-12. (Figure 5B). The optimum rate of NAA was 76.8 ppm NAA ha^{-1}. The control showed the lowest iodine value (75.0 and 85.2 for 2010-11 to 2011-12, respectively). After application of NAA, the iodine value increased in the synthesis process of fatty acid and this might have been due to the maximum amount of unsaturated fatty acids being converted to saturated fatty acids (Gangahara et al., 1990). The NAA also influenced the peroxide value. The maximum peroxide value (6.18 and 4.48 for 2010-11 to 2011-12, respectively) was found in G1 (30 ppm NAA ha^{-1}) followed by G0 (control) (Figure 5C). G5 and G3 showed the minimum peroxide value (5.11 and 3.87 for 2010-11 to 2011-12, respectively) in 2010-11 to 2011-12, respectively (Figure 5C). With the increase in the NAA level, the peroxide value was decreased. Better quality oil contains a low peroxide level.

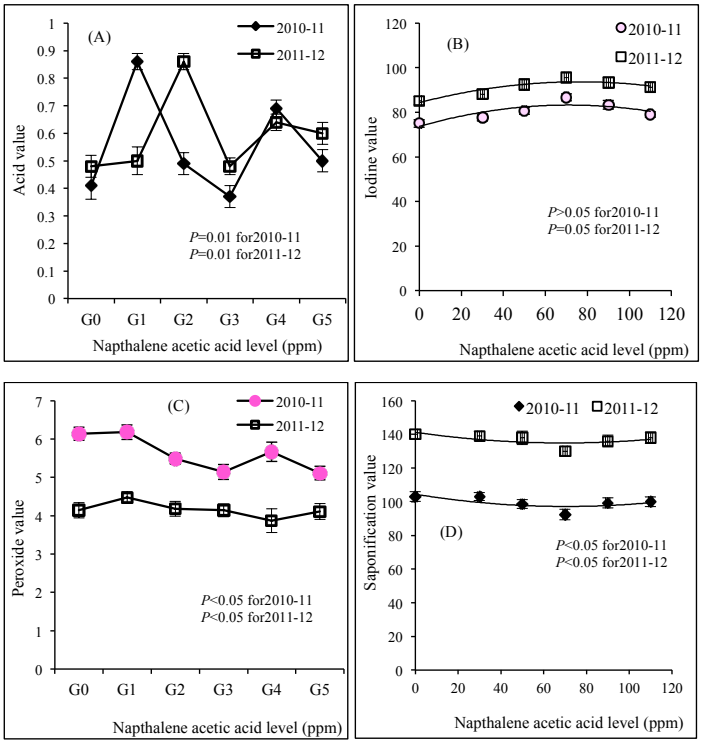

Figure 5. Acid value (A), iodine value (B), peroxide value (C) and saponification value (D) in rapeseed oil. Data are presented as mean (±SE) over replications

NAA also influenced the saponification value. The control showed the maximum saponification value (103 and 140 for 2010-11 and 2011-12, respectively), which was closely followed by G1 (Figure 5D & Table 6). The minimum saponification value (92.4 and 130 for 2010-11 and 2011-12, respectively) was found in G3 (70 ppm NAA ha^{-1}). The NAA was negatively correlated with the saponification value. With an increase in the NAA level, the saponification value decreased; this is beneficial to human health.

Fatty Acid Composition of Rapeseed Oil as Influenced by NAA

NAA significantly influenced the fatty acid composition. NAA positively influenced the palmitic acid content but the effects were not significant ($p>0.05$). The control showed the maximum palmitic acid content (1.97%) followed by G1 and G2 (Figure 6A).

Table 6
Response function of palmitic acid content, iodine value and saponification value of Rapeseed to NAA

	Regression Equation	R^2 Value	Optimum Dose (kg ha^{-1})
Iodine Value (2010-11)	$y = -0.0019x^2 + 0.2686x + 73.67$	0.7087^{NS}	70.7
Iodine Value (2011-12)	$y = -0.0016x^2 + 0.2459x + 84.297$	0.8622^{NS}	76.8
Saponification Value (2010-11)	$y = 0.0015x^2 - 0.2078x + 104.48$	0.4768^{NS}	69
Saponification Value (2011-12)	$y = 0.0013x^2 - 0.1825x + 141.23$	0.4181^{NS}	70

Note: NS, not significant

G5 (110 ppm NAA ha^{-1}) showed the minimum palmitic acid content (1.55%). The response showed a quadratic relationship in nature between the applied NAA and palmitic acid (%) value (Figure 6A). With the applied NAA level, the palmitic acid content in mustard oil was negatively correlated (R^2=955). The control showed the maximum stearic acid value (1.00 and 0.98% for 2010-11 and 2011-12, respectively) (Figure 6B). G5 and G3 showed the lowest stearic acid value (0.75 and 0.59 for 2010-11 and 2011-12, respectively) in 2010-11 and 2011-12, respectively (Figure 6B). With the applied NAA level, the stearic acid value was negatively and significantly ($p<0.05$) correlated (R^2=0.995* for 2010-11). The oil is not beneficial to health as it contains

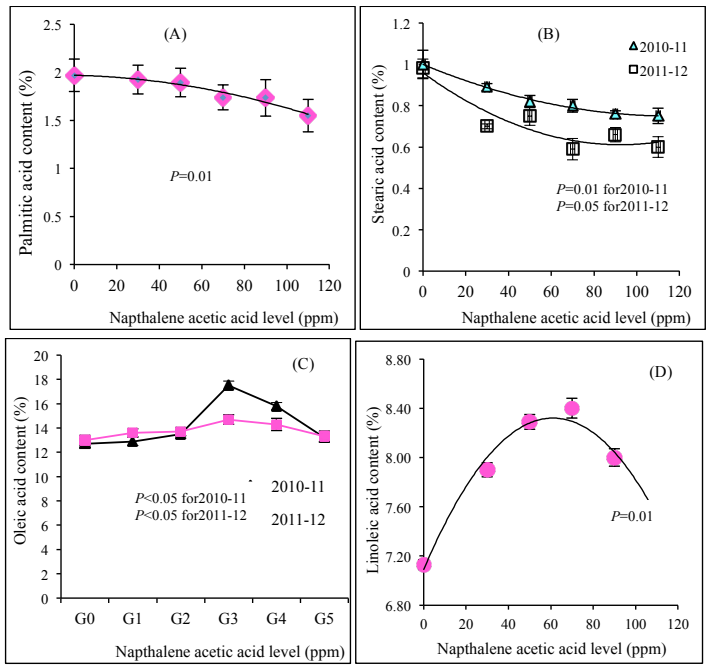

Figure 6. NAA influenced the palmitic acid (A), stearic acid (B), oleic acid (C) and linoleic acid (D) value (%) in rapeseed oil. Data are presented as mean (±SE) over replications

maximum levels of palmitic and stearic acid and it also recorded from 0-30 ppm NAA ha^{-1}. By increasing the NAA levels, these acid values were decreased; this is good for us. The NAA significantly influenced the oleic acid ($p<0.05$).

G3 showed the highest oleic acid content (17.5 and 14.7% for 2010-11 and 2011-12, respectively) followed by G4 (Figure 6C). The control showed the minimum oleic acid value (12.7 and 13.0% for 2010-11 and 2011-12, respectively). A quadratic relationship was found between the NAA level and linoleic acid content (Figure 6D). For maximising linoleic acid in rapeseed, the optimum dose of NAA would be 67 ppm NAA ha^{-1}. A positive and significant ($p<0.01$) correlation ($R^2= 0.995^{**}$) was found between the linoleic acid and NAA level. According to regression analysis, a quadratic relationship was found between the linolenic acid content and NAA (Figure 7A). The positive correlation ($R^2=0.741^{NS}$) was found between the linolenic acid value and the NAA level and the optimum dose of NAA was 68 ppm NAA ha^{-1}. The maximum ecosanoic acid content (1.2%) was found in G3. The control showed the lowest value (0.64%). A quadratic relation ($R^2=0.623^{NS}$) was found between the ecosanoic acid value and NAA level but it was not significant (Figure 7A). To receive maximum ecosanoic acid value, the optimum dose of NAA was 68 ppm NAA ha^{-1}(Table 7). The maximum ecosenoic acid content (6 and 9.16 for 2010-11 and 2011-12, respectively) was recorded in G3 and the lowest was found in the control (Figure 7C). A quadratic relation was found between the NAA and ecosenic acid value but its effect was insignificant. To get the maximum ecosenic acid, the optimum doses of NAA were 76 and 68 ppm NAA ha^{-1} in 2010-11 and 2011-12, respectively (Table 7). By increasing the NAA level, oleic, linoleic and lenolenic acid content were increased up to a certain level, after which the content of these acids decreased. To lower plasma cholesterol and lipoprotein density, a high proportion of polyunsaturated fatty acids are required. This would reduce the risk of coronary heart disease and parthenogenesis (Skoric, 1988). Erucic acid is harmful to human health. Erucic acid was also influenced by the applied NAA levels (Figure 7D). The maximum erucic acid value (53.3%) was found in the control, followed by G1 (53.2%). The minimum erucic acid content (50.3%) was noted in G4. By increasing the NAA level, the erucic acid content was decreased. This is beneficial to human health. The composition of seed oil is largely controlled by genetic factors, but environmental factors may change the pattern of fatty acid in oil of mustard seed (Mondal, 1986).

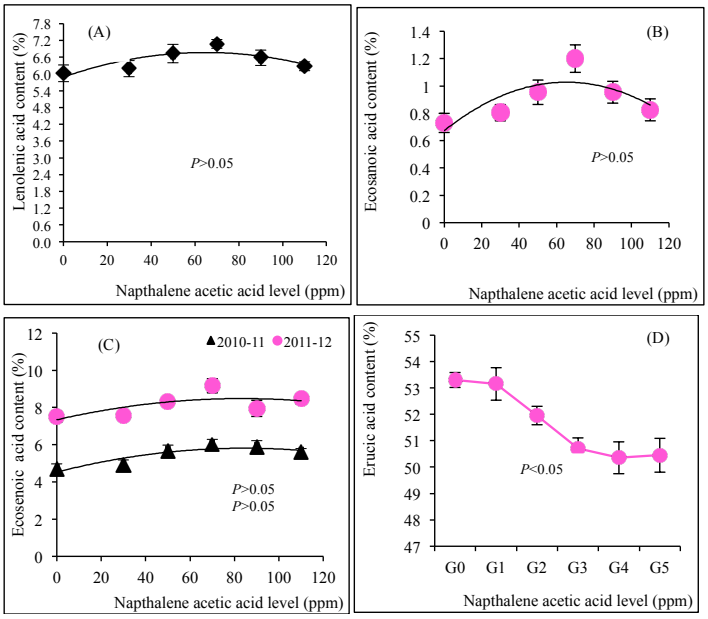

Figure 7. Linoleic acid (A), ecosanoic acid (B), ecosenoic acid (C) and erucic acid (D) content (%) in rapeseed as influenced by NAA. Data are presented as the mean (±SE) over replications

No previous report is available on the effect of NAA on fatty acid formation of oil. However, the nutritional and storage qualities of mustard depend on the relative proportion of saturated and unsaturated fatty acids in the oil.

Table 7
Influence of NAA on stearic acid, lenolic acid, lenolenic acid, ecosanoic acid, ecosenoic acid and erucic acid under different response functions

	Regression Equation	R^2 Value	Optimum Dose (ppm)
Palmitic Acid Content	$y = -3E\text{-}05x^2 - 0.0005x + 1.97$	0.9553^{NS}	-
Stearic Acid (2010-11)	$y = 2E\text{-}05x^2 - 0.0043x + 1.00$	0.9945 **	-
Stearic Acid (2011-12)	$y = 4E\text{-}05x^2 - 0.0075x + 0.96$	0.8621^{NS}	-
Lenolic Acid	$y = -0.0003x^2 + 0.0403x + 7.10$	0.9778 **	67
Lenolenic Acid	$y = -0.0002x^2 + 0.027x + 5.90$	0.7021^{NS}	68
Ecosanoic Acid	$y = -8E\text{-}05x^2 + 0.0109x + 0.67$	0.6225^{NS}	68
Ecosenoic Acid (2010-11)	$y = -0.0002x^2 + 0.0304x + 4.527$	0.83^{NS}	76
Ecosenoic Acid (2011-12)	$y = -0.0002x^2 + 0.0273x + 7.34$	0.4772^{NS}	68

Note: NS, not significant; *, significant at 5% level; **, significant at 1% level

CONCLUSION

The naphthalene acetic acid significantly influenced the biochemical character of rapeseed oil. G_3 (70 ppm NAA ha^{-1}) showed the highest chlorophyll content of rapeseed leaves at 50 days after emergence. The maximum nitrogen content in the leaves was also found in the same NAA level at 45 DAE. A value of 70 ppm NAA also showed the highest oil content. The minimum acid, peroxide and saponification values were observed in 70 ppm NAA. The same treatment also showed the highest iodine value. By decreasing the NAA level, nonessential fatty acids were increased in the mustard plant, while necessary fatty acids were highest in 70 ppm NAA ha^{-1}. So, 70 ppm NAA ha^{-1} can be used to grow quality mustard.

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The Effects of Application of Exogenous IAA and GA_3 on the Physiological Activities and Quality of *Abelmoschus esculentus* (Okra) var. Singa 979

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ABSTRACT

An experiment was conducted to investigate the effects of growth regulators on growth, yield and the quality of okra. Indole Acetic Acid (IAA) and Gibberellin (GA_3) were applied as foliar spray and stem and flower injection at concentrations of 0, 30, 60, 90, and 120 mg/L on okra plants. The results showed that foliar spray of 90 mg/L IAA, increased the number of leaves, number of branches, number of flowers and number of pods. On the other hand, spraying of 90 mg/L GA_3 increased stomatal conductance and pod weight of okra, while the highest chlorophyll content was recorded with 60 mg/L GA_3. Stem injection of 120 mg/L IAA produced the highest number of leaves, number of branches, number of flowers, number of pods and plant height. Similarly, 120 mg/L GA_3 as stem injection increased the number of branches, number of leaves, number of flowers and number of pods and total soluble solids (TSS). Flower injection of IAA at 30 and 90 mg/L increased pod size, pod weight, pod number and TSS content, while seed production was inhibited by 120 mg/L IAA. GA_3 used in a 90 mg/L treatment as flower injection increased pod size and TSS content. It is concluded that the application of 120 mg/L IAA and 90 mg/L GA_3 increased the growth, development and quality of the okra fruit and stem and that flower injection worked better than foliar spray.

Keywords: GA_3, growth regulators, IAA, injection, okra, spray

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INTRODUCTION

Okra (*Abelmoschus esculentus*) is an annual pod vegetable that grows quickly, bearing many branches. It is able to reach up to a height of 1.82 m. The common name of

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the okra plant is lady's finger, and the plant belongs to the family Malvaceae (Brouk, 1975). Balock (1994) reported that okra is an annual, herbacious and warm season vegetable. Lady's finger is a self-pollinated crop, but about 20% of pollination is cross pollination through the activity of insects and other pollinators (Grubben, 1977). The charateristics of this vegetable are indeterminate growth habit and continuous flowering, with flowering depending on nutrient supply and environmental factors. The plant starts to flower one to two months after the sowing of seeds, which can be manipulated by cultural practices (Adetuyi et al., 2008). The okra pod is a capsule that grows quickly after flowering and pollination. Fruit growth is the highest during the 4th to 6th day after pollination. Adeboye and Oputa (1996) reported that okra is the ultimate source of carbohydrate, fibre, proteins and vitamins. It has been reported that 100 g of fresh okra pod contains 89.6% moisture, 103 mg of potassium, 90 mg of calcium, 43 mg of magnesium, 56 mg of phosphorus, 18 mg of vitamin C and metals such as iron and aluminium (Markose & Peter, 1990). Recently, okra has been grown commercially in India, Turkey, Iran, Western Africa, Bangladesh, Afghanistan, Yugoslavia, Burma, Pakistan, Malaysia, Japan, Brazil, Cyprus Ethiopia, Ghana and the United States of America. India produces 70% of the total world production of okra (3.5 million tons) and ranks first in the world as a producer of the plant (FAOSTAT, 2012).

Kusvuran (2012) reported that the quality of the seed, nutrition application, environmental conditions and cultural practices are the key factors that affect the growth and quality of okra. Plant growth regulators (PGRs) and growth promoting chemicals may change the phenotype of many plants when applied at the early growth stage. PGRs stimulate or retard the natural growth regulatory systems from germination to senescence of plants (Das & Das, 1995). Plant growth regulators affect the physiological efficiency of plants including growth, photosynthesis and accumulation of assimilates. Solaimalai et al. (2001) reported that the productivity of crops is increased by stimulating the translocation of photo-assimilates. In this study, IAA and gibberellin were used to improve the physiological activities and the quality of the okra plant under field conditions. IAA and GA were applied separately to study the specific effects of the two growth regulators on the okra plant. May be the phytohormones auxin (IAA) and gibberellin (GA), which partly control overlapping processes during plant development. It has been proven that isolated or combined plant growth regulators show different responses in biometrical and productive parameters in *Solanum lycopersicum* (Choudhury et al., 2013). Growth regulators might act as a key factor for plant growth and development through various reactions to the environment in different doses, and this was of concern in the current study.

The number of seeds in the okra pod can be a deterrent for some consumers from consuming okra. Correct concentrations and suitable application methods of plant

hormones can reduce the number of seeds in okra pods. This research presents results on the effect of IAA and GA_3 on the growth, physiology and quality of okra, as this information is highly relevant to growers and researchers. This study found that the flower and stem injection technique brought better results than the spraying method.

MATERIALS AND METHOD

The present study was carried out from September 2014 to June 2015 at the field farm of the Faculty of Bioresources and Food Industry Farm, Besut Campus, Universiti Sultan Zainal Abidin. One hundred fifty okra plants were used for the treatment application. Okra seeds were sown in germination trays at the nursery and seven days after sowing (DAS) at 3-5 leaf stage, all the seedlings were transplanted to polybags containing garden soil and coco peat. Experimental plants were arranged under a completely randomised design (CRD) with five replicates. GA_3 and IAA at 0, 30, 60, 90, and 120 mg/L concentrations were applied to the experimental plant. Foliar spray, stem injection and flower injection techniques were used to apply the plant growth regulators. In using foliar spray, different concentrations of IAA and GA_3 were applied to the leaves and stems of the okra plants. A volume of 1.5 mL IAA and GA_3 was applied to the stem by injecting the okra plant stems using a surgical needle at the height of 3 cm from the ground level. Distilled water mixed with 2 mL of 1% ethanol was used in the control treatment. For flower injection, IAA and GA_3 were applied to the female okra flower before anthesis through injection using a surgical needle (Mekhled, 2011).

Plant Growth and Yield Measurements

Plant height (cm), number of leaves, number of branches, number of flowers, number of pods, pod size and weight (g) were recorded once a week after the treatment application. Plant height was measured from above the ground level up to the uppermost tip of the leaves. Numbers of leaves, branches, flowers, pods and pod weight were counted and measured on each treated and control plant. For seed production, the percentage of healthy seeds and aborted seeds were recorded and calculated using the formula (Mekhled, 2011) below:

$$\text{Healthy seed (\%)} = \frac{\text{Total number of healthy seeds}}{\text{Total number of seeds}} \times 100$$

$$\text{Aborted seed (\%)} = \frac{\text{Total number of aborted seeds}}{\text{Total number of seeds}} \times 100$$

Leaf chlorophyll content of treated and control plants was measured by SPAD meter (Minolta Japan). Stomatal conductance ($mmol/m^{-2}s^{-1}$) was measured using leaf porometer from 12 nn to 1 pm in full sunshine conditions and readings were taken a week after the treatment for three consecutive weeks. Green fruit or okra pod weight (g) was measured. A small fraction

of a homogenous mixture of okra pod was centrifuged at 4000 × g for 10 min, and the clear supernantant was evaluated for total soluble solids (TSS). The total soluble solid content of pod wax was evaluated using a hand refractometer (Atago 8469) and expressed as percentage (%) of Brix.

Statistical Analysis

All the data obtained were analysed using the IBM Statistical Package for the Social Sciences (SPSS) version 22. Significant difference of mean values were determined and analysed using one-way ANOVA and the mean differences were compared using Duncan's Multiple Range Test (DMRT) at 5% level of significance.

RESULTS

Effect of Foliar Spray of IAA on the Growth and Physiological Activities of Okra

The results showed that the 90 mg/L IAA treatment produced the highest number of leaves (35) compared to the control (26.00). The number of okra branches was significantly affected by 120 mg/L IAA applied using the spray technique, and this treatment produced 1.2 times more branches compared to control. Foliar spray of 90 mg/L IAA significantly increased the number of leaves, number of flowers, number of fruit, fruit weight and TSS content of fruit. The highest number of flowers (8), number of fruit (7), fruit weight (26 g) and TSS content (2.47% Brix) were recorded for the 90 mg/L IAA treatment (Table 1).

Table 1
Effects of spray technique on Okra growth, development and fruit quality using different concentrations of Indole Acetic Acid (IAA)

Concentration of IAA	No. of branches	No. of leaves	No. of flowers	No. of fruit	Fruit weight (g)	TSS (% Brix)
0	4.00± 0.58^{c}	26.0± 1.15^{b}	5.00±0.33^{b}	4.00±0.33^{c}	24.0±0.31^{a}	2.26±0.02^{b}
30	5.00±0.00^{bc}	28.0± 1.45^{b}	7.00±0.88^{a}	6.00±1.00^{ab}	25.0±0.31^{ab}	2.37±0.02^{ab}
60	5.00± 0.00^{bc}	30.0± 0.88^{b}	7.00±0.33^{a}	7.00±0.33^{a}	25.0±0.43^{ab}	2.43±0.02^{a}
90	6.00± 0.58^{ab}	35.0± 1.20^{a}	8.00±0.33^{a}	7.00±0.33^{a}	26.0±0.23^{a}	2.47±0.03^{a}
120	7.00 ± 0.33^{a}	28.0± 0.88^{b}	6.00±0.33^{ab}	6.00±0.33^{bc}	25.0±0.52^{ab}	2.46±0.08^{a}

All the data are the mean of three replications; ± indicates the standard of error. Different letters in the same column are significantly different at 5% level (Duncan's Multiple Range Test)

Table 2 shows that the percentage of healthy seeds of the okra pod was reduced with the increase in IAA concentrations, while the percentage of aborted seeds in the pods of okra significantly increased in all treated plants compared to in the control plants. IAA treatments produced a significant effect on the leaf chlorophyll content and stomatal conductance of the okra plants (Table 2). The highest chlorophyll content of leaves

(50 SPAD) was recorded in 30 mg/L of the IAA treatment. The highest stomatal conductance (117 mmol/$m^{-2}s^{-1}$) was obtained at the concentration of 120 mg/L IAA and the lowest conductance (82 mmol/$m^{-2}s^{-1}$) was recorded in the control, and the difference was statistically significant (Table 2).

Table 2
Effects of spray technique on the leaf chlorophyll, stomatal conductance and seed quality of Okra using different concentrations of Indole Acetic Acid (IAA) and gibberellin (GA_3)

Treatment (mg/L)	Healthy seed (%)	Aborted seed (%)	Chlorophyll content	Stomatal conductance (mmol/$m^{-2}s^{-1}$)	Fruit weight (g)
IAA					
0	95.0± 0.55^{a}	4.54± 0.55^{b}	41.9± 0.69^{d}	86.2± 0.59^{e}	----
30	94.0± 0.48^{b}	6.30± 0.48^{a}	49.7± 0.29^{a}	88.5± 1.38^{d}	----
60	93.0± 0.08^{b}	6.8± 0.08^{a}	43.7± 0.29^{c}	93.8± 0.69^{c}	----
90	95.00± 0.39^{b}	6.37± 0.39^{a}	46.6± 0.18^{b}	97.1± 1.01^{b}	----
120	93.00± 0.28^{b}	6.72± 0.27^{a}	45.8± 0.72^{b}	117.1± 0.69^{a}	----
GA3					
0	95.0± 0.48^{a}	4.7±0.50^{a}	40.6± 0.17^{d}	34.6±0.74^{e}	23.5±0.15^{e}
30	96.0±0.54^{a}	4.5± 0.48^{a}	46.5± 0.05^{b}b	90.8±0.70^{c}	28.5±0.33^{d}
60	95.0± 0.46^{a}	4.6±0.12^{a}	47.5± 0.24^{a}	88.2±1.22^{d}	32.1±0.43^{b}
90	95.0± 0.47^{a}	4.5±0.22^{a}	41.4± 0.23^{c}	156.6±1.25^{b}	33.5±0.19^{a}
120	96.0±0.55^{a}	4.4±0.18^{a}	40.9± 0.25^{d}	285.2±0.45^{a}	30.4±0.25^{c}

All the data are the mean of three replications. Different letters in the same column are significantly different at 5% level (Duncan's Multiple Range Test)

Effect of Foliar Spray of GA_3 on the Physiological Activities of Okra

Foliar spray of the 90 mg/L GA_3 produced the highest fruit weight (34 g) (Table 2). The medium concentration of GA_3, that is, the 60 mg/L treatment, increased the chlorophyll content (48). The results also showed that stomatal conductance of the okra plant leaf was significantly affected by GA_3 treatment, and the highest value of stomatal conductance (285 mmol/$m^{-2}s^{-1}$) was recorded in the 120 mg/L GA_3 treatment, whereas the control produced the lowest stomatal aperture (35 mmol/$m^{2}s$).

Effect of Stem Injection of IAA on the Growth and Physiological Activities of Okra

The results showed that the number of leaves, branches, flowers and fruit of the okra plants increased significantly when a concentration of 120 mg/L IAA was applied in the stem injection (Table 3). The highest number of leaves (37) recorded was in the 120 mg/L IAA treatment. IAA at the 120 mg/L treatment produced the highest number of branches (6) compared to other treatment. Stem injection of 120 mg/L IAA produced the the highest number of

okra flowers and pods, as seen in Table 3. The highest amount of TSS was recorded in the 30 mg/L IAA treatment (Table 3). IAA at 120 mg/L using rge stem injection treatment produced the highest plant height (48 cm). Chlorophyll content and stomatal conductance of okra plant were significantly affected by the stem injection of IAA (Figure 1). The highest amount of leaf chlorophyll content (45) was measured at 60 mg/L IAA treatment compared to the control (40). The highest stomatal conductance of the okra leaf (152 mmol/m^{2}s) was obtained in the treatment using 30 mg/L IAA (Figure 1).

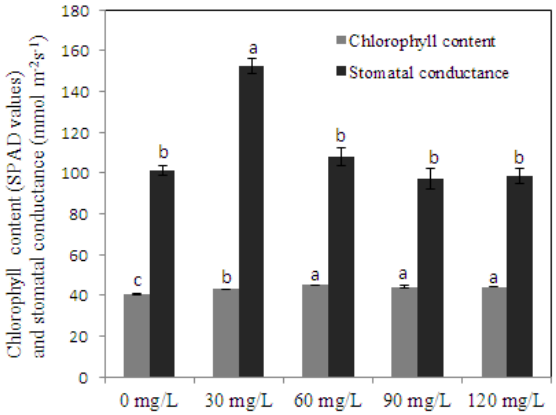

Figure 1. Effects of different treatments of IAA as stem injection on chlorophyll content and stomatal conductance of okra. Bars indicate mean ± S.E. Mean values with the same letters (a or b) are not significantly different at p=<0.05

Effect of Stem Injection of GA_3 on the Growth and Physiological Activities of Okra

Table 3 shows that all the okra plants treated with GA_3 produced the highest number of branches. At 120 and 90 mg/L, GA_3 produced the highest number of branches (6). The number of okra plant leaves was also significantly increased with the GA_3 application, and it was the highest in 120 mg/L treatment with a value of 80. Different treatments of GA_3 produced a significant effect on the number of flowers, pods, pod size, pod weight and pod TSS content of okra. The highest number of flowers (8) and pods (8) was obtained with the treatment of 120 mg/L GA_3. The results showed that fruit size of the okra plant increased with concentration up to 90 mg/L but thereafter decreased (Table 3). Pod weight of okra was the highest (44 g) in the 90 mg/l GA_3 treatment. In addition, TSS content of the okra pods also increased significantly with the stem injection of higher concentrations of GA_3. The highest TSS (2% Brix) content was recorded at a concentration of 120 mg/L GA_3 compared to the control (2% Brix) (Table 3).

Table 3
Effects of stem injection on the plant growth, flowering and fruit quality of Okra using different concentrations of Indole Acetic Acid (IAA) and Gibberellin (GA_3)

Treatment (mg/L)	No. of leaf/ plant	No. of branches	No. of flowers	No. of fruit/ plant	TSS (% Brix)	Plant height (cm)
IAA						
0	23.0± 1.45^{d}	3.0± 0.58^{b}	7.00± 0.33^{b}	6.00± 0.33^{b}	2.24± 0.03^{cd}	44.0± 2.00^{b}
30	26.0± 0.88^{cd}	4.0± 0.58^{b}	8.00± 0.58^{b}	7.00± 0.88^{ab}	2.85± 0.01^{a}	46.0± 2.00^{ab}
60	28.0± 1.15^{bc}	4.0± 0.58^{b}	8.00± 0.58^{b}	7.00± 0.88^{ab}	2.43± 0.01^{b}	45.6± 1.53^{ab}
90	31.0± 1.15^{b}	4.00± 0.33^{ab}	8.00± 0.58^{b}	8.00± 0.88^{ab}	2.21± 0.01^{d}	47.1± 1.04^{ab}
120	37.0± 2.40^{a}	6.0± 0.58^{a}	11.0± 0.33^{a}	9.00± 0.33^{a}	2.28± 0.02^{c}	48.8± 2.57^{a}
GA_3						
0	25.0± 2.40^{e}	3.00± 0.58^{b}	5.00±0.33^{b}	4.00±0.58^{b}	2.11±0.01^{c}	40.8± 2.01^{b}
30	46.0± 1.15^{d}	4.00± 0.58^{b}	7.00±0.33^{b}	7.00±0.58^{a}	2.32±0.02^{b}	45.9± 2.57^{a}
60	55.0± 2.03^{c}	4.00± 0.58^{b}	7.00±0.33^{b}	7.00±0.58^{a}	2.36±0.02^{b}	46.5± 2.40^{a}
90	66.0± 0.88^{b}	6.00± 0.58^{a}	8.00±0.33^{ab}	8.00±0.88^{a}	2.36±0.01^{b}	45.4± 1.15^{a}
120	80.0± 0.33^{a}	6.00± 0.33^{a}	9.00±0.33^{a}	8.00±0.67^{a}	2.43±0.03^{a}	45.2± 1.04^{a}

All the data are the mean of three replications. Different letters in the same column are significantly different at 5% level (Duncan's Multiple Range Test)

GA_3 increased the height of the okra plant under field conditions (Table 3). The results showed that the stem injection of the GA_3 treatment did not produce any significant effect on healthy seed percentage of the okra plant (Table 4). On the other hand, it was observed that the stem injection of 30 and 60 mg/L GA_3 increased the percentage of aborted seeds compared to the control. Leaf chlorophyll content and stomatal conductance of the treated okra plant were significantly higher in the treated plant compared to in the untreated plant, and the highest chlorophyll and stomatal conductance were recorded in the 120 mg/L GA_3 treated okra plant (Table 4).

Table 4
Effects of stem injection method on the physiology and fruit quality of Okra using different concentrations of Gibberellin (GA_3)

Treatment (mg/L)	Fruit size (cm)	Fruit wt (g)	Healthy seed (%)	Aborted seed (%)	Chlorophyll content	Stomatal conductance ($mmol/m^{-2}s^{-1}$)
0	5.13±0.03^{e}	28.6±0.18^{e}	96.0± 0.24^{b}	4.00± 0.24^{b}	43.4± 0.19^{c}	98.6± 0.85^{e}
30	14.8±0.03^{d}	29.7±0.18^{d}	95.0± 0.18^{c}	5.00± 0.18^{a}	49.6± 0.15^{b}	168.5± 0.52^{d}
60	28.5±0.23^{c}	36.5±0.18^{c}	95.0± 0.03^{c}	5.00± 0.03^{a}	49.4± 0.35^{b}	184.5± 0.29^{c}
90	43.2±0.35^{a}	44.4±0.06^{a}	96.0± 0.03^{a}	4.00± 0.03^{c}	50.5± 0.32^{a}	218.1± 0.53^{b}
120	41.4±0.22^{b}	42.6±0.21^{b}	96.0± 0.04^{b}	4.00± 0.04^{b}	50.6± 0.06^{a}	221.1± 0.47^{a}

All the data are the mean of three replications. Different letters in the same column are significantly different at 5% level (Duncan's Multiple Range Test)

Effect of Flower Injection of IAA on the Growth and Physiological Activities of Okra

Flower injection of IAA significantly affected the number of leaves and branches of the okra plant (Table 8). The number of leaves was the highest (30) in the treatments of IAA at 90 and 120 mg/L.The highest number of branches (6) was also recorded with the 120 mg/L IAA treatment compared with the control (Table 5). The number of flowers and fruit of the okra plant was significantly increased with flower injections of 90 and 120 mg/L IAA (Table 5). The highest number of flowers (10) and fruit (9) was found in the 90 mg/L IAA treatment. It was observed that flowering and fruit formation increased the concentration of IAA. The fruit size was significantly increased with the IAA concentration with the highest value, 60 mg/L IAA. The result also showed that the 30 mg/L IAA applied as a flower injection increased the fruit weight and TSS content of the okra fruit compared to other treatments and the control. The TSS value was the highest (3) in the 30 mg/L of IAA treatment (Table 5). IAA at 60 mg/L doses produced the highest plant height of okra (48 cm) compared with the lowest height, which was recorded for the control plant (42 cm).

Flower injection of IAA produced a significant effect on healthy and aborted seed percentage of the okra fruit (Table 6). In this study, the highest healthy seed percentage per plant (88%) was recorded in the control plants using the flower injection method, while the lowest heathy seed percentage was recorded in the 120 mg/L treatment (Table 6). It was also found that flower injection of higher doses of IAA reduced the production of seeds in the okra fruit. The highest stomatal conductance of the okra plant was recorded in the 60 mg/L treatment, while the highest chlorophyll content was recorded in the 60 mg/L IAA treatment (Table 6).

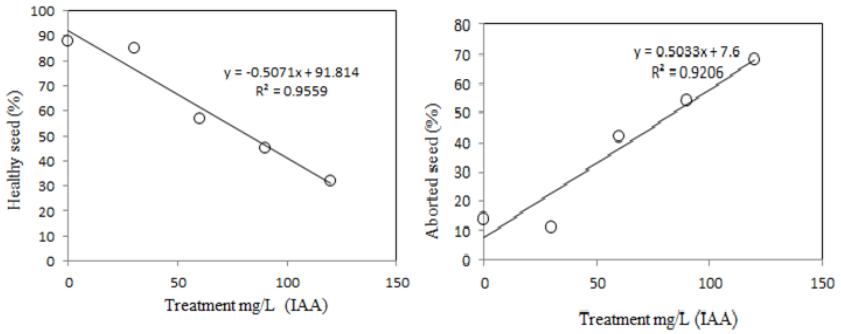

Figure 2. Correlation between concentration of IAA and % healthy seed and % aborted seeds of okra as a result of flower injection

Effect of Flower Injection of GA_3 on the Growth and Physiological Activities of Okra

Flower injection of 90 mg/L GA_3 increased the number of branches and leaves of the okra plant compared with the other treatments and the control and their mean difference was statistically significant (Table 5). The highest number of flowers (8) and fruit (7) was recorded for the 120 mg/L GA_3 treated plants (Table 5).

Table 5
effects of flower injection on the plant growth and fruit quality of Okra using different concentrations of Indole Acetic Acid (IAA) and Gibberellin (GA_3)

Treatment (mg/L)	No. of leaf/ plant	No. of branches	No. of flowers	No. of fruit/ plant	Fruit size (cm2)	Fruit weight (g)	TSS (% BRIX)
IAA							
0	23.0± 1.45^{b}	4.00± 0.58^{b}	6.00±0.33^{c}	5.00±0.33^{c}	7.70±0.06^{e}	15.3±0.12^{e}	2.22±0.01^{d}
30	28.0± 1.45^{a}	5.00± 0.58^{ab}	8.00±0.33^{b}	7.00±0.58^{bc}	32.2±0.58^{a}	34.7±0.22^{a}	3.82±0.02^{a}
60	29.0± 1.45^{a}	5.00± 0.58^{ab}	9.00±0.33^{ab}	7.00±0.58^{bc}	26.5±0.09^{b}	32.1±0.58^{b}	2.56±0.02^{b}
90	30.0± 1.45^{a}	5.00± 0.58^{ab}	10.0±0.58^{a}	9.00±0.58^{a}	23.7±0.34^{c}	30.4±0.20^{c}	2.39±0.06^{c}
120	30.0± 1.20^{a}	6.00± 0.58^{a}	10.0±0.58^{a}	8.00±0.67^{ab}	15.0±0.46^{d}	26.43±0.63^{d}	2.53±0.09^{c}
GA_3							
0	22.0± 1.53^{b}	4.00 ± 0.58^{b}	5.00±0.33^{b}	4.00±0.58^{b}	8.08±0.31^{d}	15.1±0.46^{d}	2.24±0.03^{e}
30	25.0± 0.88^{ab}	6.00± 0.58^{a}	6.00±0.58^{b}	5.00±0.88^{ab}	15.0±0.49^{c}	22.1±0.33^{c}	2.90±0.03^{c}
60	25.0± 0.88^{ab}	6.00± 0.58^{a}	6.00±0.58^{b}	5.00±0.58^{ab}	24.5±0.36^{a}	29.8±0.16^{a}	3.24±0.00^{b}
90	28.0± 1.73^{a}	6.00± 0.58^{a}	8.00±0.33^{a}	7.00±0.88^{ab}	24.4±0.25^{a}	29.9±0.41^{a}	3.56±0.17^{a}
120	28.0± 1.73^{a}	7.00± 0.58^{a}	8.00±0.58^{a}	7.00±1.15^{a}	18.3±0.08^{b}	24.6±0.17^{b}	2.60±0.02^{d}

All the data are the mean of three replications. Different letters in the same column are significantly different at 5% level (Duncan's Multiple Range Test)

On the other hand, flower injection of 60 and 90 mg/L GA_3 treatments increased the fruit size and fruit weight significantly compared with the other treatments and the control plants. TSS content of okra fruit was also affected significantly with the flower injection of 90 mg/L GA_3. The highest amount of TSS content (3.5% Brix) was recorded in 90 mg/L GA_3 treatment (Table 5). Flower injection of GA_3 did not produce a significant effect on the plant height of okra. All the treatments had a significant effect on the production of healthy seeds of okra compared with the control. Flower injection of GA_3 at doses of 60, 90 and 120 mg/L had the highest healthy seeds (95%) per fruit and this was statistically higher than in the control (84%) (Table 6). Significant variation was found between the control and the treated group in case of seed abortion intensity, which was highest in the control (6.03%) and lowest in 120 mg/L GA_3 (4%). The chlorophyll content and stomatal conductance of the treated plant were also significantly increased by GA_3 application through the flower injection method (Table 6).

Table 6
Effects of flower injection on the okra plant physiology and seed quality of Okra using different concentrations of Indole Acetic Acid (IAA) and Gibberellin (GA_3)

Treatment (mg/L)	Plant height (cm)	Healthy seed (%)	Aborted seed (%)	Chlorophyll content	Stomatal conductance ($mmol/m^{-2}s^{-1}$)
0	42.7± 1.31[b]	88.0± 0.74[b]	15.0± 0.74[d]	46.4± 0.32[c]	139.6± 2.75[c]
30	44.3± 1.58[a]	85.0± 0.15[a]	12.0± 0.16[e]	48.9± 0.50[b]	143.9± 2.11[c]
60	48.5± 1.23[a]	58.0± 0.23[c]	42.0± 0.16[e]	48.0± 0.5[b]	266.1± 0.18[a]
90	44.4± 1.12[b]	45.0± 0.46[d]	55.0± 0.46[b]	45.1± 0.95[d]	171.1± 1.19[b]
120	48.1± 1.26[a]	32.00± 0.29[e]	68.0± 0.29[a]	52.8± 0.64[a]	139.2± 7.77[c]
GA3					
0	42.1±0.32[a]	85.0± 0.34[c]	7.00±0.34[b]	43.2± 0.57[d]	95.2± 1.25[e]
30	42.4±0.12[a]	93.0± 0.06[b]	5.00±0.06[a]	44.8± 0.07[c]	138.4± 0.23[d]
60	44.5±0.23[a]	95.0± 0.11[a]	5.00± 0.11[c]	46.8± 0.05[b]	169.7± 6.44[b]
90	44.8±0.12[a]	96.0± 0.07[a]	4.00±0.07[c]	47.5± 0.02[a]	164.4± 0.34[c]
120	44.6±0.08[a]	96.0± 0.04[a]	4.00±0.04[c]	44.6± 0.38[c]	263.5± 0.25[a]

All the data are the mean of three replications. Different letters in the same column are significantly different at 5% level (Duncan's Multiple Range Test)

DISCUSSION

Two different growth regulators were used in this study for improvement of physiological activities and quality of okra. From the results, it was observed that IAA performed better than GA_3 when applied using three different techniques. The results of this study showed that IAA produced more consistent stimulation throughout spraying and stem and flower injection at different concentrations. On the other hand, flower and stem injection of GA_3 showed higher stimulatory effects than foliar spray on growth, development and quality of okra. The results showed that IAA and GA_3 application increased the branch number and plant height of the okra plant. Chhipa and Lal (1998) also reported that IAA application increased the number of branches in wheat plant. These results might be due to increase in cell division and cell elongation, which are effects of GA_3 and auxins (Ranjan et al., 2003). GAs also regulate flower initiation and induce mitotic division in the leaves. Daviere and Achard (2013) stated that GA stimulates growth by activating the degradation of DELLAs protein, a family of nuclear proteins that act as intracellular as well as growth repressors throughout the lifecycle of higher plants. It was recently found that derepression is mediated through the gibberellic acid (GA)-dependent degradation of DELLAs and the key components of the GA-DELLA signalling pathway.

From this study, it is clear that application of growth regulators increased the leaf area and chlorophyll content of the okra plant. Vamil et al. (2010) stated the similar positive effects of IAA and GA_3 on

leaf area and chlorophyll content. It has been reported that IAA application increased leaf number in onion (Hye et al., 2002). GA- has stimulatory effects on cell division and elongation, leaf area and chlorophyll content (Harrington et al., 1996). Mukhtar (2008) reported that GA_3 treatment at 100 mg/L increased the leaf number, leaf area and chlorophyll content in *Hibiscus sabdariffa* L. The results showed that both the IAA and GA_3 increased the plant height of okra. These finding are supported by the findings of Mukhtar (2008), who found that 100 mg/L GA_3 and IAA increased plant height of soybean and red sorrel applied during early seedling growth. Kaur et al. (2000) stated that enhanced plant growth by IAA and GA_3 may be mediated through changes in the activities of carbohydrate metabolism enzymes.

In general, it was found that application of IAA and GA_3 significantly increased okra growth, number of flowers, fruit size and fruit weight, and higher concentrations of 120 and 90 mg/L IAA and GA_3 were comparatively better than lower concentration. These results were parallel to those of Sarkar et al. (2002), who observed that GA_3 and NAA stimulate the fruit set of soybean when applied at 100 mg/L twice. IAA and GA_3 at 90 and 120 mg/L concentrations increased the flowering of okra at different application methods. Moneruzzaman et al. (2011) also reported that exogenous GA_3 increased the number of fruit, fruit weight and fruit quality of wax apple. On the other hand, Mekhled (2011) reported the IAA application at medium concentration (50 mg/L) promotes flowering and at higher concentration, inhibits the flowering process of okra. Awan and Alizai (1989), observed that GA_3 at 100 ppm increased seed yield in okra. Adel et al. (2011) reported that fruit quality also differred with cultural practices, growing conditions and cultivars.

Foliar spray of IAA significantly increased plant height and the number of branches, leaves, flowers and fruit weight and TSS content in the fruits. It was also observed that higher concentrations of IAA increased the chlorophyll content, stomatal conductance and aborted seed percentage and reduced healthy seed percentage. Similar effects was reported by Prajapati et al. (2015), who found that foliar application of auxin improved the growth and quality of various vegetable crops. It has been reported that IAA promotes GA biosynthesis and inhibits GA deactivation. Damian et al. (2002) stated that due to this double-barrelled effect, even moderate changes in IAA supply can lead to physiologically significant changes in GA content. They also found that IAA application induced the up-regulated expression of gibberellin biosynthesis gene and produced new wall polysaccharides so that growth may continue for longer periods. Auxin stimulates the activities of certain enzymes that are involved in biosynthesis of cell wall polysaccharides and cell wall loosening. Auxin initiates a signal transduction pathway resulting in production of secondary messengers that directly activate pre-existing H+-ATPases and stimulates the expression of several genes related to growth and development.

It has also been reported that foliar application of kinetin enhances flowering of and increases the leaf area and bract colour of the bougainvillea plant (Moneruzzaman et al., 2010). The results also showed that foliar application of GA_3increased the chlorophyll content, stomatal conductance and fruit weight of okra. Ilias et al. (2007) also reported similar results in that plant height, leaf area and biomass were significantly enhanced by the foliar application of GA_3. Ayyub et al. (2013) found that growth regulators through foliar application boosted stem elongation, number of leaves, chlorophyll content, number of pods, number of seeds, seed weight and seed yield. Spraying of GA_3 was observed to have a significant effect on the plant height of okra compared with the seed-soaking application technique (Unamba et al., 2009).

It was found that stem injection of a higher concentration of IAA and GA_3 improved the physiological characteristics of the okra plant and increased the yield parameters such as number of flowers, fruit size, fruit weight and TSS content of fruit. This improved yield and quality, probably due to the fact that GA_3 and IAA treatment might be linked to the efficiency of the photosynthetic apparatus, which leads to increase in plant productivity and quality (Azooz et al., 2004). Stem injection of IAA also increased the chorophyll content and stomatal conductance. This is contrary to what was reporte by Mekhled (2011), who found the effect of IAA on stem growth and other physiological activities of okra via stem injection of IAA and NAA to be insignificant. GA_3 stem injection significantly increased plant growth, number of flowers, fruit size and weight. It was also recorded that the GA_3 stem injection method increased healthy seed and reduced aborted seed percentage in okra pods. TSS content in the fruit and the chlorophyll content and stomatal conductance of the okra leaf were significantly increased using the stem injection method. Average plant height was observed via GA_3 stem injection. Khandaker et al. (2013) reported that localised application of GA_3 increased fruit development, fruit pigmentation and fruit quality of wax apple.

Flower injection of IAA significantly increased all physiological and reproductive parameters studied in this work. IAA at higher concentration (>90 mg/L) increased the physiological activities of the okra plant and increased the flowering and number of fruit. IAA flower injection also reduced the number of healthy seeds and increased the number of aborted seeds; this is probably the most important finding of this current study. In another study, it was reported that the application of 200 mg/L IAA decreased viable seed production (Sarkar et al., 2002). It was also observed that a lower concentration of IAA increased the chlorophyll content, fruit size, fruit weight and TSS content of okra fruit. It has been reported that application of IAA prevented the loss of chlorophyll throughout the ageing of chloroplasts. Shah (2011) reported that IAA application increased the net photosynthetic rate, leaf protein content and dry mass of black cumin.

Improved photosynthesis might increase the assimilates as well as the total soluble solid content in the fruit. For flower injection of GA_3, the same pattern of stimulatory effects was observed but plant height was not significantly affected. Mekhled (2011) stated that 200 mg/L IAA at flower injection and 100 mg/L NAA at ovary injection inhibited seed production and produced 100% aborted seeds in okra; this is known as stenospermocarpy. Comparing the three techniques of plant growth regulator applications, it can be summarised that flower injection is better than stem injection and spraying for improvement of okra production as suggested by Mekhled (2011), who stated that flower injection and ovary injection were better than stem injection.

CONCLUSION

It can be concluded that 120 mg/L IAA and 90 mg/L GA_3 were the best treatments for growth, development and quality of okra. Finally, it can be summarised that flower injection improved the pod quality of okra by reducing the healthy seeds and increasing the abortive seed percentage. Higher concentration of IAA (>90 mg/L) caused stenospermocarpy or reduced viable seed production. Flower and stem injection of IAA and GA_3 improved the physiological activities and quality of the okra plant. These two techniques can be used commercially in vegetable cultivation for improvement of quality. The flower and stem injection techniques also can reduce the use and cost of growth regulators and protect the environment from pollution due to foliar application.

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Anatomy and Histochemistry of Structures Producing Aroma in Leaves of *Sygyzium aromaticum* (L.) Merr. and *Clausena excavata* Burm. f.

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ABSTRACT

Anatomical and histochemical studies on leaves of *Syzygium aromaticum* and *Clausena excavata* have been carried out. This study was conducted in order to investigate the relationship between aroma production and a plant's secretory structures. Leaves from the two tropical aromatic plants were sampled from the Institute of Bioscience (IBS) Conservatory Park and transversely sectioned through lamina, midrib and petiole with a sliding microtome for anatomical investigation. Through light microscopy, oil cells and secretory cavities were distributed near the adaxial and abaxial epidermal layers with large in size, up to 60 µm length. Other leaf anatomical characters such as shape of petiole and midrib, pattern of vascular bundle, palisade and spongy mesophyll, the presence or absence of brachysclereids and crystals are also observed. This study also aimed to investigate the leaf's secretory structures responsible for plants' aroma production and to detect the presence of terpenes and essential oil in secretory structures histochemically.

Keywords: Aroma, secretory structures, terpenes, essential oils, oil cells, oil cavities

INTRODUCTION

Asia is well known throughout the world as the land of aromatic plants, a fame it enjoys due to its favourable climatic conditions, which are suitable for the

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growth and development of aromatic plants (Chomchalow, 2002). Aromatic plants contain volatile aromatic compounds, most of which make up the essential oils of plants. Since ancient times, aromatic plants have been the source of spices and herbs, traditional medicines as well as raw materials for essential oil extraction (Chomchalow, 2002; Joy et al., 2002). Brenes and Roura (2010) stated that a plant's secretory structures are found in every part of the plant including its leaves, flowers, fruit, bark and roots. Examples of secretory structures are oil glands, secretory cavities and glandular trichomes (Fahn, 1988; Svoboda, 2000; Cutler et al., 2008). In this study, two species of tropical aromatic plants, *Syzygium aromaticum* and *Clausena excavat*a, were studied.

Clausena excavata (Rutaceae) is classified as a small tree or shrub. It ranges in height as a shrub that is only 1-2 m tall to a small tree that can grow up to 10 m tall. The leaves appear as leaflets that are lanceolate to crescent-shaped, measuring 3-7 cm in length, while the fruit has a grape-like taste (Arbab et al., 2011). Recent studies of *C. excavata* have been restricted to investigating the chemical constituents of this medicinal plant and its contribution to antimicrobial, anti-fungal and anti-insecticide preparations (Guntupalli et al., 2012; Albaayit et al., 2015).

A previous study by Kamatou et al. (2012) recorded the presence of the chemical constituent, Eugenol, in the essential oil of *S. aromaticum* (Arras & Usai, 2001; Ayoola et al., 2008; Santos et al., 2009) and emphasised that Eugenol is the main volatile compound of extracted oil from clove buds that is used in traditional medicine, bactericides, fungicides and other preparations but no study was done on the secretory structures that produce the aromatic volatiles from the genus *Clausena*, especially *C. excavata*. Many studies on the leaf anatomy of Myrtaceae and Rutaceae have been conducted. Metcalfe and Chalk (1979) described the anatomical structure of the leaf as having basic cells such as the epidermis, mesophyll, vascular bundle, parenchyma, sclerenchyma that include specialised secretory cells. Later, histochemical studies enhanced the search for detailed information about plants' internal structures. Several studies had stated that members of Myrtaceae possess oil cavities or oil glands in their leaves (Fahn, 1988; Arruda & Victorio, 2015; Dickson, 2000). Khatijah and Ruzi (2006) revealed the presence of oil glands in the transverse section of the lamina, midrib and petiole of *S. aromaticum*. Al-Edany and Malik (2012) reported that secretory oil cavities are in the lamina near to both the adaxial and abaxial surfaces.

Essential oils are distributed at different parts of the plant including the leaves, flowers, fruit, bark and roots (Chamchalow, 2002; Chamorro et al., 2012). The leafy part of the plant possesses external secretory structures such as glandular trichomes, resin ducts or canals, whereas the internal secretory structures include lacticifers and oil cells (Cutler et al., 2008). Other studies describe secretory cells in the leaf

as idioblasts containing a variety of oil and mucilage as specialised cells contributing to the scent of the plant (Dickison, 2000). Glandular trichomes were found to secrete a number of secondary metabolites of terpenes, flavonoids, alkaloids and essential oils (Svoboda and Svoboda, 2000; War et al., 2012).

The essential oils were found accumulated at the sub-cuticular cavity of glandular trichomes. The oils diffuse outwards through the cuticle, covering the outer surface of the hair gland after the rupture of the cuticle (Svoboda and Svoboda, 2000). According to Dickison (2000), essential oils that are produced in glandular trichomes carry discrete aroma and taste to the plant part possessing them. Secretory cells are known as cells specialised to secretion of one or more, often organic substances. Cutler et al. (2008) stated that secretory cells can be single cells, groups of cells or even tissue. Morphologically, they are usually larger than the surrounding elements and sometimes resemble enlarged, densely staining parenchyma cells. Secretory cavities are spherical intercellular spaces that are lined with secretory cells and filled with secretory products including essential oils (Dickison, 2000; Svoboda & Svoboda, 2000). However, other published studies have reported the use of histochemistry to detect and locate the active components within the plant cells such as terpenoids, lipids, carbohydrates and proteins (Gersbach et al., 2001; Dubey & Trivedi, 2012; Bosabalidis, 2014; Hassan & El-Awadi, 2013). Greathead (2003) reported the oil itself is a complex mixture of secondary metabolites comprising low-boiling point and molecular weight of phenylpropenes and terpenes. Several studies on aromatic plants affirmed the presence of essential oil as the contributing factor in such essence of these plants (Joy et al., 2001; Figueirdo et al., 2008; Chamorro et al., 2012).

Studies on leaf anatomy with special reference to secretory structure in genera *Syzygium* and *Clausena* are very few. Although the leaves of *S. aromaticum* and *C. excavata* are well known for their applications of essential oil, few studies have identified the anatomical structures that produce and secrete such aroma from these plants. This study examined the relationship of the cells responsible for producing and secreting aroma as well as the histochemical identification of the secreted materials within a plant's leaf and, furthermore, the histochemical analysis determined the chemical compounds of terpenes and essential oils within the secretory structures, vascular bundle and parenchyma cells, and terpenes in the essential oil of the plants have proven to be responsible for the aroma of the two species studied.

Histochemical Study

Wick (2012) and Hassan and El-Awadi (2013) described several applications of histochemistry in plant research, which includes the detection and localisation of cellular components of active constituents. The constituents include protein, lipids, carbohydrate as well as a range of ionic elements occurring in cell solutions. Over

the past decades, a variety of chemical reagents have been used in the study of plant histochemistry. Johansen (1940) successfully came up with some such as concentrated sulfuric acid (H_2SO_4), ferric chloride ($FeCl_3$) and ruthenium red. Later, Jensen (1962) introduced reagents such as Nile Blue and Schiff's reagent following Sudan III, Sudan IV, Sudan Black B (Lison, 1960), Nadi reagent (David & Carde, 1964), Vanilin-HCl (Guerin et al., 1971) and many more. In the colour-staining technique of histochemistry, specific chemical reagents were used to test different compounds within a cell through the change in colouration (Table 1). Bakker et al. (1992) studied the distribution and systematic value of two secretory structures (idioblast) in reference to oil cells and mucilage cells for the genus *Cinnamomum* Schaeffer (Lauraceae). Later, Geng et al. (2012) performed a histochemistry test on oil cells. They localised the main chemical classes of metabolites present in the oil such as aldehydes, lipids and terpenoids. Besides the histochemistry of oil and mucilage cells, a number of other secretory structures captured the interest of researchers such as glandular trichomes.

Gersbach et al. (2001) studied the peltate and capitate glandular trichomes distributed over the adaxial and abaxial leaf surface. In addition, Christodoulakis et al. (2013) studied the localisation of secreting sites as well as the identification of the secreted material in the leaf of a Mediterranean aromatic plant. The study of histochemistry was not restricted to leaf secretory structures only, but was also applicable for secretory structures present in the flower, stem and root. Sajwan et al. (2014) revealed the presence of oil glands and clusters of calcium oxalate crystals in the parenchyma cells of the hypanthium of *Syzygium aromaticum*. They later verified a positive test on the glands containing oil globules with red colouration when stained with Sudan III.

MATERIALS AND METHOD

This study aimed to investigate the secretory structures involved in aromatic leaves. Fresh leaf samples were fixed in (FAA), then sectioned transversely (TS) through the middle part of the midrib, lamina and petiole using a sliding microtome, stained with safranin and alcian blue, dehydrated in a series of alcohol solutions and mounted in Euparal. For the histochemical study, sections were submitted to NADI reagent for detection of terpenoid compounds and Sudan Black B for detection of essential oil cells. All laboratorial procedures were conducted at the laboratory of the Biology Department, Faculty of Science (UPM) and the Laboratory of Anatomy and Microtechnique, Faculty of Science and Technology (UKM). Aromatic plants of *S. aromaticum* and *C. excavata* were sampled from the Conservatory Park, Institute of Bioscience, Universiti Putra Malaysia.

Preparation of Specimens

Preparing specimens before the sectioning process is very important for obtaining successful results. In this experiment, the fresh specimens were prepared into two main categories. One was for the leaf

anatomy study, which used fixed samples, and another for the histochemical study that directly used fresh samples with no fixation. The fixatives used in this experiment were FAA (Formalin-Acetic acid-Alcohol). The FAA mixture follows methods by Metcalfe and Chalk (1979) with 90 ml of 50% alcohol, 5 ml of 99.9% acetic acid and 5 ml of 40% formalin. Upon outdoor sampling for leaf anatomy, each leaf sample was placed in a re-sealable plastic bag filled with FAA for structural preservation. On the other hand, another batch of leaf samples was separately soaked in distilled-watered plastic bags and submitted for histochemical study.

Leaf Anatomy Investigation

Transverse sections of the lamina, midrib and petiole illustrated the anatomy and secretory structures of the two species. The method of study followed the standard method derived from Johansen (1940), Jensen (1962) and Metcalfe and Chalk (1979) with slight modifications from Khatijah and Ruzi (2006), Cutler et al. (2008) and UKM Anatomy Laboratory technicians and staff. Leaf sections of *S. aromaticum* and *C. excavat*a were hand-cut into 1 cm^2 pieces using a razor blade. The chosen part of the leaf was then manually embedded in hard polystyrene before transversely-sectioned using a sliding Microtome Reichert (model Leica Jung histoslide 200) of 15-25 µm thickness according to the plant sample (Soukup and Tylova, 2014). The sections were cleared by soaking in 20% sodium hypochlorite (NaHCl) until they turned a white colour. The sections were rinsed two to three times with distilled water before being submitted to staining procedure with Safranin and Alcian Blue. Dehydration sections were treated in a series of alcohol of increasing concentration. The sections were mounted on a microscopic slide in Euparal as permanent medium. Finally, the slides were left to dry in an oven at temperature of 60°C for two weeks. The slides were observed and viewed under light microscope (model: Olympus CH20).

Histochemical Investigation

Similarly, the leaf lamina, midrib and petiole of two species were transversely sectioned using the sliding microtome as described above and then directly submitted to the following reagents: NADI reagent for the detection of terpene compounds and Sudan Black B for the detection of essential oil and total lipids (Machado et al., 2006).

Staining Process of Histochemistry of Leaf Sample

Two types of staining process were carried out in this study. The first stain used was the NADI reagent: 5 drops of NADI reagent (5 g of α-naphtol in 125mL of 50% ethanol + 5 g of N,N-dimethyl in 125mL of phosphate buffer of pH 7) were dropped onto tissue sections in a Petri dish for 15 min. The tissue sections were rinsed twice with distilled water, then transferred onto a glass slide using a camel brush and covered with a cover slip. Observations were made under a light microscope (model: Olympus CH20). The second stain was Sudan Black B: 5

drops of Sudan Black B (500 g SBB in 20 mL acetone + 15 mL AA + 35 mL d/w) were dropped onto tissue sections in a Petri dish. The tissue sections were rinsed twice with distilled water and were then transferred onto a glass slide using a camel brush and covered with a cover slip. Observations were made under a light microscope (model: Olympus CH20). The images of the sample slides were captured using a Leitz Diaplan light microscope at magnification 4X, 10X, 20X and 40X.

Table 1
List of common chemical reagents used in plant histochemistry

Chemical Reagents	Target Compounds	Observed Colour
Sudan III & Sudan Black B	Total lipids/Essential oil	Orange red/Black
Mix of Sudan III & IV	Suberin	Red
Conc. H_2SO_4	Sesquiterpene	Orange
Ferric chloride	Polyphenols/Phenolics	Emerald-green
Nile Blue	Neutral/Acidic lipid	Red/Blue
Vanilin-HCl	Flavanoids	Yellow
Ruthenium Red	Acid Polysaccharides/Mucilage/Pectin	Red/Pink
Schiff's reagent	Water insoluble polysaccharides/Aldehydes	Magenta-red

RESULTS AND DISCUSSION

The morphology of leaf anatomy described includes the shape of the petiole, pattern of vascular bundle, cuticle, abaxial and adaxial epidermis, palisade and spongy mesophyll, ground tissue, secretory (idioblast) cells, crystals and trichomes. The leaf's anatomical characteristic is described based on Melcalfe and Chalk (1979), Bakker et al. (1992), Baruah and Nath (2006), Cutler et al. (2008), Arruda and Victoria (2011), Muntoreanu et al. (2011), Al-Edany and Malik (2012) and Geng et al. (2012).

Anatomical Features of *Syzygium aromaticum*

Petiole. The petiole outlines in transverse sections are U-shaped extending outwards in adaxial surface, whereas the abaxial surface is generally U-shaped (Figure 1A). There was an abundance of secretory cavities (up to 20) present in the ground tissue near the epidermis (Figure 1A and 1E). The general vascular system of the petiole was open type and crescent-shaped with intraxylary phloem and brachysclereid (Figure 1B). The parenchyma cells were observed to be of more than 10 layers. Idioblast tannin was present in parenchyma cells, appearing black in colour. Also present were druse and solitary crystals (Figure 1C).

Midrib. Based on observation, the adaxial surface was straight with an arc-shaped abaxial surface (Figure 3A). Secretory cavities were present in the ground tissue near the abaxial and adaxial surface (Figure 3A). *Syzygium aromaticum* has an open type and V-shaped vascular tissue with

intraxylary phloem. Sclerenchyma cells were present around the vascular bundle (Figure 3A). Druse and solitary cuboidal crystals were present in the parenchyma cells (Figures 3B and 3C).

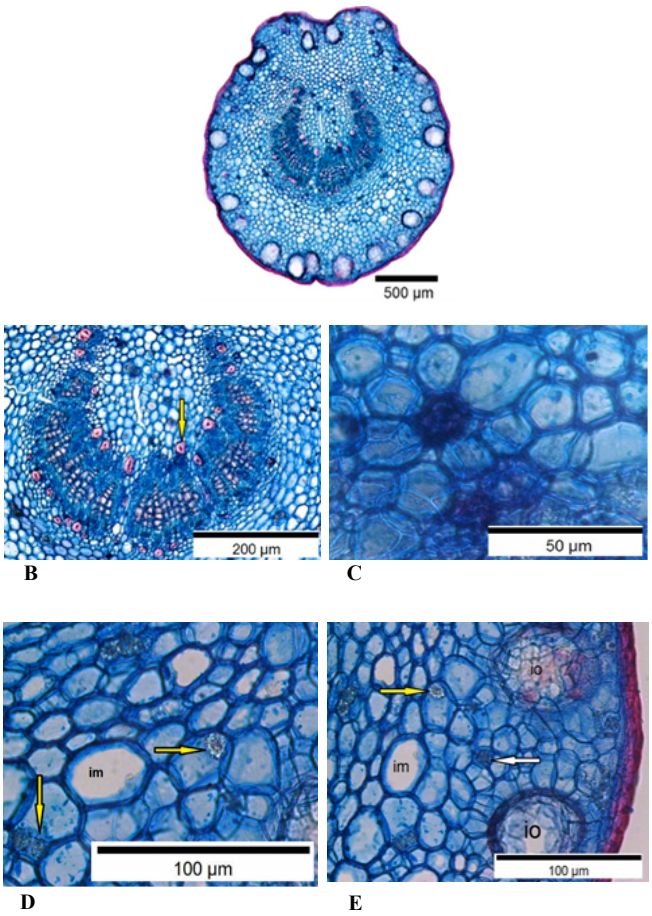

Figure 1. Transverse section of petiole *Syzygium aromaticum* (A) Sub-circular external shape of petiole, (B) Open-type vascular bundle with presence of intraxylary phloem; brachysclereid cell presence (yellow arrow); (C) Idioblast tannin cell; (D&E) Idioblast mucilage cell (im); Idioblast oil cells (io); Druse crystal (yellow arrow) and solitary crystal (white arrow) in parenchyma cell

Lamina. Based on observation, the adaxial epidermis was thicker than the abaxial epidermis. Secretory cavities were present between the palisade and spongy mesophylls (Figure 4A and 4D). The lamina also had an open-type vascular tissue (V-shaped) with intraxylary phloem (Figure 4A). In the parenchyma cells, solitary and druse crystals were present (Figure 4B and 4C).

Anatomical Features of *Clausena excavata*

Petiole. The petiole outlines in transverse section had an irregular shape of the adaxial

surface and circular abaxial surface (Figure 2A). The vascular system was organised in a open-free vascular bundle (Figure 2A). The trichomes were unicellular and multiseptate, present around the petiole especially on the abaxial epidermis (Figures 2A and 2F). Secretory cavities were present in the ground tissue near the epidermis, and were observed to be obviously larger in size than the adjacent cells in the mesophyll (Figures 2A and 2E). Idioblast tannin stained dark red-black was present in the vascular bundle (Figure 2C). Also, brachysclereid cells occurred above the vascular bundle as well as in between the cells towards the abaxial of the petiole (Figure 2B). Solitary crystals with a diamond and cuboid shape were present in the parenchyma cell (Figures 2B and 2 E).

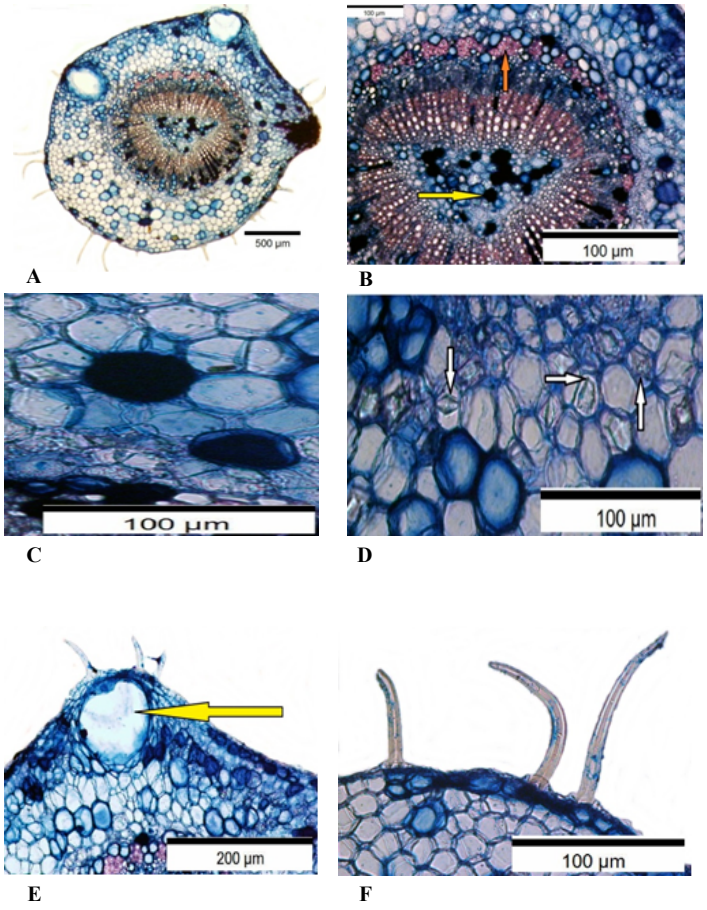

Figure 2. Transverse section of petiole *Clausena excavata* (A) Circular external shape of petiole and pattern of vascular bundle; (B) Sclerenchyma cells (brown arrow) above the vascular bundle; tannin cells (yellow arrow) in the vascular bundle; (C) Idioblast tannin in ground tissue with black colour; (D) Solitary crystals in parenchyma cells; (E) Idioblast oil cell (yellow arrow) near epidermis; (F) Unicellular (unbranched) and multiseptate trichomes

Midrib. Based on observation, both adaxial and abaxial surfaces had a convex shape (Figure 3E). The midrib had close-type vascular tissue surrounded with pericyclic fibres (Figure 5G). In the parenchyma cells, solitary rhombic crystals were found present (Figure 5F). Unicellular and non-glandular trichomes were present on the abaxial epidermis (Figure 5G). Secretory cavities were found in the ground tissue below the vascular bundle and near the epidermis (Figure 5H).

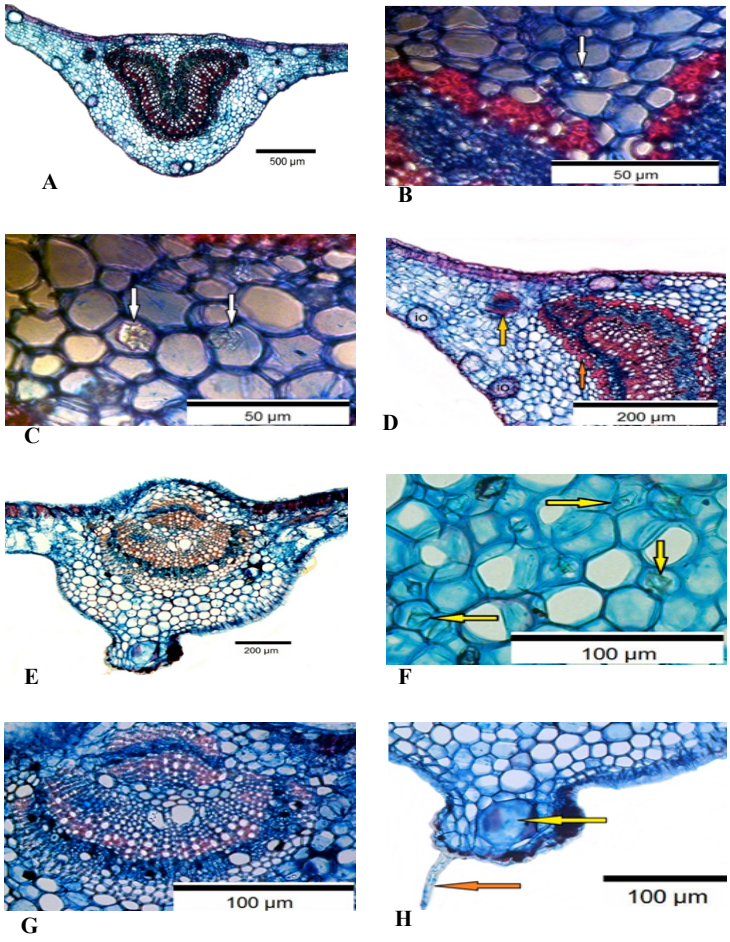

Figure 3. Transverse section in midrib of *Syzgyium aromaticum* (A) External shape of midrib and pattern of vascular bundle; open vascular tissue (V-shaped) with intraxylary phloem; (B&C) Solitary (cuboid shape) and druse crystals (white arrow) present in parenchyma cells; (D) Idioblast (oil) cells (io) in ground tissue near the epidermis; secondary vascular bundle (yellow arrow), sclerenchyma cells on vascular bundle (brown arrow), Transverse section in midrib of *C. excavata*; (E) External shape of midrib; (G) Pattern of vascular bundle; (F) Single rhombus crystal in mesophyll/parenchyma cell; (H) Idioblast (oil) cell (yellow arrow) in ground tissue near adaxial epidermis, unicelluar, multiseptate trichomes on adaxial epidermis.

Lamina. Based on observation, the adaxial epidermis was thicker than the abaxial epidermis with the presence of unicellular trichomes on both sides (Figures 4D, 4E and 4G). Large secretory cavities existed below the epidermis in between the palisade and spongy mesophyll (Figures 4D and 4F) and solitary crystals were present in the parenchyma cells (Figure 4H).

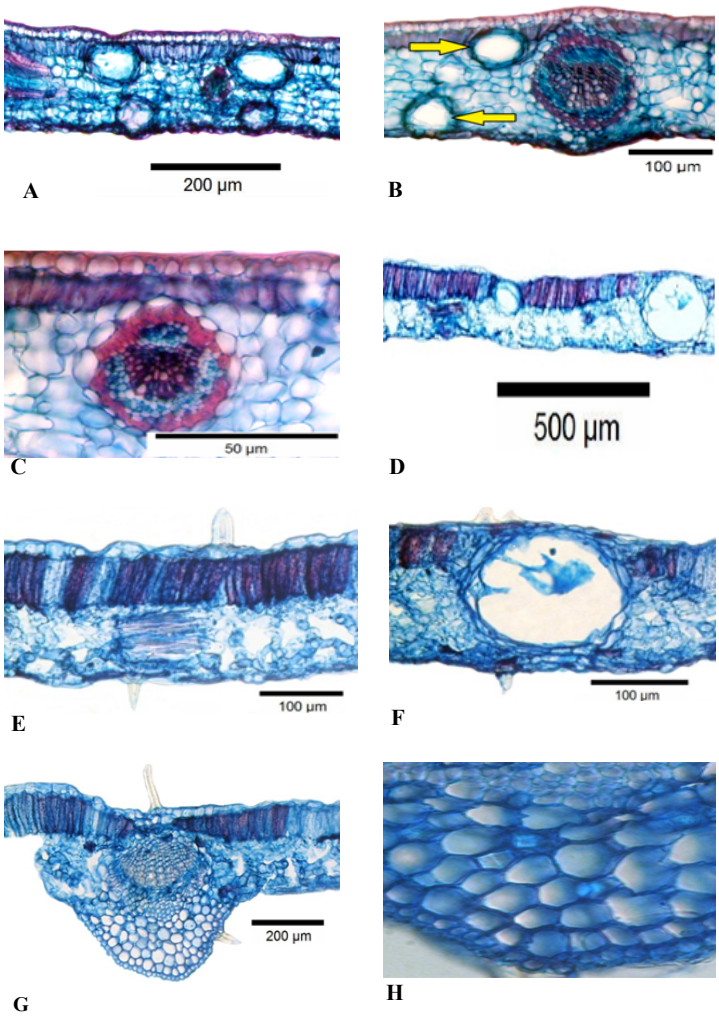

Figure 4. (A) Transverse section of lamina in *Syzygium aromaticum*; (B) Idioblast (oil) cells (yellow arrow) in palisade and spongy mesophyll; (C) Main vascular bundle with sclerenchyma tissue present; (D) Transverse section of leaf lamina *Clausena excavata*; (E) Unicellular (single) multiseptate trichome (brown arrow); (F) Idioblast oil cells in mesophyll; (G) Main vascular bundle; (H) Solitary crystal in mesophyll parenchyma (yellow arrow)

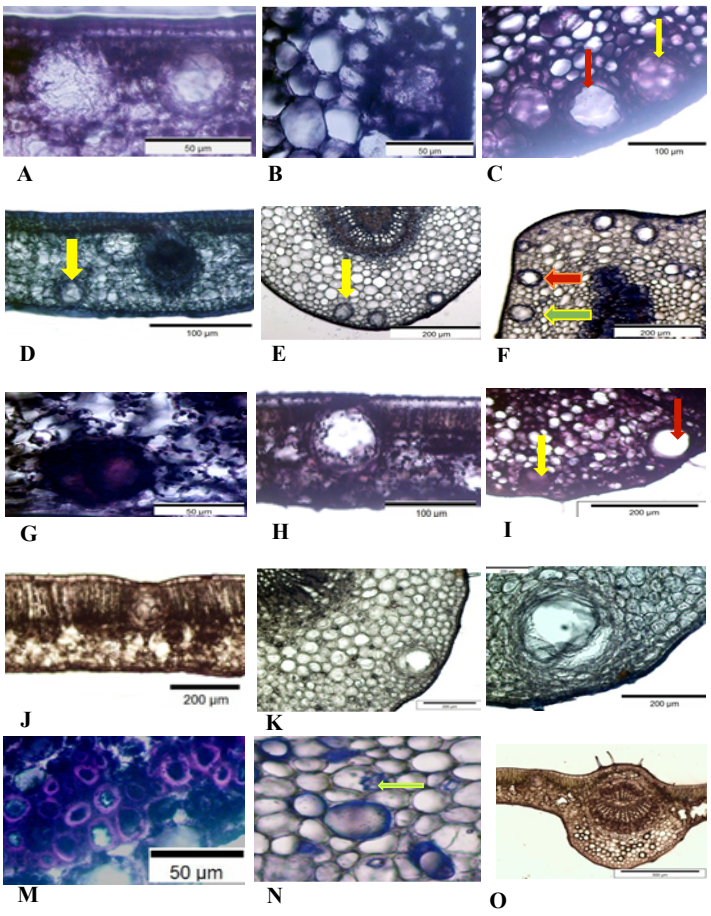

Figure 5. Photomicrographs (LM) of specimens stained with NADI reagent and Sudan Black (A-F) *Syzygium aromaticum*; (A&D) Lamina; (B&E) Midrib; (C, F) Petiole; notice full accumulation of terpenes (yellow arrow) and emptied oil cavity (brown arrow); (G-L) *Clausena excavata*; (G) Midrib; notice intense purple colouration; (O) Midrib; notice absence of oil cavity, (H, J) Lamina; black colouration; (I, K, L) Petiole; notice full accumulation of terpenes (yellow arrow) and emptied oil cell (brown arrow); (M) Sclerenchyma cells of *C. excavata* stained pinkish-violet in petiole; (N) Druse crystals of *S. aromaticum* stained blue black (green arrow) in petiole

Anatomical Analysis

The presence of secretory structures is one of the characteristics for Myrtaceae and Rutaceae (Metcalfe & Chalk, 1979). It was found that *S. aromaticum* possessed secretory cavities that secrete oil in the leaf. Morphologically, the cavities are glandular, spherical or elliptical in shape, larger than neighbouring cells and surrounded with parenchyma cells. This finding was supported by previous studies on the secretory cavities present in *S. aromaticum* (Fahn, 1988; Khatijah & Ruzi, 2006; Al-Edany & Malik, 2012; Arruda & Victorio,

2015). Although earlier studies had similar findings, they mentioned little about the distribution of the secretory cavities and the specific leaf part in which the structures were present. In the present study, the secretory cavities were found located abundantly in all parts of the leaf involving the lamina, midrib and petiole.

Clausena excavata was found to possess secretory cavities as its secretory structure in Rutaceae (Groppo et al., 2008). The glandular cavities were capitate with a unicellular stalk surrounded by secretory cells and located near the epidermis in the petiole (Figure 2). The transverse section of the midrib showed the presence of multiseptate trichomes on the adaxial epidermis (Figures 3E-H). According to Metcalfe and Chalk (1979), members of Rutaceae have both glandular and non-glandular trichomes. However, only non-glandular trichomes are found abundantly on the abaxial epidermis. This shows that certain characteristics may occur in only some members of the family and not necessarily in every genus and species.

Histochemistry Analysis

Through histochemical testing, most secretory cavities showed positive reaction to terpenes, indicating that it does accumulate within the structure. A previous study by Sajwan et al. (2014) proved the presence of oil globules located in cavities of the hyphantium region of *S. aromaticum* when tested with Sudan III. This work revealed that the oil cavities not only existed in the flower but also in the leaf of *S. aromaticum*. In addition, the oils in the cavity could also be tested using other staining reagents not necessarily Sudan III but NADI reagent and Sudan Black B. Therefore, the findings showed that the existence of volatile terpenes and essential oil in the cavity might be the contributing factor to the production of leaf aroma (Tables 2 and 3).

The absence of terpenes and essential oil in the midrib of *C. excavata* is related to the fact that the oil cells and cavity were not filled with these secreted substances. Based on observation, all the empty oil cells and cavities were colourless and unstained when treated with staining reagents. Only the area surrounding the oil cells or cavities were stained dark, whereas the centre area was brighter under transmitted light (Figures 5C, F, H, I, K, J). This shows that cells surrounding the cavity contained terpenes and lipids but not the inside of the structure. A similar observation was previously recorded by Bakker et al. (1992) when they stained oil cells using Alcian blue. The absence of essential oil in the midrib of C. excavata was due to the absence of secretory cavities in that region.

The application of NADI reagent onto each plant part not only revealed the secretory structures to be stained purple violet, but also included other cells. This can be seen in the TS of the *C. excavata* petiole where the sclerenchyma cells were also stained pinkish purple (Figure 5M). Also, the application of Sudan Black B gave a blue-black colouration to the druse crystals found in the parenchyma of *S. aromaticum* (Figure 5N). Black and violet colouration

could also be seen on the cuticle located above the adaxial layer of both of species observed. Such colouration shows that terpenes and lipids also accumulate within ground tissue and not necessarily in the secretory structures. Although druse crystals are classified as ergastic substances whose function is not yet known, it is possible that they contribute to the production of aroma in plants.

Table 2
Summary of the secretory structures in the leaves of S. aromaticum and C. excavata

Aromatic Plants	Plant Parts	Secretory Structures		
		Secretory Cavity	Secretory Cell	Glandular Trichome
S. aromaticum	Lamina	+	-	-
	Midrib	+	-	-
	Petiole	+	-	-
C. excavata	Lamina	+	-	-
	Midrib	+	-	-
	Petiole	+	-	-

Notes: +: present, - : absent

Table 3
Histochemistry of secretory structures in the leaves of S. aromaticum and C. excavata.

Aromatic Plants	Plant Parts	Compounds Investigated	
		Terpenes	Essential Oil
S. aromaticum	Lamina	++	++
	Midrib	+++	++
	Petiole	+++	+
C. excavata	Lamina	+	++
	Midrib	+++	-
	Petiole	+++	+

Notes: - : negative, + : positive, + (low), ++ (medium), +++ (high) indicating the vividness of stained colour

CONCLUSION

In this study, the morphology and anatomy of the secretory structure of two plants were determined using light microscopy. Secretory cavities were found to be the source of aroma production in *S. aromaticum* and *C. excavata*. The substances accumulated in the secretory structure were detected using histochemical testing. Terpenes, responsible for the aroma of *S. aromaticum* and *C. excavata*, were found in the secretory cells and secretory cavities in their leaves. Essential oils were found accumulated in the secretory cavity of S. *aromaticum* and *C. excavata*. Hence, the presence of either one of the volatile oils indicates they are the

source of aroma in these tropical aromatic plants. The findings of this work can be strengthened by conducting a chemical analysis of the essential oil obtained from both species.

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GC-MS Analysis of Phytochemical Compounds in Aqueous Leaf Extract of *Abrus Precatorius*

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ABSTRACT

Abrus precatorius is a flowering plant that belongs to the legume family, Fabaceae. In Malaysia, the leaves of *Abrus precatorius* are used traditionally to treat ailments such as fever, ulcer and mouth cancer. These traditional practices, however, have never been documented and usage of the plant is based on popular beliefs held by the local people. This work documented the phytocompounds that are present in the aqueous extract of *Abrus precatorius* leaves collected from a local area in Kota Bharu, Kelantan, Malaysia. The leaves were dried and then subjected to extraction using the decoction technique. The compounds were identified by gas chromatography with mass spectrometry analysis and characterised by comparison through the NIST02 and Wiley275 library search software. The GC-MS analysis showed that the classes of compounds identified in aqueous extracts of *Abrus precatorius* leaves were phenolic compounds, terpenoids and steroids.

Keywords: Abrus precatorius, aqueous extract, GC-MS

INTRODUCTION

Medicinal plants are widely used as food and medicine in traditional practice. In Malaysia, such plants are consumed as an alternative treatment for illness or to maintain a healthy lifestyle. A huge reservoir of bioactive compounds exists in over 400,000 species of plants on Earth, but only a small percentage of these compounds have been examined in research. In many developed countries,

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plant products used as Complementary and Alternative Medicine (CAM) are popular. In Malaysia, as reported in 2002, the use of CAM added up to US$500 million in cost, annually, compared to about the use of allopathic medicine, which added up to only US$300 million in cost (World Health Organisation, 2002). Medicinal plants are widely used in human and veterinary therapy, agriculture, scientific research and countless other areas. The local people generally use plants and herbs as an alternative for curing and treating various diseases and ailments.

Abrus precatorius is a flowering plant that belongs to the legume family, Fabaceae. The common names of *Abrus precatorius* include jequirity, Crab's eye, Rosary pea, precatory pea or bean, John Crow Bead, Indian licorice, 'Akar saga' and jumble bead. Phenotypically, this plant is characterised as a slender, perennial climber that twines around trees, shrubs and hedges.

Abrus precatorius is native to India and grows mostly in tropical and subtropical parts of the world. In traditional Hindu medicine, it has been used since ancient times in some regions to treat mouth ulcer by chewing the leaves. The plant was also used by other ancient cultures, including China. The leaves can also be used as a nerve tonic as well as to treat wounds and swellings due to its anti-inflammatory properties. Oil extracted from *Abrus precatorius* seeds can be used to promote hair growth, while the roots are used for treating jaundice, gonorrhoea and haemoglobinuria (Samy, Thwin, Gopalakrishnakone, & Ignacimuthu, 2008). In Malaysia, the leaves of *Abrus precatorius* are used traditionally to treat ailments such as fever, ulcer and mouth cancer. These traditional practices, however, have never been documented and so, usage of the plant is based only on popular beliefs held by the local people.

Decoction of the leaves is widely practised as a treatment for cold, coughs and colic. Juice from the leaves is applied to swellings after mixing with oil. Additionally, a mixture of rice starch and paste made from the leaves of this plant can be consumed orally to treat anthrax attacks (Pokharkar, Saraswat, Bhavare, & Kanawade, 2011). As a powdered leaf paste, the plant can also be used to treat conjunctivitis and convulsion in children (Joshi & Tyagi, 2011).

Studies undertaking phytochemical analysis of the leaves and roots of A*brus precatorius* have demonstrated the presence of glycyrrhizin (Karwasara, Jain, Tomar, & Dixit, 2010), an important compound of liquorice (Killacky, Ross, & Turner, 1976), which is widely used in the food and pharmaceutical industries. A known triterpenoid and three novel triterpenoids were identified from the acid hydrolysed methanol-soluble leaf extract (Kim, Kim, & Kinghorn, 2002) of *Abrus precatorius*. From the *n*-butanol leaves extract of *Abrus precatorius*, other compounds identified were abrusoside A (Choi, Hussain, Pezzuto, Kinghorn, & Morton, 1989), abrusosides B, C and D plus three other sweet glycosides based on the novel cycloartane-type aglycone, abrusogenin (Kinghorn & Soejarto, 2002).

Most of the studies done on *Abrus precatorius* were carried out in India (Solanki & Zaveri, 2012). Though Malaysia has an abundance of useful herbs and medicinal plants, less is known about *Abrus precatorius* in Malaysia. The aim of this study was to identify the phytochemical compounds of the aqueous leaf extract of *Abrus precatorius* by gas chromatography-mass spectrometry analysis.

METHODOLOGY

Plant Collection

Abrus precatorius leaves were collected from Kampung Sabak, Pengkalan Chepa, Kelantan, Malaysia and authenticated by Dr. Rahmad Zakaria from the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia. The voucher specimen (USM 11730) was submitted for future reference.

Preparation of Extract

The leaves of the *Abrus precatorius* were oven-dried at 50°C and macerated to fine powder using a mechanical grinder. The decoction method, which is used in traditional medicine, was applied in this experiment. An amount of 11 g of dried leaves ground to a fine powder was soaked in 450 ml water at 50°C until the water reduced to one third the initial volume. The extract was then freeze-dried for subsequent analysis.

Gas Chromatography-Mass Spectrometry (GC-MS)

A Hewlett Packard 6890 Gas Chromatograph with a 5973N Mass Selective Detector was used to carry out the GC-MS. The column was fused silica capillary, HP-5 column (30 m x 0.25 mm i.d x 0.25 µm film thickness) (Agilent Technologies, USA). The carrier gas was helium with a flow rate of 1.0 ml/min with the oven temperature programmed from 50°C (held for 2 min) to 280°C (held for 10 min) at a rate of 20°C/min. The injection and interface temperatures were set at 250°C and 280°C, respectively. A 1-ml sample was injected in splitless mode and was analysed in MS full scan mode (*m/z* 40-650). The electron ionisation was fixed at 70eV. Acquisition of data was performed using the Chemsation software.

Identification of Phytochemical Compounds

The mass spectrum of the GC-MS was interpreted based on the database of the National Institute of Standards and Technology (NIST02) and Wiley275 libraries with matches of ≥80 % to identify the phytochemical compounds.

RESULTS

The GC-MS analysis showed that the classes of compounds identified in aqueous extracts of *Abrus precatorius* leaves were phenolic compounds, terpenoids and steroids. The

GC-MS chromatogram obtained is given in Figure 1. Seventeen chemical compounds were identified, as shown in Table 1. The main class of compounds identified was phenolic compounds (2.82%). Four phenolic compounds were identified, with the major phenolic compound being 4-vinylphenol (1.17%). Other major compounds that were found in the Abrus precatorius leaves were methyl jasmonate (1.89%), decylenic alcohol (1.46%) and cis-11-Tetradecen-1-ol (1.41%). Methyl jasmonate is categorised as a fragrance that belongs to the structural groups, ketones cyclopentanones and cyclopentenones (Scognamiglio, Jones, Letizia, & Api, 2012). Decylenic alcohol also belongs to the fragrance group; it is also known as Rosalva. The chemical structure and molecular weight of each identified compound are listed in Table 2.

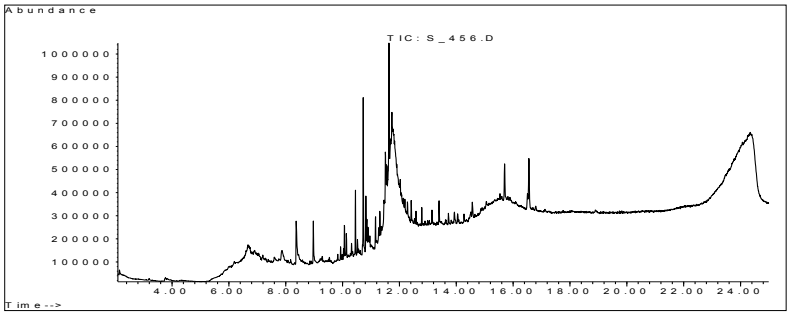


Figure 1. GC-MS Chromatogram of aqueous leaf extract of *Abrus precatorius*

Table 1
Compounds found present in the aqueous leaf extract of Abrus Precatorius using GC-MS

No	Retention Time (min)	Name of Compound	Area	*Therapeutic activity
		Phenolic Compound		
1	8.4	4-vinylphenol	1.17%	No activity recorded
2	8.9	p-Vinylguaiacol	0.68%	No activity recorded
3	10.1	β-Phenoxyethyl iso-butyrate	0.47%	No activity recorded
4	11.2	Cinnamaldehyde, β-hexyl-	0.50%	No activity recorded
		Terpenoids		
5	12.6	Phytol	0.39%	Anti-microbial, anti-cancer, anti-inflammatory
		Steroids		
6	16.5	Stigmasterol	0.31%	Anti-hepatotoxic, anti-inflammatory, anti-nociceptive, anti-ophidic, anti-viral, cancer preventive, ovulant, sedative
		Others		
7	7.8	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	0.66%	No activity recorded
8	9.8	6-Tridecene	0.13%	No activity recorded

Table 1 *(continue)*

9	9.9	β-lonone	0.17%	Anti-bacterial, anti-tumour, fungicide, pesticide, trichomonicide
10	10.7	Methyl dihydrojasmonate	1.89%	No activity recorded
11	10.9	Propanoic acid,2-methyl-3-[4-t-butyl]phenyl-	0.68%	No activity recorded
12	11.5	Decylenic alcohol	1.46%	No activity recorded
13	11.5	3-Decen-1-ol, (E)-	1.07%	No activity recorded
14	11.6	cis-11-Tetradecen-1-ol	1.41%	No activity recorded
15	13.7	Palmitic acid á-monoglyceride	0.19%	No activity recorded
16	15.7	1-Heneicosanol	0.72%	No activity recorded
17	16.6	1-Heptacosanol	1.17%	No activity recorded

**Source of reference:* Dr. Duke's Phytochemical and Ethnobotanical Databases, 1992-2016

Table 2
Compounds found present in the aqueous leaf extract of Abrus Precatorius using GC-Ms

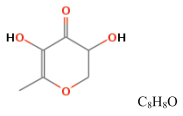
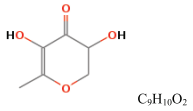
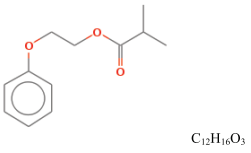
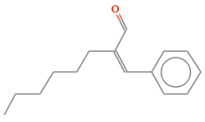
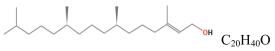
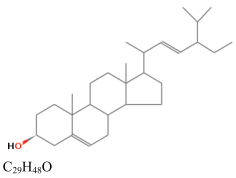
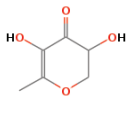
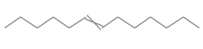
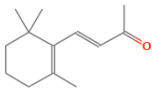
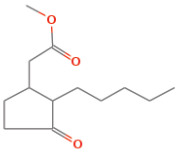
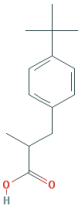
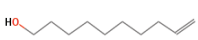
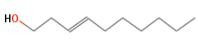
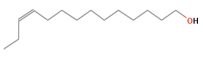
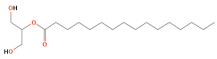
No	Name of Compound	Molecular Weight (g/mol)	Chemical Structure
1	4-vinylphenol	120.15	 C_8H_8O
2	p-Vinylguaiacol	150.17	 $C_9H_{10}O_2$
3	β-Phenoxyethyl iso-butyrate	208.25	 $C_{12}H_{16}O_3$
4	Cinnamaldehyde, β-hexyl-	216.32	 $C_{15}H_{20}O$
5	Phytol	296.53	 $C_{20}H_{40}O$
6	Stigmasterol	412.69	 $C_{29}H_{48}O$

Table 2 *(continue)*

No	Name of Compound	Molecular Weight (g/mol)	Chemical Structure
7	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	144.13	 $C_6H_8O_4$
8	6-Tridecene	182.35	 $C_{13}H_{26}$
9	β-lonone	192.30	 $C_{13}H_{20}O$
10	Methyl dihydrojasmonate	226.31	 $C_{13}H_{22}O_3$
11	Propanoic acid,2-methyl-3-[4-t-butyl]phenyl-	220.31	 $C_{14}H_{20}O_2$
12	Decylenic alcohol	156.27	 $C_{10}H_{20}O$
13	3-Decen-1-ol, (E)-	156.27	 $C_{10}H_{20}O$
14	cis-11-Tetradecen-1-ol	212.37	 $C_{14}H_{28}O$
15	Palmitic acid á-monoglyceride	330.50	 $C_{19}H_{38}O_4$
16	1-Heneicosanol	312.57	 $C_{21}H_{44}O$
17	1-Heptacosanol	396.73	 $C_{27}H_{56}O$

DISCUSSION

Phytochemical studies of the aqueous leaf extract of *Abrus precatorius* are still limited as they have only been able to identify the polar compounds. The composition of the compounds identified in the aqueous extract of *Abrus precatorius* leaves comprises a complex mixture of several classes of components, mainly phenolic compounds, terpenoids and steroids. A

study by Hussain and Kumaresan (2014) showed that only phenolic compounds and steroids were present in aqueous leaves extract of *Abrus precatorius*. Other studies have indicated that the solvent extract from *Abrus precatorius* leaves is rich in alkaloids, carbohydrates, steroids, phenolic compounds and terpenoids (Gul, Ahmad, Kondapi, Qureshi, & Ghazi, 2013; Hussain & Kumaresan, 2014; Yonemoto, Shimada, Gunawan-Puteri, Kato, & Kawabata, 2014).

One study of *Abrus precatorius* leaves showed that their biological activities were related to their active compounds such as terpenoid and phenolic compounds. Yonemoto et al. (2014) found that terpenoids isolated from *Abrus precatorius* leaves had an α amylase inhibitory effect, which is one of the therapeutic approaches for preventing diabetes mellitus. Phenolic compounds such as flavanoids and phenolic acids are well known to have antioxidant and anti-proliferative activities (Gul et al., 2013)

The main phenolic compound identified in the aqueous extract of *Abrus precatorius* leaves was 4-vinylphenol (Table 1). A recent study of 4-vinylphenol showed that this compound has anti-angiogenic activities (Yue et al., 2015). Other identified compounds that have some therapeutic activities were β-lonone, phytol and stigmasterol, as listed in Table 1. The compound β-lonone has been shown to have anti-proliferative (Faezizadeh, Gharib, & Godarzee, 2016), anti-bacterial (Kubo, Muroi, & Himejima, 1993; Patra, Das, & Baek, 2015) and anti-tumour activities (Cho, So, Chun, & Jeon, 2016; Liu et al., 2008; Sharma, Chaudhary, Arora, Saxena, & Ishar, 2013; Yu, Anderson, & Elson, 1995). In addition, it can also be used as a fungicide, pesticide and trichomonicide. Phytol has anti-microbial (Pejin et al., 2014), anti-cancer (Song & Cho, 2015) and anti-inflammatory activities (Silva et al., 2014). Stigmasterol is listed to be an anti-hepatotoxic (El-Domiaty, Wink, Abdel Aal, Abou-Hashem, & Abd-Alla, 2009), anti-inflammatory (Gabay et al., 2010), anti-nociceptive (Kamurthy, Sumalatha, Rao, & Sudhakar, 2013), anti-ophidic, anti-viral and cancer-preventive agent (Ali et al., 2015; Kasahara et al., 1994), a hypocholesterolemic agent (Barriuso, Ansorena, Poyato, & Astiasarán, 2015), an ovulant agent (Zaman, Parvez, Ali, & Sayeed, 2015) and a sedative agent (Habib, Nikkon, Rahman, Haque, & Karim, 2007).

The therapeutic activity of the aqueous leaf extract of *Abrus precatorius*, which in traditional practice is obtained by decoction, might be due to the presence of phytol, stigmasterol and β-lonone. Individually, these phytochemical compounds are known for their bioactivities; however, this does not answer how the plant works as a whole crude extract as used in traditional practice. Further experiments are needed in order to prove that this extract does have therapeutic effects when used as a whole crude extract and that its efficacy does not result from the beneficial effects of any of its individual phytochemical compounds.

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Plant Growth, Nutrient Content and Water Use of Rubber (*Hevea brasiliensis*) Seedlings Grown using Root Trainers and Different Irrigation Systems

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ABSTRACT

Rubber seedlings raised in the soil-polybag system experience root coiling and restriction and the overhead sprinkler results in much water wastage. The objective of the study was to determine the influence of root trainers and three irrigation systems on rubber seedlings grown in a peat-based medium. The irrigation systems were the overhead sprinkler (SPR), drip (DRP) and capillary wick (WCK). The fourth treatment was the control (CTRL), which required growing rubber seedlings in conventional soil-polybags that were then irrigated using the wick system. The treatments were compared with one another in terms of their influence on nutrient loss, crop water productivity and water use efficiency, plant growth parameters and plant nutrient content of the rubber seedlings. A field experiment was carried out in a rain shelter for eight months, and data collection was carried out once per month. The experimental layout was the completely randomised block design. The results showed that WCK had the lowest cumulative leachate volume and the least cumulative nutrients leached. Both DRP and WCK had the highest plant growth parameters such as total fresh and dry weight, total leaf area and girth size, water productivity and leaf nutrient content. WCK was the best irrigation system together with the peat-based growing medium for raising rubber nursery seedlings.

Keywords: Irrigation, capillary, wick, *Hevea brasiliensis*, rubber, water use, water productivity

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INTRODUCTION

The foundation for high latex production lies in the production of seedlings that are disease-free and quick to mature and that have a high field survival rate. This can be achieved by proper soil fertility management in the nursery where these seedlings are grown (Waizah et al., 2011). Rubber nurseries in the past were established mostly in newly cleared forests, which are quite rich in plant nutrients. Today, however, existing forests are protected and fertile land areas are converted to industrial use. Thus, new rubber plantations are now limited to marginal (less fertile) land (Waizah et al., 2011).

The conventional system of raising rubber seedlings in Malaysia uses the soil-polybag and the sprinkler irrigation system. The sprinkler irrigation system is common because it is cheap to install, but this system suffers from high water wastage, among other disadvantages. Lienth (1996) stated that it is difficult to irrigate a crop without over-watering and under-watering some plants. Some of the water will miss the crop altogether and fall to the ground, increasing nutrient runoff. Westervelt (2003) stated that the lack of irrigation uniformity means more water is needed to irrigate crops.

The drip system is a much better water-saving irrigation system with higher water use efficiency, but it is more expensive to install. In this system, less water is lost during application because the water is applied directly to the immediate vicinity of the plant, thus saving water (Maya et al., 2014). It has the highest uniformity (90%) in water applied to plants, yet the system could have problems that lead to poor uniformity such as low pressure inlet and clogging of the emitters (Hsiao et al., 2007).

Capillary wick irrigation system is another water-saving irrigation system where water is supplied slowly to the plant roots via the capillary action of the wick (Bainbridge, 2002) (Figure 1). The wick is usually made of an absorbing material such as cotton that will draw the water out of a water container and into the soil, thus watering the soil and plant. This system is also much cheaper to install. Capillary wick irrigation was first introduced in India, where it was used in conjunction with buried clay pot irrigation (Bainbridge, 2001). The wick system is used in areas of high evapotranspiration, such as tropical and sub-tropical countries (Ritchey & Fox, 1974). The use of wick irrigation has been demonstrated to work well in raising seedlings of perennial crops e.g. citrus (Bainbridge, 2002). The method is also useful because of its ability to maintain soil moisture (Marrone 1982; Stalder & Pestermer, 1980).

Water application methods influence the growth of nursery seedlings differently (Argo & Biernbaum, 1994). The idea of nursery irrigation is to maintain the pores filled with air and water to minimise moisture stress. However, water shortage during plant nursery growth in the container may negatively affect nutrient reserves in the plants (Scagel et al., 2011, 2012).

The conventional container used for seedling planting is the polythene bag

(polybag) filled with soil as the medium for plant growth. However, seedlings raised in polybags suffer from many disadvantages. The seedling stock produced in polybags experienced problems of root coiling, distortion and transplanting shock (Sharma, 1987). Soil-polybags also need more space and soil volume, and this makes it difficult to handle due to its large size and weight (Josiah & Jones, 1992).

A recent alternative method to growing seedlings is the use of root trainers or tubes that incorporate structural features such as vertical internal ribs designed to minimise root disturbance, to reduce root spiralling and strangulation problems, to maximise lateral root development and to shape the roots into a form that will allow more proliferation when the plant is grown into a tree. Essentially, the tree seedlings raised in containers would have lower root exposure and disturbance during field planting, thus lowering transplanting shock and allowing for higher survival and growth rates (Kinghorn, 1974).

Other than using soil as a conventional growing medium, organic materials such as compost, peat, tree bark, coconut (*Cocos nucifera* L.) coir or inorganic material such as clay, mineral wool, perlite and vermiculite (Grunert et al., 2008; Vaughn et al., 2011) can also be used. The most important physical factors of a growing medium for influencing the growth of plants are water retention and aeration of the medium. Both these properties affect not only the availability of water and air, but also the thermal properties, mineral availability and biological activity (Klock, 1997). However, physical and chemical attributes of the growing medium play a role in determining the nutritional status of natural rubber, especially during the immature stage (Salisu et al., 2013).

Because of the coarse texture of most growing media, they cannot retain the water and nutrients needed for plant growth. Consequently, water and nutrients leach through the growing medium quickly; hence, an irrigation system is required that supplies water slowly in order to minimise the leaching problem.

The aim of this study was to compare the efficiency of three irrigation systems i.e. overhead sprinkler, drip and capillary wick irrigation systems, in growing rubber seedlings in root trainers. The above treatments were additionally compared with the control, which was the wick irrigation system, for rubber seedlings grown in the conventional soil-polybag system. These treatments were compared with one another in terms of their influence on nutrient loss, crop water productivity and water use efficiency, plant growth parameters and plant nutrient content of the rubber seedlings.

MATERIALS AND METHODS

Experimental Design and Treatment Details

This experiment was done in a rain shelter facility (2° 59' 05.0 " N 101° 44' 00.9" E) at Field No. 15, Universiti Putra Malaysia, Serdang, Selangor. To avoid water supply from rain and to reduce solar radiation

reaching the young rubber seedlings, the rain shelter was partially covered with black plastic netting on the sides. The rubber seedlings were grown in a root trainer called RB900 (Humibox Sdn Bhd., Selangor) and filled with BX-1 growing medium (peat material) (Figure 2).

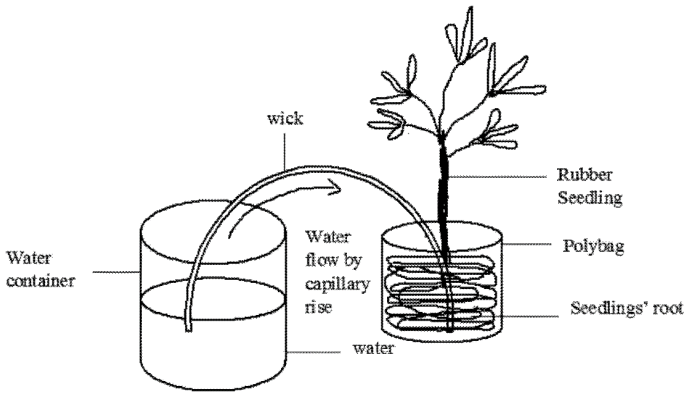

Figure 1. Capillary wick irrigation system

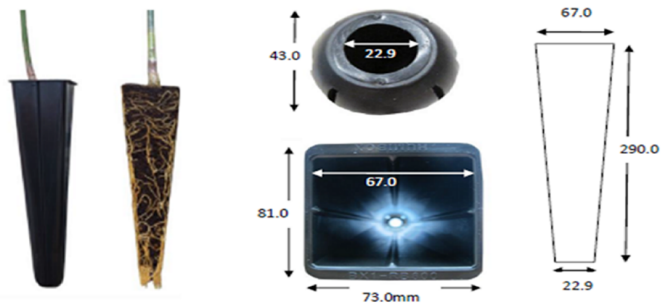

Figure 2. RB900 tube root trainer

The BX-1 medium (Peltracom, Latvia) used constituted 100% neutralised white peat treated with slow-release fertilisers. The exact formulation and ingredients of the BX-1 medium is a trade secret, so whatever information about this medium was obtained from the details given on the package. White peat is the remains of partially decomposed peat moss (*Sphagnum* sp.) of different species. BX-1 medium was used because it is enriched with nutrients and it is also lighter, so its handling is easier and therefore, the workload is reduced. RB900 root trainers were used because they can improve root growth and the containers can be reused.

This study had four treatments (with three replications each), where for the first three treatments, an amount of 230 g of BX-1 medium per RB900 tube was used in

each of the three treatments. The irrigation systems used were the overhead sprinkler (SPR), drip (DRP) and wick (WCK). The fourth treatment was the control (CTRL), which consisted of the conventional soil-polybag, while irrigation was done using the wick system. Each replication had 10 rubber seedlings; therefore, a total of 120 seedlings were used for the whole experiment. The experiment was laid out in the Randomised Complete Block (RCB) design. The rubber seedling clones used for the experiment were from the RRIM 2000 at one-month-old. For the control, the 5-kg Munchong (*Tropeptic Haplorthox*) soil series in a 15 cm x 20 cm polybag was used as the growing medium (soil-polybag system or CTRL). Munchong soil is classified as kaolinitic, very fine, isohyperthermic, Tropeptic Haplorthox (Noordin, 2013). The Munchong soil series is clayey, and strong to yellowish brown in colour. Its structure is moderate to strong with fine and medium sub-angular blocks. The soil is classified as one of the most suitable soils for rubber planting (MRB, 2009).

Each experimental plot consisted of a single tray or tube stand set that accommodated 10 rubber (*Hevea brasiliensis*) seedlings, or a polybag stand that accommodated 10 polybag seedlings as control. Each tray stand measured 50 cm wide and 150 cm long. Within each experimental block, treatments were separated from one another by a space of at least three tray stands, and blocks were separated from one another by the length of at least two tray stands. The total area, inclusive of border space, was about 9 m by 14 m or 126 m^2 (Figure 3). The water flow from the overhead sprinklers was adjusted in such a way to prevent water from falling onto neighbouring plots.

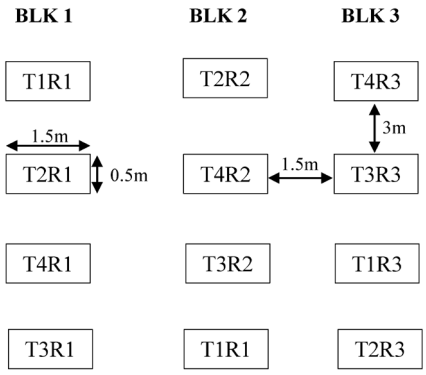

Figure 3. Experimental design [T1=SPR, T2=DRP, T3=WCK, T4=CTRL]

Watering was done once a day in the morning for the SPR and DRP treatments. The water content in the growing media was measured using a moisture meter (FieldScout TDR 100-6440FS, Spectrum Technology, Inc., USA) every day before every irrigation to monitor moisture status in the growing medium. A daily supply of 11 mm of water was provided in the DRP and SPR irrigation systems. Prior to starting the experiment, we had set up the irrigation systems so that 11 mm of water supply was equivalent to turning on the water supply from the systems for 3 min 20 s and 2 min for the DRP and SPR system, respectively. The measurement 11 mm was chosen because this was the amount of water flowing into the soil for the WCK system (11 mm was the mean daily difference in the water amount in the WCK water container).

This amount of water was supplied to maintain the media/soil moisture at about field capacity so as to avoid plant water stress. The average volumetric water content (VWC) of the BX-1 medium treatments (SPR, DRP, WCK) and soil (CTRL) were 38 m^3 m^{-3} and 27 m^3 m^{-3}, respectively, which were above field capacity (FC) for BX-1 media and slightly lower than FC for the soil (Table 1). The mean VWC values, however, showed that overall, there was no moisture stress in the treatments. A mini weather station (WatchDog 2000 series, Spectrum Technology Inc., USA) was placed inside the rain shelter to monitor the microclimate conditions under which the rubber seedlings were grown. Throughout the experiment for eight months, the average daily temperature, relative humidity and total solar irradiance under the rain shelter were kept at 27°C, 80% and 3.2 MJ m^{-2} day^{-1}, respectively. The average day length was 12 h per day. The BX-1 growing medium and the soil were analysed for physical and chemical properties.

Plant growth parameters (fresh and dry weight, leaf area, girth size, root volume, root length) were measured monthly, after which destructive samples were taken for leaf area and nutrient analysis. Fresh and dry weights were measured using a weighing scale, and root analysis was carried out using an EPSON WhinRhizo root scanner (EPSON PERFECTION V700 PHOTO, Reagent Instrument Inc., Canada). Vernier calipers were used to measure girth. Leaf area was measured using a leaf area meter machine (LI-3100C Area meter).

The concepts used by Heydari (2014) for water productivity (WP) and water use efficiency (WUE) were adopted for calculating WP and WUE based on the following formula:

$$\text{WP (g L}^{-1}) = \frac{Total\ plant\ dry\ weight\ (g)\ \text{per plant}}{Cumulative\ transpiration\ (L)\ per\ plant} \quad (1)$$

$$\text{WUE (L L}^{-1}) = \frac{Amount\ of\ water\ used\ by\ the\ plant\ (L) per\ plant}{Output\ of\ the\ irrigation\ system\ (L)\ per\ plant} \quad (2)$$

DETERMINATION OF PHYSICAL AND CHEMICAL CHARACTERISTICS OF SOIL AND BX-1 MEDIA

Physical Properties

The soil particle size was analysed using the pipette method and the particle size distribution of the BX-1 mediuma was determined by pore size distribution using sieves of different sizes in the shaking method (Teh & Jamal, 2006). Soil and BX-1 medium pH were determined by a suspension ratio of 1:5 for (soil to water) and 1:10 for (BX-1 medium to water) (McLean, 1982) using a pH meter (Meter-Toledo Delta 320 pH meter); the same suspensions were used for EC determination, as described by Rhoades et al. (1990).

Soil bulk density (Mg m^{-3}) was determined using the core method (Blake & Hartge, 1986). Total porosity (%) of soil was calculated from the measured soil bulk density values, assuming particle density of mineral soils (2.65 Mg m^{-3}) and using the following equation (Baver et al., 1972):

$$\text{Total porosity (\%)} = 1 - \frac{bulk\ density}{particle\ density} \times 100 \quad (3)$$

$$\text{Moisture content (gg}^{-1}) = \frac{weight\ of\ fresh\ soil - weight\ of\ oven\ dry\ soil}{weight\ of\ oven\ dry\ soil} \times 100 \quad (4)$$

The saturated hydraulic conductivity of both soil and BX-1 medium was determined using the constant head method (Klute & Dirksen, 1986). Water retention was determined using the pressure plate and pressure membrane described by Richards (1947). The applied pressure was 0.1, 1, 10, 33 and 1500 kPa. The samples were then oven-dried at 105 oC for 24 h, then weighed and multiplied by soil bulk density to obtain the volumetric water content (VWC).

Particle density (Pd) of the BX-1 media was obtained from an assumed formula given by Inbar et al., 1993 as follows:

$$\text{Pd (Mg m}^{-3}) = \frac{1}{\frac{\%\ organic\ matter}{100 \times 1.55} + \frac{\%\ ash}{100 \times 2.65}} \quad (5)$$

where 2.65 Mg m^{-3} and 1.55 Mg m^{-3} are the average particle density of the mineral and organic soil, respectively.

BX-1 medium bulk density was determined by oven drying a known quantity of the medium in relation to the total volume of the tube (RB900). The total porosity (TP) of the medium was computed from bulk density (Bd) and particle density (Pd) of the growing medium as they are inversely related (Beardsell et al., 1979; Hanan et al., 1980). Total porosity is defined as the total volume of pore space in a substrate.

Moisture content of the fresh media was determined by subtracting the oven-dry weight from the fresh weight and divided by the oven-dry weight of the medium. The bulk density, total porosity and gravimetric moisture content were calculated using the following formula:

$$\text{Bulk density (Mg m}^{-3}) = \frac{Weight\ of\ media,\ \ oven\ dried\ at\ 105^{o}C}{volume\ of\ fresh\ medium} \quad (6)$$

$$\text{Total porosity (\%)} = 1 - \frac{Bd}{Pd} \times 100 \quad (7)$$

$$\text{Moisture content (g g}^{-1}) = \frac{weight\ of\ fresh\ media - weight\ of\ oven-dried\ medium}{weight\ of\ oven-dried\ medium} \quad (8)$$

Transpiration by the rubber seedlings under different irrigation systems was calculated using the water-balance equation;

$$T = I - (L + E + \Delta\theta) \quad (9)$$

where T is transpiration (mm), I is irrigation (mm), L is leaching (mm), E is evaporation (mm) and $\Delta\theta$ is the change in moisture storage (mm).

The evaporation was calculated every day by weighing plantless soil and BX-1 growing systems that were watered every day with a known amount of water. Water loss was measured as that having been lost through the opening of the container only.

Soil Chemical Properties

Total C, N and S were determined using CNS analyser (LECO TruMac® CNS, USA). The leaching method by Chapman (1965) was used to determine cation exchange capacity (CEC) and exchangeable bases. Exchangeable K, Ca and Mg were determined using the leaching method with 1 M ammonium acetate buffered at pH 7. The levels of Ca, K and Mg were determined using an atomic absorption spectrophotometer (AAS) (Perkin-Elmer, 5100PC, USA). P and CEC were determined using an auto-analyser (AA) (Quikchem FIA 8000 series, LACHAT instrument, USA).

BX-1 Media Chemical Properties

Total C, N and S was measured using a CNS analyser (Nelson & Sommers, 1982). Total P, K, Ca, Mg and other micronutrients were extracted using the dry ashing method. An auto analyser (AA) was used to determine total P while K, Ca, Mg and other micronutrients were analysed using an atomic absorption spectrophotometer (AAS). The CEC of the BX-1 medium was determined using the shaking method (Fauziah et al., 1997).

Leaf and Leachate Analysis

Leaf sampling was conducted based on the Malaysian Rubber Board (MRB) guidelines. Four basal leaves from the first sub-terminal whorl were collected as a leaf sample (Rubber Research Institute of Malaysia, 1990). The sampled leaves were cut from stems and placed in a forced draft oven at 60ºC for 48 h, after which the weight was determined using a weighing machine (Multitech, GF-3000, Tokyo, Japan). Leaf samples were used for N, P, K, Ca and Mg analyses. The amount of N was determined using a CNS analyser while P, K, Ca and Mg were prepared using the dry ash method. The filtrates were sent to an Auto Analyser (AA) and atomic absorption spectrophotometer (AAS) for determination of P and K, Ca, Mg, respectively.

Leachate samples from the experimental field were collected every week using a plastic container. Enough leachate collected for each experimental unit was mixed (pooled) and sub-sampled for laboratory analysis monthly. The sample was first filtered using filter paper (Whatman No. 2, 8µm size) before it was sent to the laboratory for analyses. N and P were analysed using AA and K, Ca and Mg using AAS.

Data Analysis

Data analysis was done using the SAS system for Windows (SAS 9.4, SAS Institute Inc., Cary, NC, USA). ANOVA (Analysis of Variance) and Proc GLM were used to determine the significant treatment effect on various measured properties with the significant difference at $p<0.05$. The SNK (Student-Newman-Keuls) test and t-test for mean separation were used to detect the significant differences between the means.

RESULTS AND DISCUSSION

Physico-Chemical Properties of BX-1 Growing Medium and Munchong Soil Series

The results of the physical and chemical properties of the two different growing media (BX-1 medium and soil) are presented in Table 1. The analyses showed that the medium had a very low bulk density that was 90% lower than that of the soil, making it much easier to handle than the mineral soil. It also had a gravimetric moisture content of 0.71 g g^{-1}. The bulk density of peat depends on the plant's residue component, ash content and the degree of decomposition. The bulk density of peat is generally low, ranging from 0.1-0.5 Mg m^{-3}, with a moisture content under natural conditions exceeding 80% (Xuehui & Jinming, 2009). The properties of the media showed that the peat sample could hold more water and air than the Munchong soil. The medium had a total porosity of

Table 1
Mean (±Standard Error) physico-chemical properties of the soil and BX-1 growing media

Physical Properties	Soil	BX-1 Media
Bulk Density (Mg m^{-3})	1.43±0.03	0.14±0.01
Moisture Content (g g^{-1})	0.21±0.08	0.71±0.01
Total Porosity (%)	46.0±3.10	91.0±2.01
Saturated Hydraulic Conductivity (cm hr^{-1})	8.2±0.20	32.0±0.04
Saturation (m^3 m^{-3})	0.56±0.05	0.95±0.04
Field Capacity (m^3 m^{-3}))	0.29±0.02	0.31±0.02
Permanent Wilting Point (m^3 m^{-3})	0.21±0.07	0.20±0.01
Particle size analysis		
Sand (%)	34.54±0.02	-
Silt (%)	15.23±0.01	-
Clay (%)	50.21±0.02	-
Chemical Properties	**Soil**	**BX-1 Media**
pH	4.67±0.30	6.40±0.90
EC (dS m^{-1})	0.04±0.002	1.22±0.03
CEC (cmol+kg^{-1})	8.32±0.10	63.21±0.40
C (%) *	1.38±0.10	34.25±0.20
N (%)	0.13±0.02	1.09±0.20
C: N	10.6±0.02	27.0±0.10
S (%)	0.03±0.001	0.75±0.001
P (ug g^{-1})	8.34±1.02	680.57±8.30
K (ug g^{-1})	41.27±3.10	1779±13.21
Ca (ug g^{-1})	459.33±4.70	6223.67±17.60
Mg (ug g^{-1})	85.47±3.90	1709.33±23.70
Na (ug g^{-1})	5.43±0.30	17.93±0.92

*C and nutrient contents are expressed per unit dry weight

91%, with 20.4% macropores (>6.3 mm), 32% mesopores (2-6.3.0 mm) and 22.5% micropores (0.5-2.0 mm), following the particle size distribution of the BX-1 medium. The medium could hold more water because of the large proportion of mesopores. The percentage of macropores should be at least 20-25% (Kuslu et al., 2005). The percentage of macropores in the medium agrees with the value reported by Kuslu et al. (2005). The medium had a higher available water content of 27.3% than the soil, which showed the ability of the medium to hold more available water for the crop. The high CEC (63.21 cmol+kg^{-1}) of the medium could be attributed to industrial treatment of the medium with lime and fertiliser, as CEC is a measure of nutrient retention capacity.

The Munchong soil series is derived from sedimentary rocks (Salisu et al., 2013). The results of the analysis showed that the main content of this soil was clay. The result also showed that the soil had plant available water, low pH (4.6), low CEC (8.35 cmol+kg^{-1}) as well as little available nutrients, which are characteristics of highly-weathered Ultisols and Oxisols (Shamshuddin & Fauziah, 2010). The predominant chemical properties of these soils include low soil acidity (pH<5) and low inherent fertility, which make the soils less productive for crops (Shamshuddin & Fauziah, 2010).

Cumulative Nutrient Leaching Losses, Water Productivity, Water Use Efficiency and Elemental Ratios

The statistically significant ($p<0.05$) results for the cumulative leachate volume and cumulative nutrient leachates are shown in Figure 4. The results showed that overall, the better treatments were the ones using WCK and CTRL because these had the lowest cumulative leachate volume as well as the lowest cumulative leachate for the individual nutrients. The SPR and DRP treatments, on the other hand, had the highest amounts of cumulative leachate volume and cumulative leachate of nutrients. In terms of cumulative leachate volume, the SPR and DRP systems had 39% and 80% higher cumulative leachate volume than the WCK and CTRL systems, respectively. The lowest cumulative leachate was recorded for CTRL.

SPR and DRP had the highest cumulative leachate volume because the required amount of water, aided by the higher hydraulic conductivity of the medium, was applied for a shorter period than for WCK and CTRL (Table 1). This means in the DRP system, the water flowed down from the point of entry and rapidly downwards to the bottom, without having to wet the entire or a large part of the medium first. On the other hand, the higher leachate volume in SPR was due to the nature of the irrigation system, which supplied water over the entire

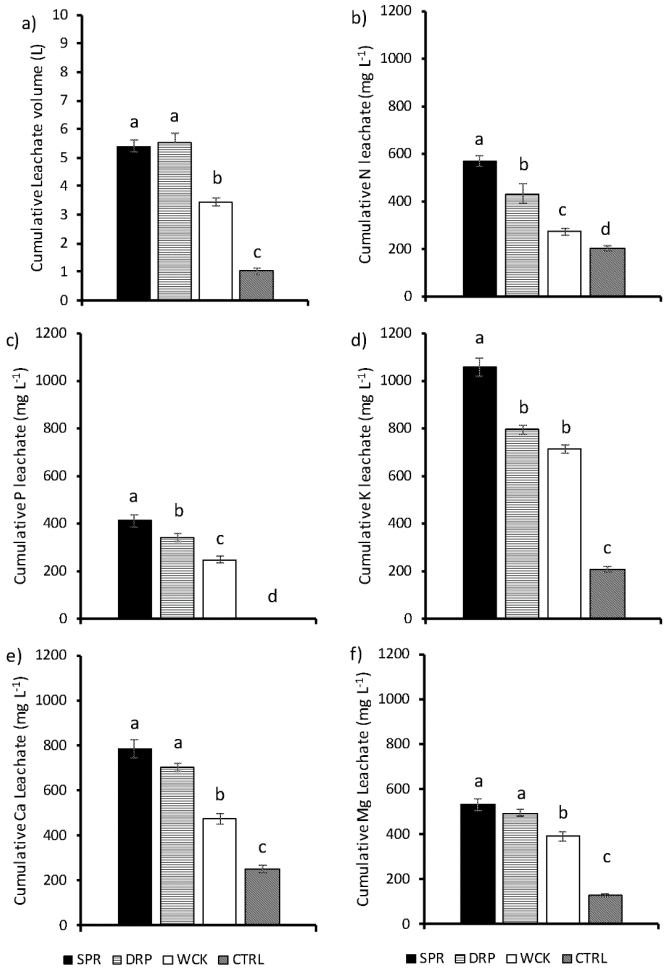

Figure 4. Means (±standard error) of different irrigation systems effect on cumulative (a) leachate volume, (b) N leachate, (c) P leachate, (d) K leachate, (e) Ca leachate, and (f) Mg leachate. (SPR=Overhead sprinkler, DPR=Drip, WCK=Capillary wick, CTRL=Soil-polybag with capillary wick irrigation). Means with same letters in the same chart are not significantly different from one another at 5% level of significance

opening area of the container. This caused more leaching in the system. DRP started to leach when wetted over a small area, so less N and K were leached than by SPR, which leached when most of the medium was wetted. WCK had low leaching of nutrients because the water flowed from wick to medium more slowly and gradually. Water was absorbed by the surrounding medium first before leaching out, and this was reflected in the high leaf nutrient content (Table 2). The lowest cumulative leachate volume in CTRL was due to higher soil compaction and volume and lower hydraulic conductivity of the soil than was the case for the medium (Table 1).

Table 2
Effect of different irrigation systems on the leaf nutrient content (per dry weight)

Treatments	N (%)	P (%)	K (%)	Ca (%)
SPR	4.01 ± 0.10b	0.25 ± 0.003b	0.63 ± 0.001b	0.47 ± 0.003b
DRP	4.27 ± 0.10a	0.28 ± 0.01b	0.87 ± 0.01a	0.53 ± 0.04a
WCK	4.22 ± 0.01a	0.32 ± 0.01a	0.90 ± 0.10a	0.50 ± 0.03a
CTRL	3.82 ± 0.20c	0.19 ± 0.003c	0.53 ± 0.03b	0.43 ± 0.03c
*Sufficiency level (%)	3.71-3.91	0.21-0.27	1.10-1.60	0.60-0.70

*According to Noordin (2013)
Means (± Standard Error) for the Same Column and Same Parameter, Followed by Same Letter, are not Significantly Different from One Another at the 5% Significant Level by SNK. (SPR=Overhead Sprinkler, DRP=Drip, WCK=Capillary Wick, CTRL= Soil-Polybag with Capillary Wick)

Nitrogen concentration leached out by the samples using the different irrigation systems was related to the volume of water leached out (Zotarelli et al., 2009). The low cumulative N leachate under CTRL was attributed to the lower soil N level (Table 1). Andrisse (1988) found a substantial amount of the total available K in soil solution in peat soil. Hence, K is strongly mobile and prone to leaching. In addition, K fixation is almost absent in peat despite its high CEC, and peat does not also readily adsorb exchangeable K. This experiment agreed with Andrisse's (1988) findings as more K was leached than other nutrients (Figure 4). The effect of the different irrigation systems on the seedlings' water use efficiency and water productivity was significant ($p<0.01$) (Figure 5). The most important month was the final month of the experiment, which was the eighth month, the month that the seedlings were due for field transplanting. The highest cumulative WP of the seedlings was recorded for the WCK and DRP systems, and it was 30% higher than for SPR and CTRL. The higher WP for DRP and WCK was due to their highest plant dry weight of the seedlings recorded for the systems (Table 3). The lowest WP, recorded for CTRL, was due to the lowest seedling plant dry weight achieved in this treatment and possibly due to the higher bulk density of the soil, which might have led to root growth restriction. CTRL had the highest transpiration value of 11.84 L of water compared to the other treatments, with their transpiration value in the range of 8-10 L of water, cumulatively over a period of eight months. In spite of the higher transpiration values, dry matter yield was low due to the polybag's root growth restriction of the seedlings. Potential root growth was restricted due to root coiling as it prevented development of lateral roots (Josiah & Jones, 1992). The highest WUE was obtained in CTRL, which allowed 25% higher WUE than did DRP and WCK and 96% higher WUE than did SPR. The poorer SPR had only 3% water utilisation in the last month. The highest WUE in CTRL was due to its lowest cumulative percolation loss (Figure 4a), which resulted in higher

transpiration. The amount of water supplied was almost equal to the irrigation need of the seedlings that resulted in the least amount of leachate and it could also be a result of the higher soil quantity used.

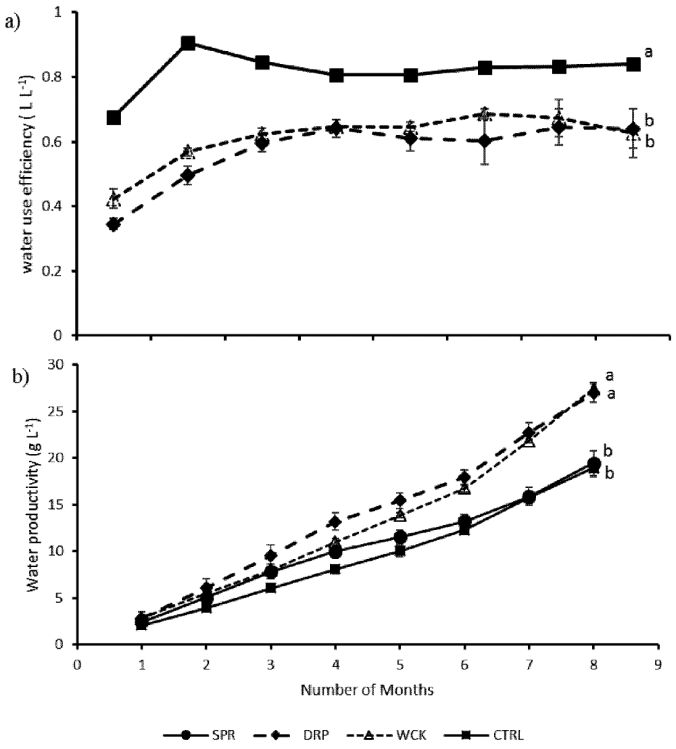

Figure 5. Means (±standard error) of (a) water use efficiency and (b) water productivity of rubber seedlings as influenced by different irrigation systems and month. (DPR=Drip, WCK=Capillary wick, CTRL=Soil-polybag with capillary wick system). At Month 8, means with same letters are not significantly different from one another at 5% level of significance. SPR (overhead sprinkler) had a WUE of less than 0.04 LL^{-1} throughout the eight months

The results of the WP and WUE were similar to those obtained by Teh et al. (2015), who reported that the wick irrigation system had the highest WP and WUE than two other irrigation systems on the growth of water spinach (Ipomoea reptans). Salemi et al. (2011) claimed that irrigating crops with less water increased water productivity in their study.

Water use efficiency (WUE) is among the most important indices for determining optimal water management practices (Kharrou et al., 2011). The lowest water use efficiency in the SPR system was due to the lack of uniformity and efficiency of the system, which resulted in much water wastage. El-Rahman (2009) reported high water use efficiency (WUE) in wheat using the drip irrigation saving system.

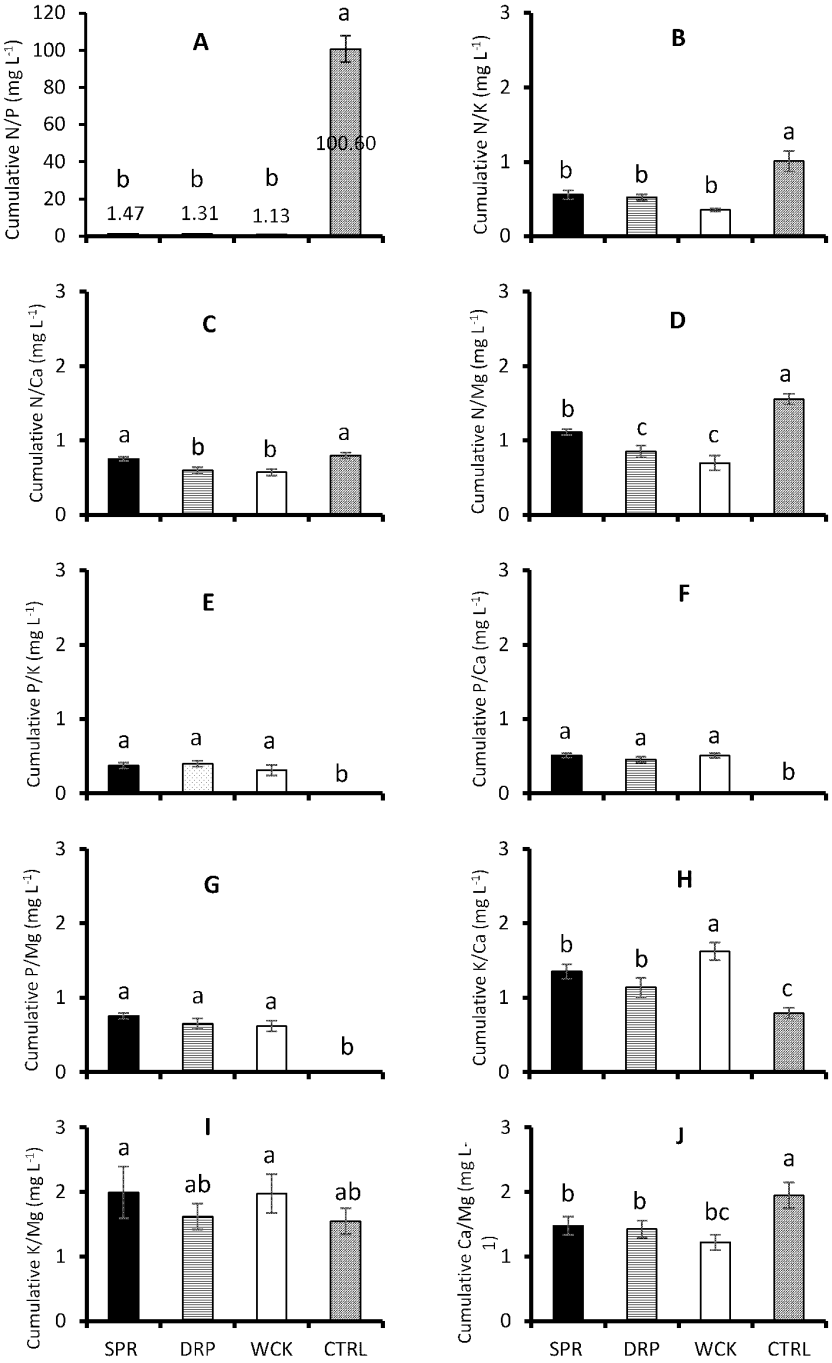

Figure 6. Means (±standard error) of different irrigation systems' effect on cumulative leachate ratios of (A) N/P (B) N/K (C) N/Ca (D) N/Mg (E) P/K (F) P/Ca (G) P/Mg (H) K/Ca (I) K/Mg (J) Ca/Mg (SPR=Overhead sprinkler, DPR=Drip, WCK=Capillary wick, CTRL=Soil-polybag with capillary wick irrigation). Means with same letters in the same chart are not significantly different from one another at 5% level of significance

Elemental Ratios

The results of the elemental ratios are shown in Figure 6. In the eight months of the research, CTRL had the highest N/P, N/K, N/Ca, N/Mg and Ca/Mg, with a range of 30-90% higher than the BX-1 treatments (SPR, DPR, WCK), despite CTRL having the lowest leachate of the elements (Figure 4). This was due to the lower cumulative leachates of P, K, Ca and Mg in relation to the N by the CTRL treatment as shown in Figure 4. The BX-1 treatments exhibited higher percentages of P/K, P/Ca, P/Mg and K/Ca ratios, which did not differ significantly ($P<0.05$) from one another except in N/Ca and N/Mg with the highest (50%) percentage recorded for SPR, which could be attributed to the higher amount of nutrients leached out by SPR (Figure 4).

The higher elemental ratios recorded in the BX-1 treatments were due to the higher nutrient content of the BX-1 media. There were no significant differences between them in terms of most of the elemental ratios because the BX-1 medium was uniform in nutrients (Table 1). Statistically similar elemental ratios among the BX-1 treatments could be the reason why they did not differ significantly in terms of growth parameters and leaf nutrient content (Table 2), but SPR had the lower of the parameters mentioned not because of the nutrient content of the growing medium as was the case for CTRL, but because of the nature of the irrigation system.

Plant and Root Growth

The interaction between the different irrigation systems and the duration (eight months) of the experiment for seedlings' water content, total fresh and dry weight, total leaf area and girth size were significant ($p<0.05$) and the results are presented in Table 3. In the last month of the experiment (Month 8), the overall better treatments in terms of highest plant growth parameters were DRP and WCK. There was no significant difference between these two treatments, but they differed from SPR and CTRL (the poorer treatment being SPR). The interaction for plant height and number of leaves was not significant ($p>0.05$), but the main effect of time (months) was significant ($p<0.05$), which

Table 3
Effect of different irrigation systems on the growth of seedlings (per plant basis).

Treatments	Water Content (g)	Total Fresh Weight (g)	Total Dry Weight (g)	Total Leaf Area (cm^2)	Girth Size (mm)
SPR	68.2 ± 0.50b	103.7 ± 2.62c	35.5 ± 0.20b	1275.0 ± 4.41b	13.9 ± 0.31b
DRP	82.0 ± 0.60a	126.2 ± 1.60a	44.2 ± 2.40a	1508.1 ± 61.12a	17.7 ± 0.21a
WCK	84.0 ± 0.51a	131.1 ± 1.21a	47.1 ± 0.41a	1602.1 ± 8.12a	18.0 ± 0.10a
CTRL	76.7 ± 3.52b	114.5 ± 3.80b	37.8 ± 2.61ab	1323.5 ± 48.91b	14.6 ± 0.30b

Means (± Standard Error) for the Same Column and Same Parameter, Followed by Same Letter, are not Significantly Different from One Another at the 5% Significant Level by SNK. (SPR=Overhead Sprinkler, DRP=Drip, WCK=Capillary Wick, CTRL= Soil-Polybag with Capillary Wick)

of course showed a continuous increase of the parameters as plant growth progressed.

The DRP and WCK systems had the highest plant water content, fresh and dry plant weight, total leaf area and seedling girth size, while the lowest values of these parameters were recorded for the SPR system. The SPR parameters did not differ significantly from those of CTRL (Table 3). Increase in plant growth parameters is a function of water and sunlight, so the higher growth parameters recorded for DRP and WCK could be due to the influence of the irrigation systems, since the treatments were treated and raised in the same conditions of temperature and solar radiation. The higher values for DRP and WCK were due to the ability of the irrigation systems to dissolve the BX-1 medium nutrients into solutions for the seedlings' use, as moisture is non-limiting in the systems on the one hand and due to the availability of nutrients in the medium on the other hand. The lower values recorded for CTRL could also be attributed to the lower nutrient content of the soil compared to that of the growing medium (Table 1). Girth size was lowest for the SPR and CTRL treatments; girth size for rubber is very important because it determines the amount of latex flow and latex quality (Salisu et al., 2013).

Higher growth of the seedlings was obtained for DRP and WCK (Table 3) because of the lower leachate of nutrients in the treatments with a consequent higher WP of the systems as well as higher nutrient content in the leaves (Table 2). CTRL had the lowest water and nutrient leaching, and this was translated in the higher WUE of the treatment. However, WP and growth parameters of the seedlings were low because of the lower nutrient content of the soil.

Table 4 shows the interaction between the treatments and the months for root length, root volume, root surface area and root diameter, which were significant ($p<0.05$) for nearly all the months. The most important was the eighth month of the experiment, when there was no significant difference in the seedlings that were raised in the BX-1 growing system (SPR, DRP and WCK) in terms of the seedlings' root length and root volume.

Table 4
Root growth of seedlings (per plant basis) at month 8.

Treatments	Root Length (cm)	Root Volume (cm^2)	Root Surface Area (cm^2)	Root Diameter (mm)
SPR	779.30 ± 5.90a	4.80 ± 0.06a	123.10 ± 0.80b	0.62 ± 0.01c
DRP	819.12 ± 3.50a	4.31 ± 0.20a	193.50 ± 10.00a	0.89 ± 0.01b
WCK	798.71 ± 6.80a	4.92 ± 0.08a	172.00 ± 1.04a	0.92 ± 0.01a
CTRL	532.08 ± 17.01b	3.20 ± 0.10b	158.20 ± 2.93ab	0.92 ± 0.01a

Means (± Standard Error) for the Same Column and Same Parameter, Followed by Same Letter, are not Significantly Different from One Another at the 5% Significant Level by SNK. (SPR=Overhead Sprinkler, DRP=Drip, WCK=Capillary Wick, CTRL= Soil-Polybag with Capillary Wick)

At Month 8, the BX-1 system treatments (SPR, DRP and WCK) had an average root length and volume of 765.67 cm and 3.7 cm^3, respectively, which were significantly different from those achieved in the conventional system treatment (CTRL), which achieved 532.03 cm and 3.2 cm^3 for root length and volume, respectively. However, there was no significant difference in terms of root surface area and root diameter among the BX-1 system treatments (161.3 cm^2 and 0.82 mm) and the conventional system (158.3 cm^2 and 0.92 mm). The BX-1 system had an increase of root length and root volume of 30% and 15% respectively, more than those recorded for the CTRL system. Krizek et al. (1985) stated that when moisture is non-limiting, restricting the growth of roots can mimic the effect of soil moisture stress on plant growth.

Nutrient Content of Rubber Seedlings Leaves

The interaction between different irrigation systems and Month 8 was significant ($p<0.05$) for the N, P, K and Ca content of the seedlings' leaf tissue, as can be seen in Table 2. Only the interaction for the Mg leaf content (0.4%) was not significant ($p>0.05$). The better treatments in terms of leaf nutrient content were obtained from the seedlings that were raised in the DRP and WCK irrigation systems, while CTRL had the lowest seedling leaf nutrient content. The higher nutrient content recorded for DRP and WCK can be attributed to the lower amount of nutrients leached, especially N and K. Overall, the lowest nutrient tissue content was recorded in the CTRL despite its having the lowest nutrient leachate because of the lower nutrient content of the soil by more than 10 times (Table 1).

CONCLUSION

This study showed that water application methods influenced the growth of rubber seedlings. The conventional method for irrigating rubber seedlings in Malaysia is the sprinkler system, and this research showed that this system was a weak irrigation system as it yielded the highest amount of nutrient leachate, the lowest growth parameters and poorer water productivity and water use efficiency. The sprinkler system had another limitation i.e. higher water loss due to canopy interception of water. The study indicated that the drip and capillary wick systems had the highest plant growth parameters and leaf nutrient content than the sprinkler and control systems. This was because the drip and wick systems could supply water to the growing seedlings more slowly and steadily. The capillary wick system proved to be the best irrigation system used together with a root trainer and the BX-1 growing medium, recording the lower amount of leachate, the highest plant and root growth parameters and the highest water productivity. By using the root trainer rather than a polybag, a smaller amount of growing medium was used, but it was sufficient to sustain the growth of the seedlings for eight months with better growth than the conventional system. The research also suggested that farmers have an alternative growing system for nursery

rubber seedlings. Using a root trainer and the better physical and chemical properties of the BX-1 growing medium gave better seedling growth and leaf nutrient content in this study than did the conventional system (soil-polybag).

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Potential Mangrove Species in Porong River Estuary as Inhibiting Agent of Heavy Metal (Pb, Cu and Zn) Pollution

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ABSTRACT

This study investigated the ability of the mangrove species to accumulate heavy metals such as lead (Pb), copper (Cu) and zinc (Zn). Concentrations of these metals in sediment, roots and leaves of *Avicennia alba, Sonneratia alba, Avicennia marina* and *Rhizopora mucronata* found in the mangrove ecosystem of the Porong River estuary, Sidoarjo, East Java, Indonesia were measured. The bio-concentration factor (BCF) and translocation factor (TF) were calculated. The results showed that Pb concentrations in the roots and leaves of mangroves were 0.0038-0.0505 mg/kg and 0.0042-0.0395 mg/kg, respectively, while Cu concentration in the roots of mangroves was 0.2658-0.3390 mg/kg and in the mangrove leaves was 0.0655-0.1490 mg/kg. The average concentration of Zn found in mangroves ranged from 1.327-3.1380 mg/kg in the roots and 1.767-3.674 mg/kg in the leaves. Among all the mangroves, the highest BCF values for Pb, Cu and Zn were found in *Avicennia alba*. However, the highest TF for Pb and Zn was found in *Sonneratia alba*. On the other hand, the highest TF for Cu was found in *Rhizopora mucronata*. The capability of *Avicennia alba* to accumulate Pb, Cu and Zn heavy metals compared with other species is highly important for determining a suitable species for phytoremediation.

Keywords: Bioaccumulation, bio-concentration, heavy metals, mangrove wetland, phytoremediation

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INTRODUCTION

Rising populations around the world the development of industry may result in anthropogenic discharges that pose a threat

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to ecosystems. Industries listed as causing heavy-metal pollution are the battery, electronic, chemical, electroplating and paint industries (Marg, 2011). Sidoarjo Regency in Indonesia is one of the many cities that contribute to environmental pollution due to industrial activities such as cement, battery, chemical, electroplating and plastics manufacturing. Consequently, this area is prone to heavy-metal pollution. The strongest impact is experienced by the estuarine and coastal waters since heavy metal discharge from industries and urban areas from incoming freshwaters and tidal water is accumulated in the estuary (Tam & Wong, 2000). This situation is expected to occur in the Porong River estuary. The increasing concentrations of Pb, Cu and Zn in this estuary will have long-term implications to the biota.

Cu and Zn act as essential plant micronutrients, being involved in enzyme mechanisms, while Pb is a non-essential element that is toxic to the biota. Zinc is often found in contaminated sediment of estuarine areas at high concentrations of up to 800 µg/g (Luoma, 1990). The presence of these heavy metals in high-level concentrations may cause harm to metabolic processing in the cellular level, inhibit enzyme reaction and the anti-oxidative process, delay growth and cause mortality of biota (Vangronsveld & Clijsters, 2008). Furthermore, these heavy metals are continuously found at high concentrations in contaminated estuarine areas, including the Porong River estuary. A previous study by Lapindo Mud Flow reported that discharge from the Porong River contained Cu and Pb at levels of 24.5 and 17.8 mg/L, respectively (United Nations Environment Program/Office for the Coordination of Humanitarian, 2006). In 2009, Pb was found in the estuary of the Porong River ranging from not detected to 0.0490 mg/L while Cu was determined to be 0.083-1.310 mg/L (Juniawan, Rumhayati, & Ismuyanto, 2012; Parawita, Insafitri, & Nugraha, 2009). Zn reached 0.03±0.01 mg/L in 2005 in pond waters in the Porong estuary (Kohar, Budiono, Indriany, & Wilujeng, 2005).

Mangrove estuary ecosystems have the ability to reduce heavy-metal contamination due to the ability of their roots to absorb and re-translocate heavy metals into other parts of the plant (Mejías, Musa, & Otero, 2013). In addition, it has been concluded by a previous study that mangroves have high tolerance to heavy metal pollution; the LC_{50} for Cu, Zn and Pb in mangroves was 566, 580 and 400 µg/g, respectively (MacFarlane, Koller, & Blomberg, 2007). The capability of mangroves to accumulate heavy metals from their environment makes them a suitable candidate for heavy-metal pollution remediation. This capability is applied in terms of the phytoremediation method. It is a method that employs plants for undergoing remediation of pollutants. By taking advantage of the ability of mangrove to absorb and accumulate heavy metals, phytoremediation is considered a cost effective and ecologically friendly technology (Etim, 2012).

However, despite its advantages, application of the phytoremediation technique using mangrove plants especially in the Porong River estuary faces difficulties.

The selection of the most appropriate mangrove species for remediation in this area is currently under discussion. Furthermore, studies regarding the ability of the mangrove species to inhibit Pb, Cu and Zn, particularly in the Porong River estuary, are limited.

This study tried to determine the concentration of heavy metals, Pb, Cu and Zn, in sediment and parts (roots and leaves) of the mangrove plant found in the Porong River estuary, and finally to reveal the potential of the mangrove species in the Porong River estuary as a suitable inhibiting agent of Pb, Cu and Zn pollution.

MATERIALS AND METHOD

The research was conducted in the mangrove ecosystem of the Porong River estuary in September 2014. Three sampling stations were chosen to represent the difference in heavy metal input from the river mouth (Stations 1 and 2) and close to the open sea (station 3). Four mangrove species were identified in this area: *Avicennia alba, Sonneratia alba, Avicennia marina* and *Rhizopora mucronata*. Mangroves more than 2 m in height and 15-20 cm in diameter, as well as their surface sediment, were collected. Sampling sites are presented in Figure 1 and Table 1.

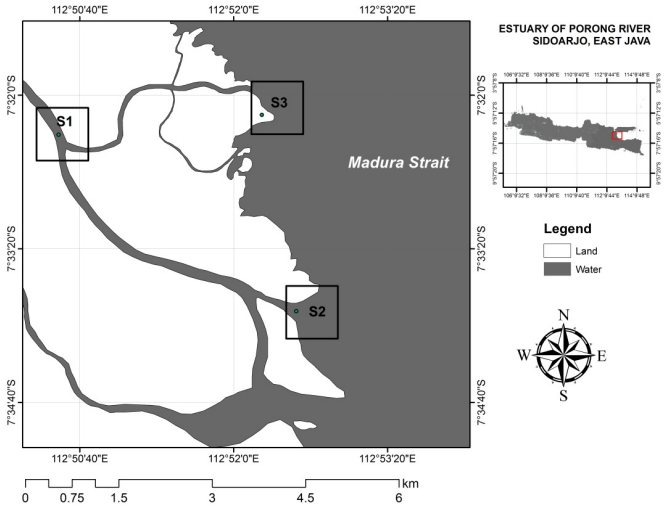


Figure 1. Location of sampling sites

Table 1
Sampling sites position and dominated mangrove species in mangrove ecosystem of Porong

Study Area	Mangrove Species
S 07° 53' 90" E 112° 84' 10"	*Avicennia alba, Avicennia marina,* and *Sonneratia alba*
S 07° 56' 40" E 112° 87' 60"	*Avicennia marina* and *Sonneratia alba*
S 07° 53' 60" E 112° 87' 10"	*Rhizopora mucronata* and *Avicennia marina*

Each species was sampled in duplicate for its sediments, roots and leaves from the same individual tree. A 10-cm diameter PVC pipe was used to sample the sediment at the mangrove roots at 5-10 cm deep from the surface layer. Next, about 20-30 pieces of the leaves were taken from middle-aged mangrove trees, while the roots were removed with a knife at the part buried in the sediment. Prior to laboratory analyses, all samples of roots, leaves and sediments were placed in plastic bags and stored in a cool box. The samples were then dried in an oven at 105 °C as the samples were measured for heavy metal content (Pb, Cu and Zn) based on dry weight calculation. Water quality parameters i.e. temperature, salinity, pH and DO were measured *in situ* as the supporting data for environmental conditions.

The analysis was conducted in the Water Quality Laboratory, Jasa Tirta Malang. Atomic Absorption Spectrophotometry (AAS) Shimidzu AA-6800 was used to measure the concentration of heavy metals in the sediments, roots and leaves of the mangrove. To monitor the performance of the instrument, blank and standard solutions were used to obtain data quality by developing calibration curves. Heavy metal in the sediments and mangrove parts (leaves and roots) were analysed using the APHA method (USEPA-311B and 3050B).

To determine the potential of mangrove species for accumulation of heavy metals Pb, Cu and Zn in the study area, BCF and TF were calculated. BCF is a ratio between metal concentrations in the leaf to metal concentration in the sediments, while TF is generated from the ratio between metal concentration in the leaf to metal concentration in the roots (Etim, 2012). The statistical method was applied to determine the significance difference of the mean of heavy metal concentrations in the sediment among the stations using one-way ANOVA. SPPS 16.0 and Microsoft Excel 2007 software were utilised for the data analysis.

RESULTS AND DISCUSSIONS

Concentration of Pb, Cu and Zn in the Surface Sediment

The average concentration of Pb, Cu and Zn in the surface sediment of the Porong River estuary is presented in Figure 2. Pb was found between 0.059-0.068 mg/kg, which is below the Interim Sediment Quality Guideline of the Canadian Council of Ministers of the Environment (CCME) level of 35 mg/kg. The concentration of Pb in the surface sediment was considerably lower than the concentration of Pb in the sediment at the mangrove ecosystem of Jakarta Bay, which was between 18.640-29.570 mg/kg as reported by Hamzah and Setiawan (2010). Jakarta Bay is surrounded by significantly increasing anthropogenic activities. Waste, including heavy metal pollution, comes from five river mouths to Jakarta Bay (Takarina, 2011). While the Porong River estuary is also an urban area, environmental stress is lower than at Jakarta Bay.

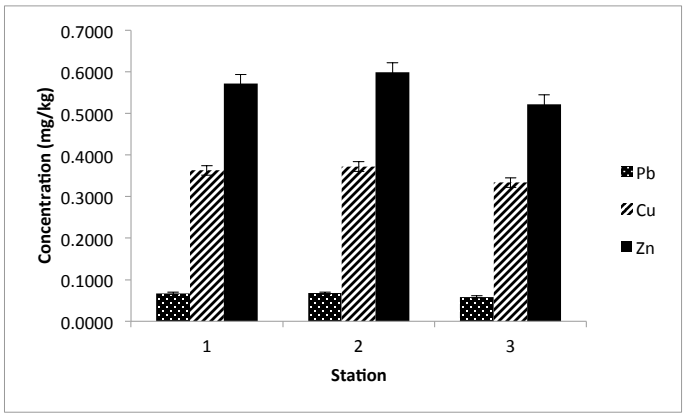


Figure 2. The concentration (mean and standard error) of Pb, Cu and Zn in mangrove sediments at Porong River estuary (n=4)

Concentration of Cu in surface sediment of the study area ranged from 0.3338-0.3723 mg/kg. Compared to that of the mangrove ecosystem in Tapak, Semarang with an average of 0.275 mg/kg (Kariada & Irsadi, 2014), Cu concentration in this study was higher. However, it did not exceed the Interim Sediment Quality Guideline of CCME (0.019 mg/kg).

Subsequently, the average concentration of total Zn in the Porong Estuary Mangrove Sediment was 0.584 mg/kg, which was significantly lower than the Interim Sediment Quality Standard. Zn of CCME (123 mg/kg) found in the mangrove sediment of the Porong River estuary was considerably less than at other locations such as Mai Po, Hong Kong (240 mg/kg) and Hawksbury, Australia (94 mg/kg) (MacFarlane, 2002). In Indonesia, Zn concentration of mangrove sediment in the Porong estuary was lower than in the Mahakam Delta (74.95 mg/kg) (Budiyanto & Lestari, 2013), Jakarta Bay (64.2-209.4 mg/kg) (Sindern et al., 2016) and Dumai (31.490–87.110 mg/kg) (Amin, et al., 2009).

The average concentration of Cu and Zn was not significantly different among the stations (F_{Cu}=1.14 and F_{Zn}=1.02, p value>0.05, n=4) in the Porong River estuary. However, there was a significant difference in Pb concentration among the stations (F_{Pb}=4.76, p value>0.05, n=4). The highest concentration of Pb was at Station 2, while the lowest concentration for Pb was found at Station 3. Mangrove at Station 2 was located near incoming water streams that may have been contaminated as a result of the development of industrial areas and harbour activities as well as increasing population around the upper water stream of the Porong River. Consequently, mangrove swamp sediment in these areas retain and accumulate more heavy metals compared to Station 3, which is located near the open sea. It has been noted by MacFarlane and Burchett (2002) that mangrove sediment has significant ability in trapping heavy metals

originating from tidal waters, downstream rivers and river run-off.

Furthermore, Zn was the most prevalent heavy metal in the sediment of this estuary, followed by Cu and Pb (Pb<Cu<Zn). Among the eight metals found there (Fe, Mn, Ni, Pb, Cu, Zn, Cr, Cd), Zn was present in abundance, that is at 105 ppm in the mangrove sediment, Punta Mala Bay, Pacific Panama (Defew, Mair, & Guzman, 2005). In aquatic environments, Zn and Cu are classified as nonpoint sources i.e. originating from anthropogenic activities; for example, urban and agricultural runoff and boating activities are common sources of Cu in water (Joseph & Kundig, 1998).

It is very important to measure Pb, Cu and Zn concentration in mangrove sediment as concentration of the heavy metals, Pb, Cu and Zn, influence their concentration in parts of the mangrove. A high concentration of metals in sediment due to high bioavailability is associated with the concentration of these metals in the mangrove plant (Marchand, Fernandez, & Moreton, 2016).

The concentration of heavy metals Pb, Cu and Zn in mangrove sediment is high in environments that have significant human-induced stressors. A high concentration of Pb, Cu and Zn was found in the sediment of the mangrove ecosystem near Yaibu City, the Red Sea Coast of Saudi Arabia, resulting from industrial effluent, domestic runoff and sewage (Abohassan, 2013). Maldonado-Román et al. (2012), who conducted a similar study in Peninsula La Esperanza in the northern coast of Puerto Rico, reported that mangrove vegetation presence in coastal areas tended to accumulate high amounts of heavy metals including Pb, Cu and Zn in their sediment, predictably from clandestine dumpsites and thermo-electrical refineries. Furthermore, with regards to anthropogenic input, the concentration of Pb, Cu and Zn found in the surface sediment of Sundarban Mangrove Ecosystem, Bangladesh, in 2012 was significantly associated with non-point sources (agricultural activities) and point-sources (jetties and boat activities) (Kumar et al., 2016).

Concentration of Pb, Cu and Zn in Mangrove Parts

It has been proven that mangrove tissue has high capacity for absorbing and accumulating heavy metals from sediment. Excess metals in sediment tend to be distributed among mangrove tissue depending on mobility of heavy metals (Kumar, Sajish, Kumar, George, & Viyol, 2010). The roots of four studied mangrove species accumulate heavy metals Pb, Cu and Zn in varying concentration, in the proportion Zn>Cu>Pb. This pattern is likely due to the concentration of metals in the sediment near their roots. Moreover, the higher value of Zn and Cu in roots of all studied mangrove species found in this estuary was likely due to their function as an essential element of mangrove plants. Pb was found at low concentration in the roots, ranging from 0.004-0.051 mg/kg, because Pb content in the sediment (0.059-0.068 mg/kg) is also low. This is due to the fact that Pb is a non-essential element, and it is more

immobilised in the sediment. In addition, Pb has low solubility in acidic sediment such as the mangrove ecosystem (Usman, Alkredaa, & Al-Wabel, 2013). Concentration of Zn, Cu and Pb in the roots and leaves of four mangrove species found in the Porong River estuary is listed in Table 2 and Table 3, respectively.

Table 2
Average of metal concentration (mg/kg dry weight) in roots of mangrove species at Porong River estuary

Species	Pb	Cu	Zn	Species	Pb	Cu	Zn
	Mangrove Roots Tissue				Mangrove Roots Tissue		
	Avicennia alba				*Avicennia marina*		
Mean	0.0505	0.3390	1.6385	Mean	0.0437	0.2917	3.1375
SD	0.0672	0.1428	0.0940	SD	0.,0520	0.1918	3.9769
Maximum	0.0980	0.4400	1.7050	Maximum	0.1390	0.6100	11.2000
Minimum	0.0030	0.2380	1.5720	Minimum	0.0030	0.0700	1.0260
% CV	133.02	42.13	5.74	% CV	119.11	65.78	126.75
	Sonneratia alba				*Rhizopora mucronata*		
Mean	0.0038	0.2658	1.3270	Mean	0.0320	0.0865	1.6385
SD	0.0010	0.0689	0.1859	SD	0.0240	0.0332	0.0799
Maximum	0.0050	0.3440	1.5990	Maximum	0.0490	0.1100	1.6950
Minimum	0.0030	0.1780	1.2060	Minimum	0.,0150	0.,0630	1.5820
% CV	25.53	25.92	14.01	% CV	75.13	38.42	4.88

Table 3
Average of metal concentration (mg/kg dry weight) in leaves of mangrove species at Porong River estuary

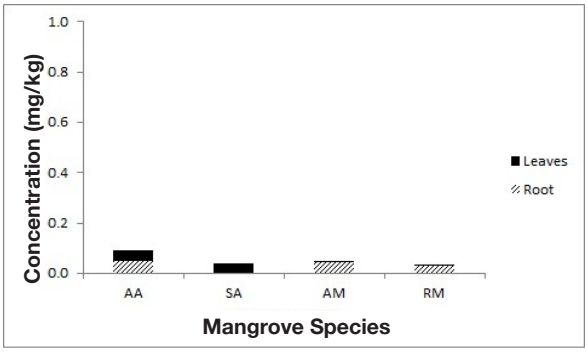
Species	Pb	Cu	Zn	Species	Pb	Cu	Zn
	Mangrove Leaves Tissue				Mangrove Leaves Tissue		
	Avicennia alba				*Avicennia marina*		
Mean	0.0395	0.1490	3.6735	Mean	0.0072	0.1908	1.9198
SD	0.0163	0.0325	0.9383	SD	0.0059	0.0988	0.9832
Maximum	0.0510	0.1720	4.3370	Maximum	0.0190	0.3180	3.3280
Minimum	0.0280	0.1260	3.0100	Minimum	0.0044	0.0400	0.8830
% CV	41.17	21.83	25.54	% CV	81.71	51.80	51.21
	Sonneratia alba				*Rhizopora mucronata*		
Mean	0.0348	0.1145	3.0250	Mean	0.0042	0.0655	1.7665
SD	0.0197	0,0635	2.5536	SD	0.0003	0.0445	1.3739
Maximum	0.0620	0.2080	6.7630	Maximum	0.0044	0.0970	2.7380
Minimum	0.0150	0.0660	1.3020	Minimum	0.0040	0.0340	0.7950
% CV	56.60	55.50	84.42	% CV	6.73	68.01	77.78

The concentration of Pb, Cu and Zn in the roots of *A. marina* in the Porong River estuary are significantly lower than those reported in the Sydney estuary (153, 189 and 378 mg/kg for Cu, Pb and Zn, respectively). The average concentration of these metals in sediment is 95, 42 and 196 mg/kg for Pb, Cu and Zn, respectively (Chaudhuri, Nath, & Birch, 2014). *A. alba* in Cochin, India was observed to accumulate Cu, Pb and Zn in its roots as much as 53.6, 3.3 and 1.0 mg/kg (Harish & Murugan, 2011). This concentration is also higher than for *A. alba* in the Porong River estuary. The heavy metal content in sediments in Cochin, India is also higher than in the Porong River estuary. It can be concluded that bioavailability of metals in sediments seem to be in line with their concentration in the mangrove plant.

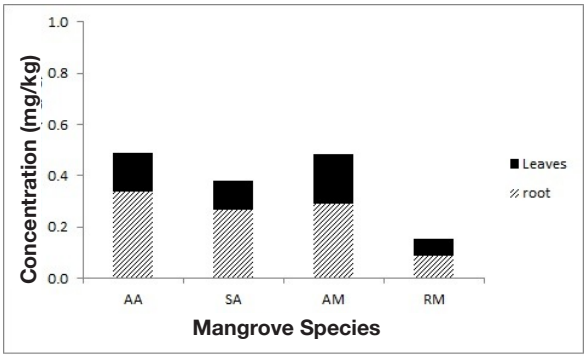
As seen in Table 3, there are similar trends for accumulation of Pb, Cu and Zn in Porong River estuary sediments in mangrove roots and leaves. The highest concentration in leaves were of Zn, followed by Cu and Pb. This trend is similar to that reported in another study (Mn>Cr>Zn>Ni>Cu>Pb>Cd) (Lotfinasabasl & Gunale, 2012). The difference among concentration of the metals Pb, Cu and Zn in the roots and leaves of the mangrove species found in the Porong River estuary is illustrated in Figure 3.

Among the four studied mangrove species, a greater amount of Pb is accumulated in the roots of *A. alba, A. marina* and *R. mucronata*. The genus Avicennia is proven to accumulate a large amount of heavy metals including Pb, Cu and Zn (Harish & Murugan, 2011). Only the species *S. alba* distributes Pb among its leaves more than among other parts. Genus Sonneratia readily translocates Pb and Zn to the upper parts such as the leaves (Lotfinasabasl & Gunale, 2012). In contrast, Cu is primarily accumulated in the roots of all the species found in the Porong River estuary, while the concentration of this metal in the leaves is approximately 1.5-2 times lower than it is in the roots. Zn is accumulated more in the leaves of the species *A. alba, S. alba* and *R. mucronata*. However, *A. marina* preferred to accumulate Zn more in its roots compared to in its leaves. In line with this, another study found that the highest concentration of Zn in A. marina from mangroves in the Red Sea was also in the roots, approximately 36.8 mg/kg (Usman, Alkredaa & Al-Wabal, 2013).

Variability of heavy metal absorption in different parts of mangrove might be influenced by several factors. A previous study reported that the factors of sampling time, tissue age, morphology and physiology may cause varying rates of accumulation in mangrove parts (Lewis, Pryor, & Wilking, 2011).



(a)



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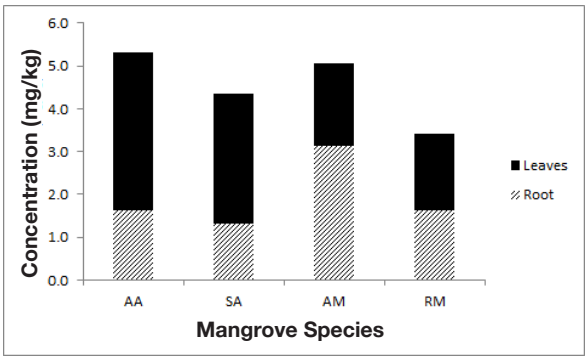


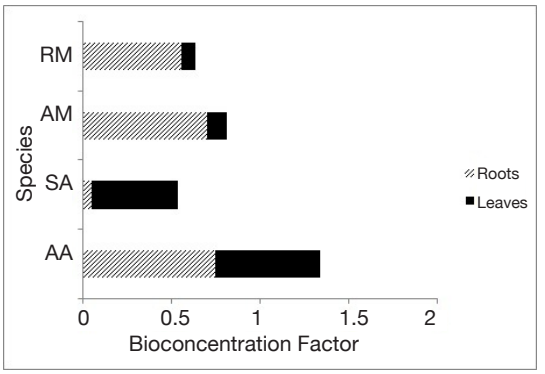
(c)

Figure 3. Comparison between concentration of heavy metals (a) Pb, (b) Cu and (c) Zn in mangrove parts in Porong River estuary (AA : *Avicennia alba*, SA: *Sonneratia alba*, AM: *Avicennia marina* and RM: *Rhizopora mucronata*)

The BCF and TF of Pb, Cu and Zn in Mangrove Plants

To evaluate pollutant absorption capability of mangroves, the bio-concentration factor, BCF, was used (Wu et al., 2014a). The BCF of four mangrove species is presented in Figure 4. Genus Avicennia had the highest BCF in the roots for Pb and Cu. Specifically,

A. alba accumulated significantly higher levels of Pb in both its roots and leaves compared with the other mangrove species. Moreover, this study also revealed that the highest accumulation of Cu in the leaves was also by *A. marina*. The presence of Cu in leaves is influenced by the metabolic need for essential micronutrients such as Cu (Qiu, et al., 2011). The Avicennia genus accumulates Cu in greater amount compared



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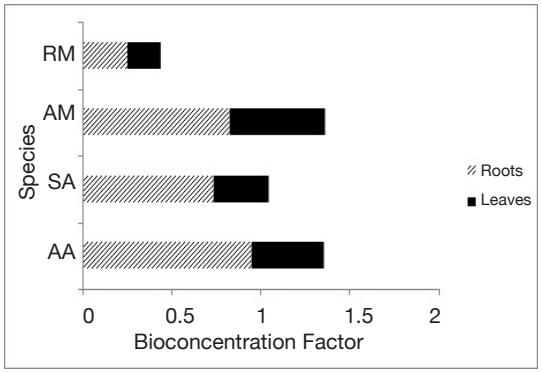

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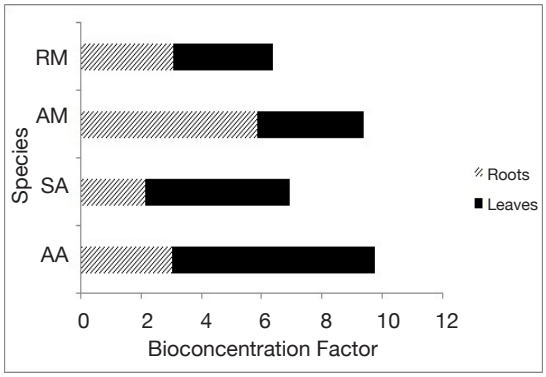

(c)

Figure 4. Comparison between bioconcentration of (a) Pb, (b) Cu and (c) Zn in mangrove parts in Porong River estuary (AA: *Avicennia alba*, SA: *Sonneratia alba*, AM: *Avicennia marina* and RM: *Rhizopora mucronata*)

to other mangrove species. This concurs with the findings of Parvaresh et al. (2011), who found that *A. marina* accumulated Cu to a greater extent than did the other species. For Zn absorption, the average BCF at the roots of *A. marina* was considerably higher than in the other species, while in the leaves, it was greater in *A. alba*. Hence, the BCF for Zn of *A. alba* was only slightly higher than the BCF of *A. marina*. However, the BCF for Zn in the mangrove species *R. mucronata* and *S. Alba* was lower than the BCF of genus Avicennia.

The BCF for Cu in the mangrove species *Rhizopora stylosa* from Dumbea, New Caledonia in the main roots and leaves was found to be 0.86 and 0.52, while the BCF for Zn was found to be 0.48 and 0.16 (Marchand, Fernandez & Mareto, 2016). This number is lower than the BCF of Cu in the roots of the genus Avicennia from this study. Moreover, the leaves of *R. stylosa* from Dumbea have a BCF of Cu that is higher than the BCF of Cu in the leaves of *A. alba, S. alba* and *R. mucronata* in this study. However, the BCF of Zn in the roots and leaves of *R. stylosa* from Dumbea is lower than the BCF of Zn in the roots and leaves of all studied species in the Porong estuary.

TF values indicate heavy metal movement rate (from roots to leaves) throughout the plant. TF of the four Porong River estuary mangrove species for Pb, Cu, and Zn ranged from 0.1375-9.2667, 0.4309-0.7572 and 0.6119-2.2796, respectively (Figure 5). The highest TF for Pb and Zn belong to *S. alba*, while *R. mucronata* had the highest Cu TF value.

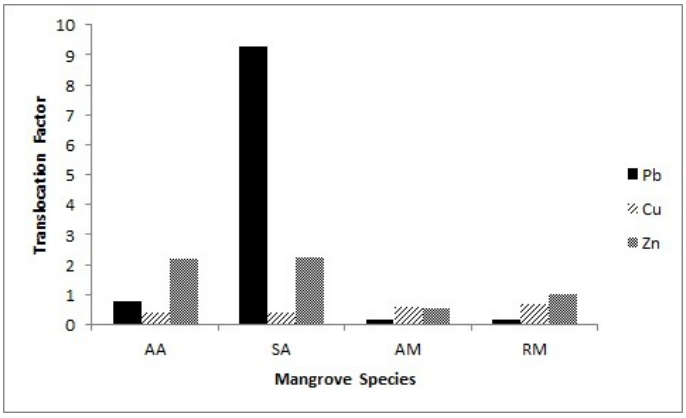

Figure 5. Translocation factor in mangrove species in Porong River estuary

TF in the mangrove species *Sonneratia apetala* and *Cyperus malaccensis* in Nansha Mangrove, South China is 0.205 and 0.110 for Cu and 0.364 and 0.049 for Pb (Wu et al., 2014b). TF for Cu in both mangrove species endorsed by Wu et al. (2014b) is lower than that of all studied mangroves in this study. However, *S. alba* and *A. alba* have higher concentration of TF for Pb than the species *S. apetala* and *C. malaccensis* in Nansha Mangrove.

The high BCF and TF values indicate that these species are able to accumulate heavy metals effectively. The highest BCF for Pb and Cu was in the roots of *A. alba.* However, the BCF values of these metals were almost less than 1. Moreover, the highest BCF for Zn was in the leaves of the same plant. Thus, it can be concluded that Pb and Cu are not accumulated in the mangrove effectively, while Zn is confirmed to be the metal most accumulated in parts of the mangrove plant.

CONCLUSION

Pb, Cu and Zn concentration in sediment of the Porong River estuary was below the sediment quality guidelines. *A. alba* accumulated high concentration of these metals in both the roots and leaves, recording the highest BCF among all the mangrove species. However, the mobility of Pb and Cu was greater in *S. alba*, while Zn was mostly translocated in *R. mucronata*. The capability of *A. alba* of accumulating Pb, Cu and Zn was significantly higher compared to that of other species in the East Java Porong River estuary, indicating its suitability for phytoremediation of heavy-metal pollution in the study area.

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Patterns of Biomass Allocation in Upland Rice Cultivars Grown on Soils along a Toposequence

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ABSTRACT

Biomass allocation to root, culm, leaf, and grain of 20 upland rice cultivars was assessed in pots set up in an open field experiment. The cultivars consisted of 18 upland NERICA rice (N 1 to N 18) and Funaabor 1 and 2. The rice cultivars were grown under rainfed condition on 5 kg soils collected along a toposequence designated as Upper Crest (UC), Middle Slope (MS), and Valley Bottom (VB) soils. Plants were harvested at maturity for biomass allocation pattern into various organs. Toposequence soils and cultivar significantly ($p<0.01$) influenced all the fractions of biomass and standing biomass to different organs except root dry weight (RDW) and root mass fraction (RMF) for the latter. The lowest standing and fraction of biomass to root (3.64g and 0.08 respectively), culm (17.92g and 0.42 respectively), and leaf (3.59g and 0.08 respectively) of the rice cultivars were observed on VB soils. Principal Component (PC) biplot accounted for 95.67% and 97.38% of the total variation in standing biomass and fractions of biomass to organs respectively. Higher grain weight per plant was observed in NERICA 2 and 15 and was closely associated with standing biomass to root than to culm and leaf. Upland rice grown on UC soil accumulated more biomass to vegetative parts with concomitant decrease in fraction of biomass allocated to reproductive structures unlike rice cultivars grown on VB soils. Hence, growing upland rice on VB of a toposequence promotes increase of biomass allocation to grains.

Keywords: Biomass fractions, PC biplot, standing biomass, toposequence, upland rice

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INTRODUCTION

The proportion of biomass partitioned to various organs of a plant is a key feature in its survival strategy (Sultan, 2001; Poot & Lambers, 2003; Grigg et al., 2010; Pichancourt & van Klinken, 2012). The roots, stems, leaves, and grains are the main organs within which biomass accumulated by plants are being partitioned and this is done at the expense of other organs involved in the partitioning process. The proportion of total biomass that is partitioned to these organs is influenced by plant size, its growth environment and species (Niklas, 1994; Reich, 2002).

A widely observed feature of tropical upland rice ecology is the unpredictability of rainfall as well as hot and dry conditions which create variable drought conditions at different stages of crop growth. Reduced biomass allocation to leaves as compared to stems or roots is one of the features of plants adapted to these conditions (Callaway et al., 1994; Bazzaz & Grace, 1997; Roa-Fuentes et al., 2012). The NERICA rice cultivars are released for cultivation in similar environments where continuous access to water during growth cycle is almost impossible. Ability to cope with periodic drought through the deep root system is the major characteristics of these rice cultivars. Increasing allocation of biomass to the root is one of the ways by which plants achieve extensive root system development. Based on the Optimal Partitioning Theory (OPT), plants partitioned biomass to various organs depending on the need and demand for growth by the latter and at a proportion that ensures balance in growth of all organs (McCarthy & Enquist, 2007; Zhang et al., 2015). The difference in potential of upland rice cultivars for biomass yield and allocation to different organs, especially root when grown in different soils, therefore, needs to be explored.

Soils play a major role in influencing biomass allocation to roots. Soil condition can influence biomass allocation to different component of plants (Chaudhary et al., 2015). The fraction of biomass allocated to reproductive structure, otherwise termed harvest index, is also highly influenced by environmental factors like soil conditions (Yoshida, 1981; Dalling, 1985). Physical, chemical, and biological properties of soil can influence root growth (Bengough et al., 2011) and this can influence biomass accumulation and distribution in plants. Variation in edaphic properties of different soil types related to topography can influence productivity of annual crops (Cambardella et al., 2004; Ontl et al., 2013). Soil texture is one of the important soil physical properties that influences root growth (Alameda et al., 2012; Kobaissi et al., 2013) and is characterised by relative proportion of sand, silt, and clay. The proportion of these soil components determines the amount of available pores within which root grows (Giménez et al., 2002; Dexter, 2002; Keith & Buchan, 2002). Soil physical and chemical properties change with changing slopes due to deposition of sediments carried from upper slopes to lower slopes along a toposequence (Cambardella et al., 2004). Due to reduced penetration resistance, plants

grown on light soils with larger number of pores have the tendency to grow more roots unlike heavy soils with reduced soil pores and high penetration resistance (Bengough et al., 2011). The survival of rice plant, especially under moisture limitation, is expected to be influenced by type of soil on which it is grown.

Biomass allocation and partitioning are used interchangeably in literature referring to the standing biomass in roots, stems, and leaves at a particular point in time and dividing accumulated biomass to root, stem, and leaves (Reich, 2002; McCarthy & Enquist, 2007). Many studies have also been conducted on biomass allocation in plants but only a few of such studies were reported on upland rice cultivars at harvest. In this study, we focused on standing biomass and fractions of biomass to these various organs of upland rice cultivars.

The use of biomass fractions in the analyses of allocation patterns has been criticised; nevertheless, it provides an easy-to-understand scaling relations between biomass allocated to organs (Poorter et al., 2012). Analysing fractions of biomass allocated to different organs could provide a means of linking plant biomass investment to different plant functions under contrasting environmental condition (Poorter & Nagel, 2000; Poorter et al., 2012; Kumordzi et al., 2016). Modification of biomass allocation to different organs is one opportunity to improve yield in wheat (Xie et al., 2016). Differences in allocation of biomass to different organs of rice could also be the major contributor to differences in yield. Therefore, understanding the proportion of biomass allocated to different organs of rice is a prerequisite to identifying upland rice with potential for higher yield. The objectives of this study are to address the following hypotheses, namely (1) Biomass allocation to different organs are the same in upland rice cultivars, (2) Soils collected along a toposequence influence biomass allocation to different organs similarly among upland rice cultivars and, (3) Grain weight in upland rice is influenced by standing biomass or fractions of biomass to other organs

MATERIALS AND METHODS

NERICA rice

NERICA rice are selections from crosses of *Oryza sativa* and *Oryza glaberrima*, which are known for their high yields and disease resistance respectively. NERICA 1 to 7 were released in 2000 (Kaneda, 2007; Manneh & Ndjiondjop, 2008) while 11 NERICA cultivars i.e. NERICA 8 to 18 were released in March 2005 (Semagn et al., 2006). The 18 NERICA rice cultivars were bred for upland condition and are released to cope with the present erratic rainfall that characterised most part of the tropics (Wainaina et al., 2015). NERICA 1 to 18 and the two other upland rice cultivars, Funaabor 1 and Funaabor 2, also called "Ofada white" and "Ofada Gold" respectively, are mostly cultivated in rainfed areas. The NERICA cultivars were sourced from African Rice (WARDA) Ibadan station while the two Funaabor cultivars were sourced from

Federal University of Agriculture, Abeokuta (FUNAAB).

Experimental sites

The study was conducted in an open field in the premises of College of Agricultural Sciences, Olabisi Onabanjo University Ayetoro Campus. The Campus is located in derived savannah ecology of South Western Nigeria (6.5°N, 10°E). The area is characterised with rainfall pattern different from its neighbouring town. The topography of the area (where the soils were collected) slopes towards a river which is found in the western part of the area.

Experimental method

Soil sample collection and analyses. Soils were collected along the toposequence which stretched from the upper to the lower of the slope near the river side. Soil samples were taken from the upper, middle, and valley bottom of the slope designated as upper crest (UC), middle slope (MS), and valley bottom (VB) respectively. The different toposequence soils were scooped with shovel up to 15 cm depth, homogenised, bagged, and taken to nursery station within the field where 5 kg each of the soil samples were later potted. Sub-sample of each toposequence soil was collected, air dried, and sieved with 2 mm sieve for routine analyses in soil laboratory. Particles size distribution was determined by the hydrometer method (Bouyoucos, 1951). The pH of the soils in soil water ratio of 1:2 was determined using electrode pH meter. Using 1N ammonium acetate (NH_4OAc pH 7.0), exchangeable bases –Calcium (Ca), Magnesium (Mg), Potassium (K), and Sodium (Na), were extracted after which Ca and Mg were subjected to reading on Atomic Absorption Spectrophotometer (AAS) while K and Na were determined with flame photometer. The effective cation exchange capacity (ECEC) was estimated using summation method. The ratio of sum of exchangeable cations to ECEC expressed in percentage was used in calculating base saturation. Total N were determined by micro-Kjeldahl method. Organic carbon content was determined using dichromate ($K_2Cr_2O_7$) as an oxidising agent (Walkley & Black, 1934). Available P was determined colourimetrically using Bray-1 method.

Seedling establishment and plant sample collection. The 20 upland rice cultivars were sown in pots containing 5 kg soils. The twenty upland rice cultivars were each sown in pots prepared for the nursery. The rice cultivars were nursed for 21 days in pots using the same soil collected from the upper slope in order to ensure uniformity in growth conditions of seedlings at initial stage of growth. The seedlings were later transplanted into pots already prepared using the different soils collected along the toposequence. Two weeks after transplanting (WAT), NPK 15:15:15 (15% N: 15% P_2O_5: 15% K_2O) fertiliser was applied at a recommended rate of 40 kg: 40 kg: 40 kg ha^{-1}. At reproductive stage (4 WAT); the

second dose of nitrogen fertiliser at 60 kg ha^{-1} of nitrogen was applied using urea. The experiment was rainfed and supplemental irrigation was applied at 40mm of water/pot/day for 21 days- a long dry spells that characterised the area during the cropping period. At harvest maturity, whole plant parts were carefully recovered from the pots by dipping the root with soils in water to loosen the soils. Roots and other plant parts were carefully removed and were separated into root, culm, leaf, and grain. Plant parts were oven dried at 65°C to constant weight after which they were weighed again and recorded. Fractions of total biomass for each organ were estimated as follows:

$$\text{Organ biomass fraction (g/g)} = \frac{\text{Dry weight of organ (g/plant)}}{\text{Total biomass including grains (g/plant)}}$$

Statistical analyses

Data collected on weight of different organs were subjected to Analyses of Variance (ANOVA) using Genstat software package, 12th Edition (Payne et al., 2009). The design used was the Complete Randomized Design (CRD). Significant treatment means for toposequence soils and cultivars were later separated using Duncan Multiple Range Test (Gomez & Gomez, 1982). Data on fractions of biomass that violates the assumption of ANOVA were transformed by using square root transformation. Principal Component Analysis (PCA) was conducted to assess the new variables that formed among RDW, CDW, LDW, and yield in contributing to the variation (explanation rates) in standing biomass, and among RMF, CMF, LMF, and HI for fraction of biomass variation. The PCA was also descriptively used to assess the relationships among the recorded variables.

RESULTS

Table 1 shows the physico-chemical properties of different soils used in growing the rice cultivars. The textural class of UC and MS soil is loamy-sand while that of VB was sandy-loam and sand proportions among them were 83.20%, 85.20% and 74.20% respectively. The upper crest and middle slope soils had similar proportion of silt (12.60 and 10.40) and clay (4.20 and 4.40) respectively while valley bottom soil had highest silt (13.60) and highest clay (12.20) content than the other two toposequence soils. The pH of the soils was moderately acidic to neutral (6.35 to 7.00). Effective cation exchange capacity (ECEC) was higher in upper crest soil (26.24 cmol kg^{-1}) than the other two toposequence soils (20.27 in MS and 16.81 cmol kg^{-1} in VB). Total organic carbon (Total Org.C) and total nitrogen (Total N) was high in UC soil (3.59 and 0.24%) compared with MS (1.67 and 0.14%) and VB (1.69 and 0.15%) soils respectively. Available phosphorus was high in UC soil (9.31 mg kg^{-1}) when compared with VB soils (3.25 mg kg^{-1}).

Table 1
The physico-chemical properties of soils collected along a toposequence

Soil Property	Upper Crest soil	Middle slope soil	Valley bottom soil
Sand (%)	83.20	85.20	74.20
Silt (%)	12.60	10.40	13.60
Clay (%)	4.20	4.40	12.20
Textural class	Loamy sand	Loamy sand	Sandy loam
pH (H_2O)	7.00	6.35	6.55
Ca (cmol kg^{-1})	19.36	15.26	11.83
Mg (cmol kg^{-1})	4.38	3.54	3.34
Na (cmol kg^{-1})	1.60	0.94	1.04
K (cmol kg^{-1})	0.85	0.46	0.54
Al+H (cmol kg^{-1})	0.05	0.07	0.06
ECEC (cmol kg^{-1})	26.24	20.27	16.81
Base saturation %	99.81	99.65	99.64
Total N (%)	0.24	0.14	0.15
Total Org. C. (%)	3.59	1.67	1.69
Available P. (mg kg^{-1})	9.31	5.83	3.25

The mean square values of all the standing biomass as well as fractions of biomass to different organs for toposequence soils and cultivars are shown in Table 2. Toposequence soils and cultivars exhibited significant effect ($p<0.01$) on all the standing biomass and fractions of biomass with the exception of RDW and RMF for cultivar effect. Interaction between toposequence soils and cultivars were significant only for total biomass (TB) and yield per plant among the variables.

Differences in toposequence soils and cultivars were observed in all the variables with the exception of RMF and RDW for cultivar effect (Table 3). The lowest biomass and fractions of biomass were recorded in valley bottom with the exception of harvest index (0.41) and yield $plant^{-1}$(17.75 g). Funaabor 2 had the highest CDW (29.32 g) and leaf dry weight LDW (7.18 g) as well as highest LMF (0.15) and CMF (0.59). The cultivar however, recorded the lowest harvest index HI (0.15) and grain (7.70 g).

Figure 1 shows the interactions of cultivars by toposequence soils on total biomass (TB) of upland rice cultivars grown on soils collected along a toposequence. The TB accumulated by the rice cultivars across the toposequence soils was similar with the exception of few cultivars. NERICA 9 accumulated the highest TB when grown in UC and MS than in VB while NERICA 16 accumulated higher biomass when grown in UC than in MS.

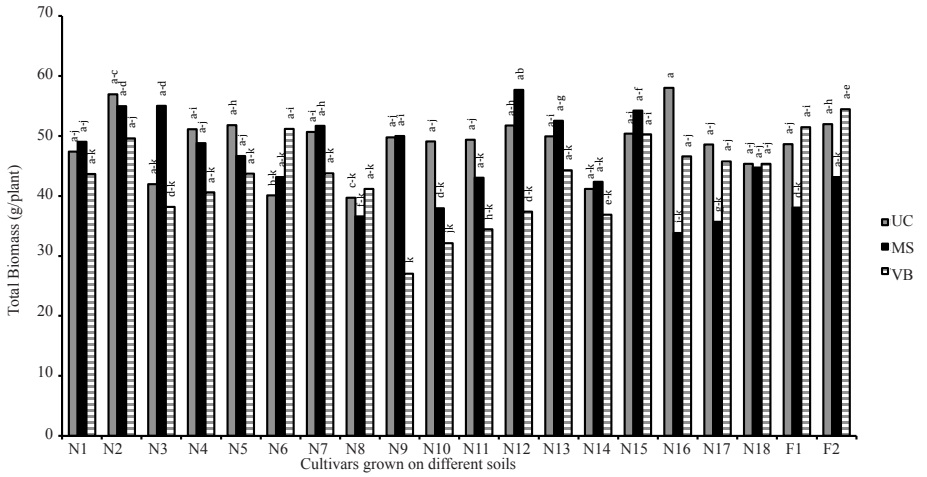


Figure 1. Interactions of Cultivars x Toposequence soils on Total Biomass of upland rice cultivars grown on soils collected along a toposequence

Note: UC= Upper Crest; MS= middle slope; VB= valley bottom; N1 to N18= NERICA 1 to 18; F1 & 2 = Funaabor 1 and 2. Bars with the same letter(s) are not significantly different from one another using Duncan Multiple Range Test (DMRT) at p=5% among all treatment combinations of soils x cultivars

Interaction of toposequence soils by cultivar on yield plant-1 of the rice cultivars when grown on soils collected along a toposequence is shown in Figure 2. The grain weight per plant of the rice cultivars were similar across the toposequence soils with the exception of NERICA 4, 16, 17 and 18 where grain produced when grown in UC soil were higher than those grown in MS soil. NERICA 6, and Funaabor 1 and 2 however, produced higher grain weight per plant when grown in VB than in UC and MS. Table 4 shows the Eigen vector loadings

Table 2

Mean square values of standing biomass and fractions of biomass to organs of upland rice cultivars grown on soils collected along a toposequence

Source of variation	RDW	CDW	LDW	RMF	CMF	LMF	TB	HI	Yld plt^{-1}
Topo_soil	90.41**	1056.67**	52.21**	0.021**	0.330**	0.020**	712.73**	0.720**	1129.47**
Cultivar	7.81ns	100.18**	9.15**	0.002ns	0.020**	0.004**	185.41*	0.050**	95.32**
Topo_ soil*Cultivar	12.56ns	38.74ns	2.62ns	0.003ns	0.010ns	0.001ns	158.75*	0.010ns	44.24*
Residual	9.06	37.29	2.27	0.002	0.01	0.001	98.50	0.01	29.05

Note: **,* significant at 1% and 5% level of probability respectively. The mean square values for RMF, CMF, LMF, and HI were based on non- transformed data but the associated significances are based on square root transformed data

Topo_soil= Toposequence soil; RMF= root mass fraction; CMF= culm mass fraction; LMF= leaf mass fraction; HI= harvest index; RDW= root dry weight; CDW= culm dry weight; LDW= leaf dry weight; Yld plt^{-1}= yield plant^{-1}; and TB=Total biomass.

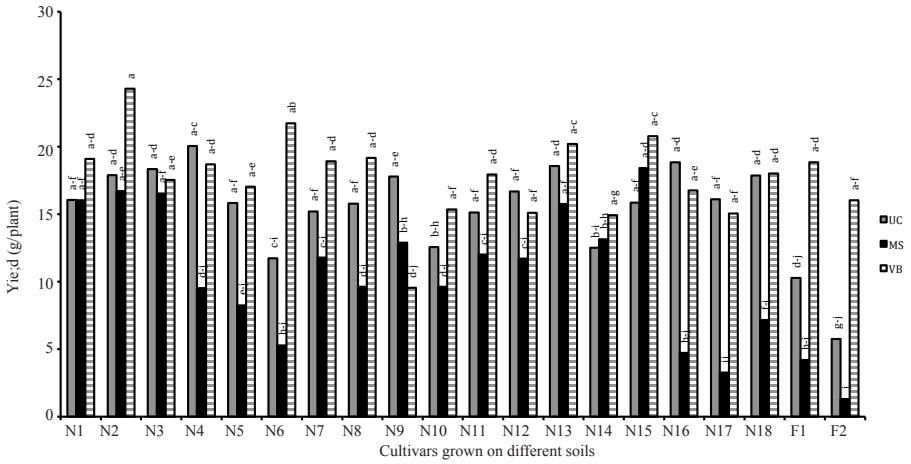


Figure 2. Interactions of Cultivars x Toposequence soils on Yield Plant-1 of upland rice cultivars grown on soils collected along a toposequence

Note: UC= Upper Crest; MS= middle slope; VB= valley bottom; N1-N18= NERICA 1to 18; F1 & 2 = Funaabor 1 and 2. Bars with the same letter(s) are not significantly different from one another using Duncan Multiple Range Test (DMRT) at p=5% among all treatment combinations of soils x cultivars

and explanation rates from PCA for the rice cultivars across and on each toposequence soil. Principal component 1 (PC 1) contributed about 64% explanation rate (ER) of the biomass standing. Based on the eigen vector loadings, PC 1 was basically determined by CDW, yield, and LDW in that order. On the other hand, principal component 2 (PC 2) that was majorly determined by yield and CDW contributed 32% variation of biomass standing. Summing up the PC 1 and PC 2, both had contributed 96% variation in the biomass standing. Culm dry weight, yield and to certain extent of LDW had caused variation in biomass standing. Practically speaking, the RDW had little contributory role for the above variation. As for fraction of biomass variation, PC 1 had almost played total role in the variation, i.e. with ER of 92%. Principal component 1 was determined by HI and CMF. This may indicate partitioning was to culm and grain (trade-off). Other principal components (PC 2 - PC 4) had very little or insignificant role in the variation of fraction of biomass across all types of soil.

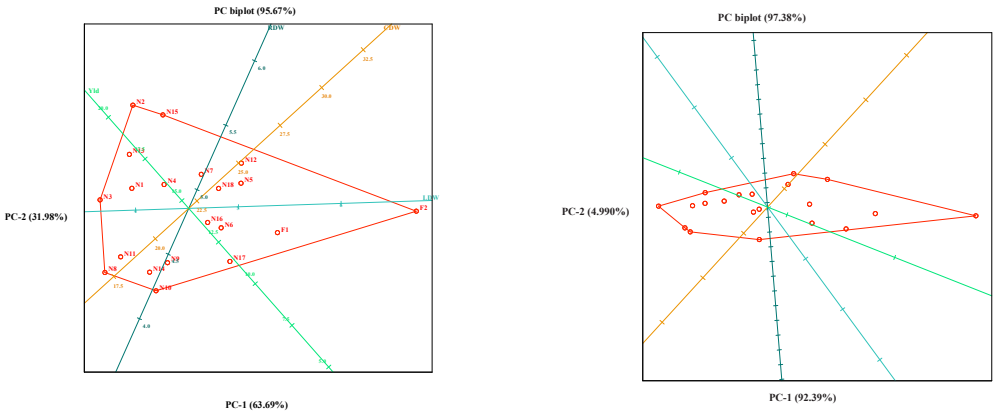
In the case of UC soils, PC 1 contributed 60% ER of the biomass standing. Based on the eigen vector loadings, PC 1 was basically determined by CDW, yield, and LDW in the same order as it was observed in PCA across soil types. On the other hand, PC 2 was majorly determined by yield, CDW, and RDW and contributed 24% variation of biomass standing. The first two principal components had contributed 84% variation in the standing biomass. Basically, RDW and LDW contributed little to the variation in the first and second principal components respectively. As for fractions of biomass variation, PC 1 contributed 74%

and was majorly determined by HI and CMF which also cuts across other toposequence soils. Principal component 2, however, contributed an additional 20% variation in fraction of biomass with RMF, CMF, and HI causing the variation in that order. Summing up these two components, PC 1 and PC 2 contributed 94% of the variation in fraction of biomass for UC soils.

The first and second principal component of MS soil contributed 59 and 34% variations in the biomass standing respectively. Based on the eigen vector loadings, PC 1 was basically determined by yield and CDW in that order and vice-versa for PC 2 while LDW and RDW played little role in the two above variations. Summing up the PC 1 and PC 2, both had contributed 94% variation in biomass standing. As for fractions of biomass, PC 1 alone contributed 88% while PC 2 contributed additional 9% of the variation with RMF and CMF being the main determinants. Summing these two components, both had contributed 97% variation in the fraction of biomass to organs.

In the case of VB soil, PC 1 contributed 64% ER of the biomass standing and based on eigen vector loading, CDW, yield per plant, and RDW are the main determinants of this component. In PC 2, yield per plant and CDW were the main determinants and it contributed 33% of the variation. Summing up the two components, both had contributed 96% of the variation in standing biomass. The PC 1 and 2 for fraction of biomass in VB soils were respectively similar to that of MS soil with PC 1 and 2 also contributing 88 and 9% variation, respectively. Summing up the two components, both contributed 97% variation in fraction of biomass.

Figures 3a and b show the PC biplot for standing biomass and fractions of biomass to various organs of rice respectively. NERICA 2 and 15 had the highest yield which was closely related to RDW than CDW and LDW (Figure 3a). The lowest yield was recorded by Funaabor 2 but with



(a) (b)

Figure 3. PC biplot of (a) standing biomass and (b) fractions of biomass to organs of upland rice cultivars grown on soils collected along a toposequence over all soils

higher LDW. Wider angle of separation was observed between HI axis and CMF axis than that between CMF and LMF axes with Funaabor 2 recording the highest value for CMF and LMF (Figure 3b and Table 3.)

Table 3
Means of standing biomass and fractions of biomass to organs of upland rice cultivars grown on soils collected along a toposequence

Sources of Variation		RDW	CDW	LDW	RMF	CMF	LMF	TB	HI	Yld plt^{-1}
		g/plant			g/g			g/plant	g/g	g/plant
Toposequence Soils	Upper Crest	5.29a	23.23a	4.91a	0.11b	0.47b	0.10b	48.87a	0.32b	15.44b
	Middle Slope	5.63a	24.88a	5.05b	0.12a	0.55a	0.11a	45.96ab	0.22bc	10.40c
	Valley Bottom	3.64b	17.92b	3.59c	0.08c	0.42c	0.08c	42.90b	0.41a	17.75a
Cultivars	Nerica 1	4.15a	20.69b-e	4.78b-e	0.09a	0.44c-e	0.10b-e	46.68a-d	0.37a-d	17.06a-d
	Nerica 2	6.86a	22.98b-e	4.37b-e	0.12a	0.42de	0.09ef	53.84a	0.37a-d	19.64a
	Nerica 3	4.52a	19.35b-e	3.71c-e	0.10a	0.42e	0.08ef	45.05a-d	0.41a	17.48a-d
	Nerica 4	4.54a	21.98b-e	4.23b-e	0.09a	0.47b-e	0.09d-f	46.84a-d	0.35a-e	16.04a-e
	Nerica 5	4.96a	24.68a-c	4.07c-e	0.10a	0.53ab	0.09d-f	47.40a-d	0.28d-g	13.70b-e
	Nerica 6	5.57a	22.54b-e	4.77b-e	0.10a	0.50b-d	0.11b-e	44.81a-d	0.29b-g	12.91c-e
	Nerica 7	5.28a	23.40b-e	4.72b-e	0.10a	0.47b-e	0.10b-f	48.71a-d	0.32a-f	15.30a-e
	Nerica 8	3.47a	17.49 e	3.33d-e	0.09a	0.45b-e	0.09d-f	39.16d	0.37a-d	14.87a-e
	Nerica 9	4.42a	19.59b-e	4.86bc	0.09a	0.46b-e	0.11b-d	42.28b-d	0.33a-f	13.41b-e
	Nerica 10	5.51a	18.43de	3.26e	0.14a	0.46b-e	0.09d-f	39.71d	0.32a-g	12.51d-e
	Nerica 11	4.47a	18.17de	4.62b-e	0.10a	0.42e	0.11b-e	42.28b-d	0.38a-c	15.03a-e
	Nerica 12	4.45a	25.27ab	4.74b-e	0.09a	0.51b-d	0.10b-f	48.96a-d	0.31a-g	14.50a-e
	Nerica 13	4.88a	21.64b-e	4.23b-e	0.10a	0.44c-e	0.08ef	48.92a-d	0.38ab	18.17a-c
	Nerica 14	3.87a	18.93c-e	3.82c-e	0.10a	0.48b-e	0.09c-f	40.15a-d	0.34a-f	13.53b-e
	Nerica 15	5.60a	24.02a-d	4.07c-e	0.11a	0.47b-e	0.07f	51.64ab	0.36a-e	18.35ab
	Nerica 16	5.84a	22.04b-e	4.81b-d	0.12a	0.50b-e	0.11b-e	46.13a-d	0.28e-g	13.44b-e
	Nerica 17	5.22a	21.68b-e	4.97bc	0.12a	0.51bc	0.12a-c	43.35b-d	0.25fg	11.48ef
	Nerica 18	3.76a	23.83a-d	4.47b-e	0.08a	0.51bc	0.10b-f	46.41a-d	0.31b-g	14.35b-e
	Funabor 1	5.10a	24.12a-d	5.73b	0.11a	0.53ab	0.13ab	46.06a-d	0.23g	11.11ef
	Funabor 2	5.65a	29.32a	7.18a	0.12a	0.59a	0.15a	49.86a-c	0.15h	7.70f

Means followed with same letter within a column for each factor are not different at p=5% by DMRT. The mean comparisons (rankings) associated with RMF, CMF, LMF, and HI are based on the square-root transformed data. RMF= root mass fraction; CMF= culm mass fraction; LMF= leaf mass fraction; HI= harvest index; RDW= root dry weight; CDW= culm dry weight; LDW= leaf dry weight; Yld plt^{-1}= yield plant^{-1}; and TB=Total biomass

Table 4
Eigen vector loadings and explanation rates from Principal Component Analyses (PCA) of standing biomass and fractions of biomass based on culm, leaf, root, and grains of upland rice cultivars grown on soils collected along a toposequence

Toposequence soils	Rice organs	Standing Biomass				Organs biomass fraction	Fractions of Biomass			
		PC 1	PC2	PC3	PC4		PC 1	PC2	PC3	PC4
Over all soils	CDW	0.72	0.66	0.16	0.14	CMF	0.56	0.61	0.25	0.50
	LDW	0.21	0.01	-0.09	-0.97	LMF	0.19	-0.25	-0.81	0.50
	RDW	0.06	0.14	-0.98	0.10	RMF	0.06	-0.68	0.54	0.50
	Yld plt^{-1}	-0.65	0.74	0.05	-0.14	HI	-0.81	0.32	0.02	0.50
	%ER	63.69	31.98	2.86	1.47	%ER	92.39	4.99	2.62	0.00
Upper Crest	CDW	0.76	0.60	0.23	-0.12	CMF	0.57	0.44	0.49	0.50
	LDW	0.20	-0.07	0.05	0.98	LMF	0.18	0.13	-0.84	0.50
	RDW	0.06	0.30	-0.95	0.06	RMF	0.06	-0.84	0.18	0.50
	Yld plt^{-1}	-0.61	0.74	0.21	0.17	HI	-0.80	0.27	0.17	0.50
	%ER	60.04	23.79	12.90	3.28	%ER	74.29	19.46	6.25	0.00
Middle Slope	CDW	0.20	0.97	-0.14	0.08	CMF	0.56	0.60	0.27	0.50
	LDW	-0.04	0.11	0.18	-0.98	LMF	0.16	-0.19	-0.83	0.50
	RDW	0.03	0.12	0.97	0.19	RMF	0.08	-0.72	0.48	0.50
	Yld plt^{-1}	0.98	-0.19	0.00	-0.07	HI	-0.81	0.31	0.07	0.50
	%ER	59.26	34.45	4.97	1.32	%ER	88.38	9.11	2.52	0.00
Valley Bottom	CDW	0.91	0.32	0.25	0.03	CMF	0.64	0.58	0.09	0.50
	LDW	0.16	0.10	-0.68	0.72	LMF	0.06	-0.39	-0.77	0.50
	RDW	0.23	-0.08	-0.68	-0.69	RMF	0.06	-0.60	0.62	0.50
	Yld plt^{-1}	0.29	-0.94	0.13	0.08	HI	-0.76	0.40	0.06	0.50
	%ER	63.71	32.71	2.66	0.92	%ER	88.22	9.08	2.70	0.00

Note: RMF= root mass fraction; CMF= culm mass fraction; LMF= leaf mass fraction; HI= harvest index; RDW= root dry weight; CDW= culm dry weight; LDW= leaf dry weight; Yld plt-1= yield plant-1; %ER = Percentage explanation rates

DISCUSSION

Earlier findings (Cambardella et al., 2004; Alameda et al., 2012; Kobaissi et al., 2013; Ontl et al., 2013) had established the important role of soils on plant growth and development. This important role of soils is also established in this study. Significant amount of variations in standing biomass and fractions of biomass to organs were captured by toposequence soils as indicated by high mean square values with high significant effect ($p<0.01$) recorded by the soil (Table 2). The toposequence soils belong to different textural classes (loamy sand and sandy loam, due mainly to reduced proportion of sand coupled with increased proportion of clay) observed in valley bottom soil. The higher clay content observed in valley bottom soils could have caused restriction in root growth, especially

with limited moisture and concomitant high bulkiness, resulting in reduced fraction of biomass allocated to the root and other vegetative organs of the rice cultivars as well as standing biomass accumulated to these organs. The increased biomass generated by the rice cultivars grown on upper crest and middle slope soil could be linked to reduced restriction to root growth in these soils compared with valley bottom soils which resulted in overall increase in biomass accumulated to other vegetative parts of rice grown on the soils. Soil exploration by roots is limited by strong mechanical resistance in the soil which is the most common physical limitation to plant roots (Hodge et al., 2009) and it can result in reduced biomass accumulated by upland rice.

Toposequence soils varied more in chemical properties than physical properties and this could have also contributed to variation in standing biomass and fractions of biomass to various organs of rice observed in this study. The higher total organic C, N, ECEC, and available P of UC soils and corresponding low values observed in VB soils appears to be some of the major soil chemical properties influencing biomass fractions and standing biomass to organs of rice (Table 1). Strong relationship between topography and total organic C and N had earlier been reported (Wood et al., 1990; Senthilkumar et al., 2009) and may likely be due to soil redistribution (Pennock et al., 1994). Increased biomass accumulated by the rice cultivars and fractions of biomass to various organs of rice grown in UC soils as compared with reduced biomass and fractions of biomass observed in VB soils are based on the trend of soil nutrient availability. It has been shown that root productivity responds positively to nutrient concentration within soils as predicted by optimal partitioning theory and could vary among plant species (McCarthy & Enquist, 2007). The increased root productivity in UC and MS soils could have contributed to higher biomass observed in the rice cultivars. This is in contrast with the findings of Ontl et al. (2013) who noted non-significant influence of topography or soil properties on root productivity of annual plants.

The variations in standing biomass as well as fractions of biomass to different organs of the rice cultivars indicate differences in potential of the rice cultivars to allocate biomass to different organs. Going by the non-significant effect observed for toposequence soil by cultivar interaction (Table 2), the differences in standing biomass and fractions of biomass to organs among the rice cultivars can be concluded to be relatively similar across toposequence soils meaning that each soil along the toposequence is distinct in its influence on standing biomass and fractions of biomass among various organs of upland rice cultivars. This could as well imply that standing biomass and fractions of biomass to organs could be a reliable means of assessing biomass yield potential of upland rice under fairly contrasting soil environments.

The trend of biomass distribution among various organs of the rice cultivars grown on soil collected along the toposequence revealed the possibility of increased fractions

of biomass to reproductive structure for rice grown on VB soil (Table 3). With higher proportion of clay and lower total N, C, ECEC and available phosphorus in soils collected down the gradient, the amount of biomass accumulated by the rice cultivars decreased coupled with increase in fractions of biomass allocated to reproductive structures (Table 1). This was revealed by higher harvest index in rice cultivars grown on valley bottom soils. It can be deduced that conditions that reduce the amount of biomass allocated to vegetative parts such as higher clay content and lower total N, C, ECEC, and available P of the soil could amount to increased fractions of biomass to the grains (less vegetative growth in VB).

There was a trade-off in grain weight per plant and standing biomass to other organs in rice grown on upper crest soil and between LDW and other standing biomass in middle slope soil under PC1 while no trade-offs in standing biomass between the organs were observed in valley bottom soil (Table 4). However, trade-offs between harvest index (HI) and fractions of biomass to other organs were consistent across the toposequence soils under PC1 while under PC2, trade-offs were between LMF, RMF and CMF, HI. Among the various fractions of biomass assessed, HI contributed most to yield increase of upland rice cultivars. This was also confirmed by the trade-offs between HI and other fractions of biomass i.e. root, culm, and leaf mass fractions as explained by the PC1. Harvest index has been considered as a measure of biological success in biomass allocation to harvestable products (Donald & Hamblin, 1976; Hay, 1995; Sinclair, 1998) and was given high priority in the identification of best genotypes (Tardieu, 2013). The fraction of biomass allocated to the reproductive part was therefore, an important trait to be explored in the identification of high yielding upland rice. The second principal component (PC2) loadings further revealed the trade-offs between CMF and HI, and other fractions of biomass implying that CMF was next to HI among organ's fractions of biomass that could be considered in the identification of high yielding cultivars. This observation confirms previous findings that amount of biomass allocated to culm contributed significantly (about 10-40%) to grain weight of rice at harvest (Gebbing & Schnyder, 1999; Takai et al., 2005) and an inverse relationship exists between these two fractions (Figure 3b).

The trade-offs between standing biomass to grain and other organs of the rice cultivars was comparable to trade-off observed between HI and fractions of biomass to other organs (Table 4). However, this was not consistent across the toposequence soils for standing biomass. This could imply that fraction of biomass allocated to reproductive parts was key in the determination of higher grain weight in upland rice cultivars at harvest and can be more important than actual biomass allocated to this organ. Atlin et al. (2008) reported that grain weight was associated with biomass production and fractions of biomass to grain (harvest index) at vegetative and reproductive stages respectively.

The highest yield observed in NERICA 2 and 15 provided information on the ability of the cultivars to produce reasonably well when grown on contrasting soil environment (Figure 3a). The potential of these two cultivars can be attributed to higher total biomass accumulated across toposequence soils coupled with increased fraction of biomass allocated to the grains. The ability of a cultivar to combine increased biomass with increased fraction of biomass to reproductive parts is therefore, a useful trait to be considered in the breeding of high yielding cultivars.

CONCLUSION

Biomass accumulated by rice and its differential distribution to other organs of the rice plant was a reliable way of estimating plant performance on different soils. Toposequence soils influenced biomass allocation into various organs similarly among upland rice cultivars with most biomass allocated to the culm. However, total biomass and yield were influenced differently by toposequence soils among the rice cultivars. Grain weight per plant is greatly influenced by fractions of biomass compared with standing biomass to organs. Fractions of biomass to other organs, especially the reproductive part, could be a more reliable estimate for identifying rice cultivars with higher yield potential at harvest. Soil environment that promotes greater allocation of biomass to reproductive structure through restriction in expansion of vegetative organs is well suited for upland rice cultivation.

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Enhancement of the Contents of Anticancer Bioactive Compounds in Mutant Clones of Rodent Tuber (*Typhonium flagelliforme* Lodd.) based on GC-MS Analysis

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ABSTRACT

Rodent tuber (*Typhonium flagelliforme* Lodd.), which is a well known herbal plant from the Araceae family, is know for its anticancer activities. The genetic variation of rodent tuber is low due to its commonly applied vegetative propagation methods. Thus, its genetic variation has to be increased to obtain a new and superior plant containing a high amount of anticancer compounds. The aim of this study was to analyse the chemical compounds of the leaves and tubers of rodent tuber' mutant and non irradiated (control) plants by GC-MS method. In this study, *in vitro* calli of rodent tuber was irradiated with 6 Gy of gamma ray which produced mutant plantlets which was genetially different from non irradiated (control) plants. Mutant plantlets had been acclimated and propagated in the greenhouse to obtain the 6th generation vegetative mutant clones (MV6), which are stable superior mutants. The results indicated that MV6 contained six new anticancer compounds in the leaves and four new anticancer compounds in the tubers which have not been detected in control. The new anticancer compounds present in leaves and tubers were identified by GC-MS. They are hexadecanoic acid methyl ester, octadecadienoic acid, phytol, gamma-sitosterol, eicosane, geranylgeraniol, squalene, octacosane and 7-pentadecyne. MV6, is the new superior variety and a potential source of anticancer drugs.

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INTRODUCTION

Rodent tuber (*Typhonium flagelliforme* Lodd.) is a medicinal herbal plant from Indonesia that belongs to the Araceae family (Essai, 1986). Biologically active chemicals in this plant are alkaloids, saponins, steroids, and glycosides (Syahid, 2007). A study has documented the effectiveness of ethanolic fraction of the rodent tuber' extract in inhibiting the growth of T47D breast cancer cell line (Nurrochmad et al., 2011) while another has show the efficacy of its dichloromethane fraction against MCF-7 breast cancer cell line (Putra & Winarto, 2012). The rodent tuber's extract has also been found toinhibit the proliferation of human T4 lymphoblastoid (Mohan et al., 2008) and NCI-H23 non-small lung carcinoma cell line (Lai, et al., 2008). Anticancer compounds could be found in all parts of the plant, such asthe roots, tubers, stems, and leaves (Choo et al., 2001). The hexane extract of rodent tuber was proven to be cytotoxic against *Artemia salina* larvae (Sianipar et al., 2013a). This plant also has antibacterial and antioxidant properties (Mohan et al., 2008).

The main obstacle in the development of rodent tuber into drugs are its low genetic diversity and low organic compounds content (Syahid, 2008). The low genetic variation is due to commonly practiced vegetative propagation methods, mainly through conventional buds separation (Essai, 1986). Although vegetative propagation methods could produce seedlings, these methods rarely cause genetic recombination. Thus, the genetic variation in species level is low and reduce the creation of new genotypes (Syahid & Kristina, 2007). Genetic variation of rodent tuber has to be increased in order to obtain superior mutant clones which contain a high amount of anticancer compounds. Mutation induction is an effective way to its increase genetic diversity (Wulan, 2007). The mutation could be induced by irradiating the respective sample with physical mutagens, such as gamma ray (Poespordasono, 1988). *In vitro* embryogenic somatic cell population or calli of rodent tuber has been induced, proliferated, and regenerated with single node culture method (Sianipar et al., 2011).

In an earlier study, the rodent tuber calli were irradiated with gamma ray. They were regenerated into in vitro plantlets that showed various growth responses based on the observation of the number of shoots, number of leaves, and plant's height (Sianipar et al., 2013b). Rodent tuber calli was irradiated at many doses to induce mutation. Rodent tuber mutation induction successfully done at the dose 6 Gy. The 1st generation plantlet of rodent tuber through gamma rays irradiation can detect genetic changes of mutant by using RAPD (Sianipar et al., 2015b).

In the first generation of vegetative mutant (MV1 generation) 37 mutant clones were discovered which had several different morphological characteristics from control plants (Sianipar et al., 2013c). Out of those, 17 clones had genetic differences with control plants according to PCR-RAPD molecular marker analysis (Sianipar et al., 2015a). These 17 mutant clones were

propagated and regenerated into the 6th generation of vegetative mutant (MV6).

Extraction involves isolation or purification of chemicals from raw samples (Mohrig, 2010). Mutant clones contain a lot of bioactive compounds with various functional groups and polarities (Hota, 2007). Hydrophilic substances were extracted with polar solvents, such as ethanol (Rostagno et al., 2013). Genetic mutation could affect the relative abundances of plant's bioactive compounds. Gas Chromatography-Mass Spectrometry (GC-MS) is able to analyse the metabolomic profile of an organism. GC uses gas as its mobile phase to separate various chemicals. The MS could separate chemicals based on their molecular weight (Kayser & Quax, 2007). The GC-MS is a powerful device to identify chemicals by referring to their databases (Kayser & Quax, 2007) and it has been applied to analyse phytochemical and bioactive compounds of herbal plants, such as *Melia orientalis* (Marimuthu, 2013) and *Maranta arudinacea* L. (Nishaa, 2013). The GC-MS has also been employed to identify chemical compounds in the nonpolar fraction of rodent tuber from Malaysia (Mohan et al., 2011). The aim of this study is to analyse the bioactive anticancer compound content in the polar fraction of rodent tuber stable superior mutant clones by using GC-MS.

MATERIALS AND METHODS

Plant material

The 1st generation of mutant plantlets irradiated by gamma rays (MV1) were acclimatised and propagated in the greenhouse to obtain the 6th generation of vegetative mutant clones (MV6). The 6th generation of vegetative stable superior mutant population (MV6) from Bogor (Sianipar et al., 2013), is in the patenting process. There were 8 mutant MV6 clones (6-3-3-6, 6-1-1-2, 6-3-2-5, 6-9-1, 6-2-5-2, 6-1-2, 6-9-4, 6-2-6-3) and 1 (non-irradiated) for control. Each sample had 9 replicates. The leaves and tubers of rodent tubers underwent metabolite extraction.

Preparation of extract from rodent tuber

The rodent tuber was dried and macerated in 96% ethanol overnight. The solvent was removed after it was filtered through Whatman filter paper No. 1. The concentrated extract was collected and used for GC-MS analysis.

Identification of chemical content with GC-MS

The concentrated ethanol extracts were injected into the GC column. Injection volume was 5µl with 5:1 split ratio and the injection temperature was 250°C. Helium was used as carrier gas with velocity 0.8 µl per minute. Column temperature was set at 70°C with 5°C per minute increment. At 200°C, the temperature was kept constant for 1 minute before it was increased to 280°C at the rate of 20°C per minute. The temperature remained constant for another 28 minutes. Mass spectrometer wasused in electron impact ionisation mode with 70 eV voltage.

The mass spectrum of GC-MS was identified by referring to the National Institute Standard Technique (NIST) database with ≥ 90% fit factor. The content of the compound was calculated by comparing its average peak area to the total area.

RESULTS AND DISCUSSION

The results of fresh and dry weight for each clone (Table 1) are different. Mutant clones 6-1-2, 6-9-4, 6-2-6-3, and 6-2-5-2 had higher stem, leaves and tuber weight than control. Mutant clone 6-2-5-2 had the highest fresh and dry weight compared with control. The highest tuber dry weight was obtained by clones 6-1-2 and 6-2-5-2. The differences in fresh and dry weight are due to genetic changes or mutation, but the increase in fresh or dry weight did not occur in all mutant clones. Genetic changes are not always followed by morphological changes. Sianipar et al. (2013) have shown irradiated calli can lead to genetic changes. These genetic changes are evidenced by genetic variation in 1st generation mutant of the rodent tuber (MV1), 3rd generation mutant (MV3), 4th generation mutant (MV4) by using RAPD (Sianipar et al., 2015a; 2016; 2017).

In vitro culture treatment and gamma ray irradiation could induce chromosomal abberation i.e. the modification of chromosomal number and structure (Surya & Soeranto, 2006; Pillay & Tenkouano, 2011). These modifications can alter morpho-physiological properties of mutant clones (Table 1). The DNA mutation will lead to the generation of new genotypes and affect transcription, translation, protein synthesis and enzyme expressions that leads to production of secondary metabolites (Gorbunova & Levy, 1997; Kovacs & Keresztes, 2002).

Table 1
Fresh and dry weights of rodent tuber's control and mutant clone

Plant	Total fresh weight (g)	Dry weight (g)		
		Total	Leave and stem	Tuber
Field control	150	38,8	8,7	32
In vitro control	100	22,7	NM	19
6-9-1	100	17,3	7,4	13
6-3-3-6	100	26	8,4	20
6-3-2-5	50	20,1	2,5	20
6-1-1-2	50	23	5,7	21
6-1-2	162,5	34,48	14	37
6-9-3	50	17,4	NM	NM
6-9-4	100	38,2	8,9	30
6-1-3-4	50	22	NM	NM
6-2-6-3	200	34,5	13,5	29
6-2-4-1	150	22	NM	NM
6-2-5-2	300	54,8	17,9	37

Note: NM stands for 'Not Measured'

Chemical composition of the leaves, tubers of rodent tuber's control and mutant plants were successfully identified with GC-MS (Figure 1, Tables 2-5), which showed that there were phytochemical profile differences between control and mutant clones as well as between each of the mutant clones. Leaves and tubers of control plants contained 19 and 26 different chemicals respectively (Tables 2 and 3). Five most abundant chemicals in the leaves of control plant were 9,12,15-octadecatrienoic acid or linolenic acid, hexadecanoic acid, stigmasterol, (2E)-3,7,11,15-tetramethyl-2-hexadecen-1-ol, and campesterol (ergost-5-en-3-ol). Meanwhile, five most abundant compounds in the tubers of control plant were (9E,12E)-9,12-octadecadienoic acid, hexadecanoic acid/palmitic acid, methyl (9z,12z)-9,12-octadecadienoate, stigmasterol and ergost-5-en-3-ol.

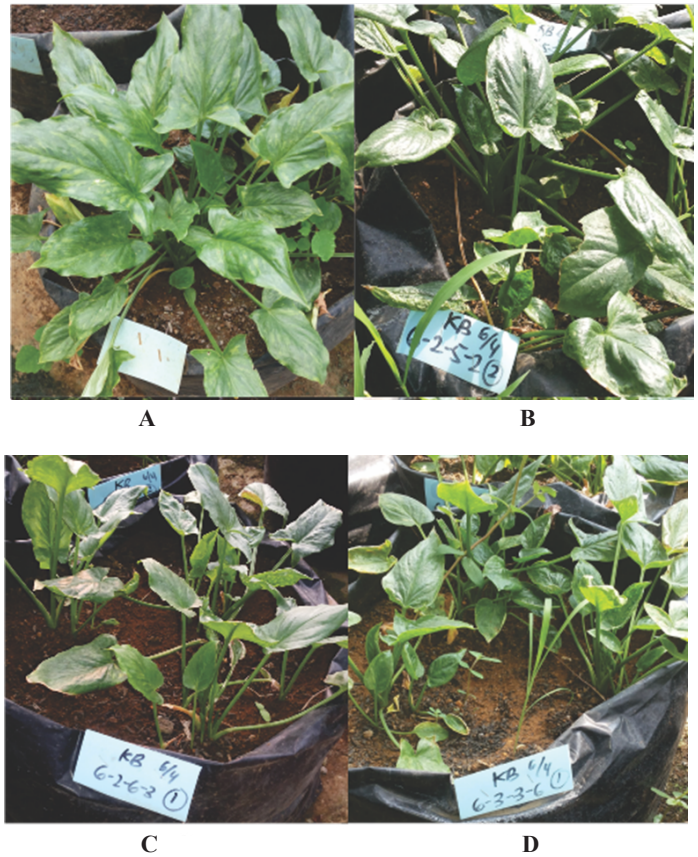

Figure 1. Control and stable superior mutant clones of rodent tuber at 8th week in greenhouse
Note: A = Control; B = 6-2-5-2; C = 6-2-6-3; D = 6-3-3-6

Table 2
Chemical compounds in leaves of rodent tuber control plant based on GC-MS

RT	Relative abundance (%)	Chemical compound
30,051	2,98	2,6,10-trimethyl,14-ethylene-14, pentadecne (neophytadiene)
30,34	0,94	2,6,10-trimethyl,14-ethylene-14, pentadecne (neophytadiene)
30,534	1,24	2,6,10-trimethyl,14-ethylene-14,pentadecne (neophytadiene)
31,54	0,2	Hexadecanoic acid, ethyl ester
31,678	20,77	Hexadecanoic acid
32,216	0,36	9,17-octadecadienal
32,257	0,86	9,12,15-octadecatrienoic acid, methyl ester (linolenic acid methyl ester)
32,375	5,87	(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol
32,602	1,05	Ethyl (9z,12z)-9,12-octadecadienoate
32,795	41,16	9,12,15-octadecatrienoic acid (linolenic acid)
33,278	0,9	Methyl 8,11,14-heptadecatrienoate
35,043	0,12	Oleic acid (9-octadecadienoic acid)
35,746	0,6	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl (squalene; spinacene)
36,146	0,36	Octacosane
39,587	3,16	Campesterol (ergost-5-en-3-ol)
39,939	7,67	Stigmasterol
40,746	1,96	Beta-sitosterol
44,8	2,87	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-
50,158	0,25	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-

Note: Compounds were identified by comparing retention time data with authentic standard database of NIST/EPA/NIH fit factor ≥ 90%. Relative abundance was determined based on area percentage of each compound

Table 3
Chemical compounds in tubers of rodent tuber control plant based on GC-MS

RT	Relative abundance (%)	Chemical compound
31,168	0,08	Farnesol, methyl ether
31,954	22,08	Hexadecanoic acid (palmitic acid)
32,526	0,73	Hexadecanoic acid (palmitic acid)
32,581	0,22	Hexadecanoic acid (palmitic acid)
32,616	0,26	Heptadecanoic acid (potassium heptadecanoate)
32,699	0,2	n-hexadecanoic acid (palmitic acid)
32,919	0,85	linoleic acid ethyl ester (ethyl linoleate)
33,078	38,37	(9E,12E)-9,12-octadecadienoic acid
33,616	4,34	Methyl (9z,12z)-9,12-octadecadienoate
33,85	1,15	(9E,12E)-9,12-octadecadienoic acid

Table 3 *(continue)*

33,953	0,89	4-(4-ethylcyclohexyl)-1 pentyl-1-cyclohexene
34,036	1,27	2-aminoethanol hydrogen sulfate (ester)
34,36	1,08	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-
34,422	1,05	z,e-3,13-octadecadien-1-ol acetate (9e,12e)-9,12-octadecadienoic acid
34,795	1,24	9,12-octadecadienoic acid (grapeseed oil)
36,084	0,73	farnesol isomer A
36,656	0,34	Eicosane (Icosane)
36,994	0,25	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-
37,705	0,19	Stigmast-5-en-3-ol
38,753	1,78	Peri-xanthenoxanthene-4,10-dione, 2,8-bis (1-methylethyl)-
39966	0,61	Solanesol
40,532	1,55	campesterol (ergost-5-en-3-ol)
40,573	2,38	Ergost-5-en-3-ol
40,966	4,05	Stigmasterol
41,876	1,92	Gamma-sitosterol
41,945	1,63	Stigmast-5-en-3-ol

Note: Compounds were identified by comparing retention time data with authentic standard database of NIST/EPA/NIH fit factor ≥ 90%. Relative abundance was determined based on area percentage of each compound

One of the clones with highest amount of anticancer compounds was 6-1-2 (Tables 4 and 5). Either in the leaves or tubers of this clone, the most abundant compound was 9,12-octadecadienoic acid (Figure 2). Octadecadienoic acid could induce apoptosis of various cancer cells (Yoo et al., 2007). The other most abundant compounds in leaves of 6-1-2 were 9,12-octadecadienoic acid, hexadecanoic acid, phytol, neophytadiene, and stigmasterol. While in its tubers were (9E,12E)-9,12-octadecadienoic acid, hexadecanoic acid, hexadecanoic acid ethyl ester, and ethyl (9Z,12Z)-9,12-octadecadienoate.

The amount of some anticancer bioactive compounds in mutant clones were higher than control. Mutant clones also contained several new anticancer compounds which were not found in control (Tables 6 and 7). Leaves of mutant clones had at least six anticancer compounds and in greater quantities compared with control. The amount of hexadecanoic acid was the highest in mutant clone 6-9-4, about 15.04% compared with control. Also known as palmitic acid, it has cytotoxic effect against MOLT-4 leukimia (cancer cell line) by interacting with DNA topoisomerase I to induce apoptosis (Kwan et al., 2014). Palmitic acid has also been reported to exhibit antitumour activity in vivo (Harada et al., 2002).

Table 4
Chemical compounds in leaves of rodent tuber mutant clone 6-1-2 based on GC-MS

RT	Relative abundance (%)	Chemical compound
30,147	5,43	2,6,10-trimethyl,14-ethylene-14, pentadecne (neophytadiene)
30,416	1,04	2,6,10-trimethyl,14-ethylene-14,pentadecne (neophytadiene)
30,602	1,89	(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol
31,051	0,23	Hexadecanoic acid, methyl ester
31,588	0,13	Hexadecanoic acid, ethyl ester
32,023	14,44	Hexadecanoic acid
32,278	0,94	Hexadecanoic acid
32,319	1,62	Hexadecanoic acid
32,478	6,73	Phytol
32,678	1,03	9,12-octadecadienoic acid, ethyl ester
33,085	36,01	9,12-octadecadienoic acid
33,354	1,96	13-tetradece-11-yn-1-ol
33,443	1,84	Methyl 8,11,14-heptadecatrienoate
33,692	1,01	(9E, 12E)-9,12-Octadecadienoic acid
34,588	0,16	8-(2-octylcyclopropyl)octanal
36,263	0,27	Nonacosane
39,966	3,7	Ergost-5-en-3-ol
40,387	3,71	Stigmasterol
40,408	4,68	Stigmasterol
41,249	4,32	Cholest-5-en-3-ol,23-ethyl-, (3 beta 23s)
45,489	2,97	5,5-dimethyl-7,8-epoxyspiro (3.5) nonan-1-one
51,737	0,03	5,9-dimethyl-4,10-octadecadiene

Note: Compounds were identified by comparing retention time data with authentic standard database of NIST/ EPA/NIH fit factor ≥ 90%. Relative abundance was determined based on area percentage of each compound

Table 5
Chemical compounds in tubers of rodent tuber mutant clone 6-1-2 based on GC-MS

RT	Relative abundance (%)	Chemical compound
31,912	8,26	Hexadecanoic acid, ethyl ester
31,968	22,8	Hexadecanoic acid
32,568	1,41	9,12-octadecadienoic acid, methyl ester (linoleic acid, methyl ester/ methyl linoleate)
32,954	6,67	Ethyl (9Z,12Z)-9,12-octadecadienoate
33,03	24,29	9,12-octadecadienoic acid
33,112	23,4	(9E,12E)-9,12-octadecadienoic acid
33,616	1,4	Tricosane
34,064	0,83	Z,Z-10,12-hexadecadien-1-ol acetate
34,147	1,84	9,12-octadecadienoic acid

Table 5 *(continue)*

34,505	0,56	Eicosane
35,464	0,89	Eicosane
36,263	1,05	(6E,10E,14E,18E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene
36,705	1,71	Heptacosane, 1-chloro
38,456	0,83	Nonacosane
41,063	1,03	Stigmasterol
41,152	0,04	Stigmasterol

Note: Compounds were identified by comparing retention time data with authentic standard database of NIST/EPA/NIH fit factor ≥ 90%. Relative abundance was determined based on area percentage of each compound

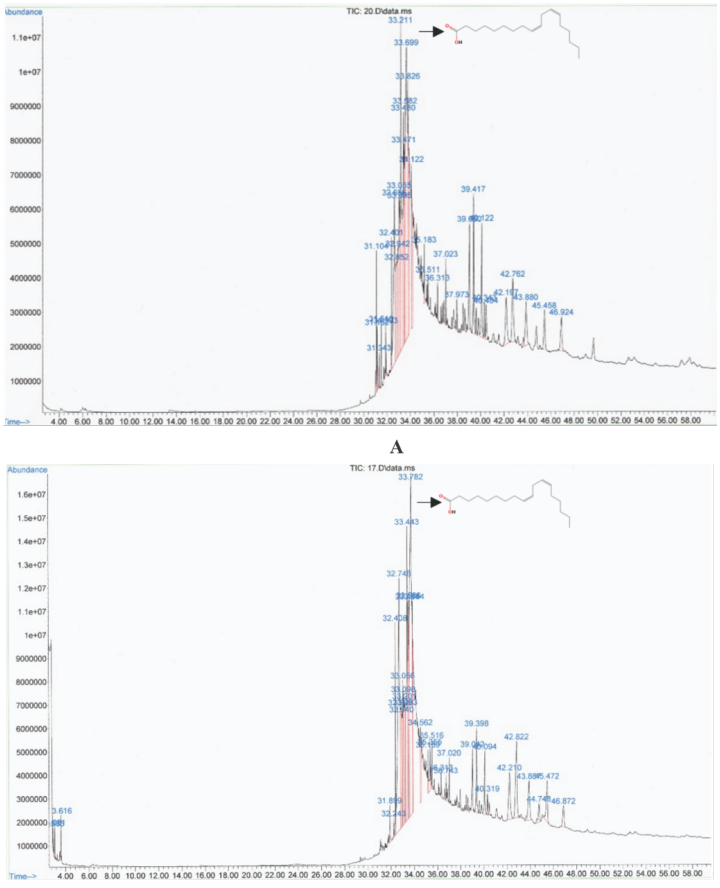

Figure 2. GC-MS chromatogram of leaves and tubers of mutant clones. X-axis represents retention time while Y-axis represents relative abundance. The chemical structure of 9,12-octadecadienoic acid, which has the highest relative abundances are shown (chemical structure obtained from NIST database) here
Note: A = leaves of 6-1-2, B = tubers of 6-1-2

Table 6
Comparison of the relative abundances of anticancer compounds in the rodent tuber's control and mutant plants based on GC-MS analysis

	Name of compound	Relative abundance (%)								
		Control	6-3-3-6	6-1-1-2	6-3-2-5	6-9-1	6-2-5-2	6-1-2	6-9-4	6-2-6-3
1.	Hexadecanoic acid ethyl ester	0,2	0,41	0,91	1,43	0,52	0,52	0,13	0,93	0,84
2.	Hexadecanoic acid methyl ester	NA	0,46	0,36	NA	0,29	NA	0,23	NA	NA
3.	Hexadecanoic acid	20,77	25,35	23,91	22,26	19,87	17,81	17	35,81	18,79
4.	Octadecadienoic acid	NA	0,22	1,4	NA	0,98	NA	37,02	NA	NA
5	Squalene	0,6	1,79	1,21	2,34	1,46	0,98	NA	1,77	1,04
6.	Campesterol (ergost-5-en-3-ol)	3,16	0,21	NA	0,71	1,93	3,42	3,7	NA	3,93
7.	Stigmasta-5,22-dien-3-ol (3 beta) (stigmasterol)	7,67	0,35	6,48	4,48	5,49	8,69	8,39	8,74	9,63
8.	Stigmast-5-en-3-ol (beta-sitosterol)	1,96	0,11	0,97	1,28	NA	NA	NA	NA	NA
9.	Phytol isomer	NA	NA	5,41	NA	NA	9,77	6,73	NA	8,78
10.	Gamma sitosterol	NA	NA	0,68	NA	1,63	NA	NA	NA	NA
11.	Pyridine-3-carboxamide,oxime	3,12	NA	0,41	NA	0,73	6,38	NA	NA	2,04
12.	Eicosane (Icosane)	NA	NA	1,61	NA	NA	NA	NA	NA	NA
13.	Geranylgeraniol	NA	NA	NA	0,63	NA	NA	NA	NA	NA

Note: Chemicals were identified by comparing the retention time with authentic standard database of NIST/EPA/NIH (fit factor ≥ 90%). NA is not available, which means that the quantity of the compound is too low to be detected by GC-MS. The quantities of anticancer bioactive compounds in MV6 mutant clones which are higher than control are indicated by the grey highlights

Leaves of mutant clones contained six new anticancer compounds, namely hexadecanoic acid methyl ester, octadecadienoic acid, phytol, gamma-sitosterol, eicosane, and geranylgeraniol. Hexadecanoic acid methyl ester has been known to to inhibit the growth and induce apoptosis of human gastric cancer cells (Yu et al., 2005). Phytol is an antitumour chemical which could induce the apoptosis of human gastric adenocarcinoma (Song & Cho, 2015) and hepatocellular carcinoma cells. Therefore, it has good potential as a medicine for liver cancer (Kim et al., 2015). Additionally, phytol is cytotoxic against the MCF-7 breast adenocarcinoma cells, but it did not harm normal cells in humans (Peijin et al., 2014).

Gamma-sitosterol has anticancer activity against MCF-7 breast cancer cell and A549 lung cancer cell lines by inhibiting their growth, stopping the cell cycle, and inducing apoptosis (Peijin et al., 2014). Eicosane is a derivative of methyl ester which could inhibit the growth of SGC-7901 gastric cancer cell (Yu et al., 2005). Geranylgeraniol can lead to fragmentation of the DNA of HL-60 leukemia and inhibit the proliferation of DLD1 colon

Table 7
Comparison of the relative abundances of anticancer compounds in the rodent tuber's control and mutant plants based on GC-MS analysis

	Name of compound	Relative abundance (%)								
		Control	6-3-3-6	6-1-1-2	6-3-2-5	6-9-1	6-2-5-2	6-1-2	6-9-4	6-2-6-3
1.	Hexadecenoic acid ethyl ester	NA	32,32	33,07	29,37	3,37	3,94	8,26	28,18	23,61
2.	Octadecadienoic acid	40,76	51,58	44,68	40,36	44,27	42,82	49,53	3,27	38,7
3.	Squalene	NA	0,86	0,94	NA	0,59	0,63	NA	NA	0,54
4.	Stigmasta-5,22-dien-3-ol (3 beta) (stigmasterol)	4,05	NA	4	5,15	3,84	3,68	1,07	3,99	3,37
5.	Stigmast-5-en-3-ol (beta-sitosterol)	1,82	NA	NA	0,39	0,59	1,33	NA	2,36	0,39
6.	Eicosane (Icosane)	0,34	0,73	1,62	1,69	NA	1,3	1,45	1,61	2,42
7.	Octacosane	NA	0,58	0,69	NA	2,23	NA	NA	NA	NA
8.	7-pentadecyne	NA	NA	NA	NA	NA	NA	NA	0,48	NA

Note: Chemicals were identified by comparing the retention time with authentic standard database of NIST/EPA/NIH (fit factor ≥90%). NA means it is not available, which means that the quantity of a compound is negligible and cannot be detected by GC-MS. The quantities of anticancer bioactive compounds in MV6 mutant clones which are higher than control are indicated by the yellow highlights

adenocarcinoma (Yoshikawa et al., 2009). Mutant clones' tubers contain greater amounts of at least 5 different anticancer compounds compared with control. The highest increase from control was observed in the chemical profile of clone 6-3-3-6 which contained octadecadienoic acid, 10.82% higher compared with control plant. This compound could induce apoptosis of colon cancer cells (Yoo et al., 2007).

Tubers of mutant clones contain four new anticancer compounds, namely hexadecanoic acid ethyl ester, squalene, octacosane, 7-pentadecyne, which were not found in control plant. Hexadecanoic acid ethyl ester has antioxidant and antimicrobial properties (Bodoprost & Rosemeyer, 2007). In addition, it could reduce the risk of coronary heart disease and cancer (Lai et al., 2008; Bodoprost & Rosemeyer, 2007). Squalene has been proven to be able to inhibit the carcinogenesis of various cancer cell lines, such as colon cancer (Rao et al., 1998). Octacosane was cytotoxic against B16F10-Nex2 skin cancer cells based on *in vitro* experiment (Figueiredo, 2014). The chemical structure of 7-pentadecyne was similar to protein kinase C activator, so it has the potential to be developed into an effective anticancer drugs (Block, 2012). The anticancer activity of 7-pentadecyne has also been confirmed by (Kozikowski et al., 2001).

Tables 6 and 7 also show that mutant clones contain greater amounts of some other chemicals compared with control

plants. Stigmast-5-en-3-ol (3.beta,24s) (beta-sitosterol) is a phytosterol with various biological activities, such as reducing cholesterol level in cells, modify membrane lipid profile (Awad, 1996), anti-diabetic (Sujtha et al., 2010), and inhibit cancer cells (Fraille et al., 2012; Von et al., 1998). Ergost-5-en-3-ol (3 beta) (campesterol) is a phytosterol that could prevent carcinogenesis in lung (Mendilaharsu et al., 1998), gastric (De et al., 2000) and ovarium (Mccann et al., 2003).

Stigmasta-5,22-dien-3-ol (3 beta) (stigmasterol) is antiproliferative against PC3 prostate cancer cells by inducing its apoptosis (Traber & Atkinson, 2007). Stigmasterol could reduce the number of Ehrich Ascites Carcinome (EAC) and is an antioxidant because it could reduce lipid peroxidation and increase glutathione, superoxide dismutase, and catalase in the liver of EAC mice (Ghosh et al., 2011). Another anticancer bioactive compounds of rodent tuber are phenolic compounds (Mohan et al., 2011), pyridine carboxamide (Surjana et al., 2012), octadecanoic acid (Habib et al., 1987), and geranygeraniol (Fernandes et al., 2013).

Rodent tuber's MV1 has undergone protein expression changes compared with control based on 1D and 2D SDS-PAGE analysis (Sianipar et al., 2016). The greater amount of anticancer compounds in mutant clones compared with control has also been observed in MV1 (Sianipar et al., 2016). Leaves and tubers of MV1 contain greater amounts of 3 and 4 anticancer compounds compared control, respectively. Leaves and tubers of MV1 each contains four new anticancer compounds.

According to (Yaycili & Alikamanoglu, 2012), genetic modification of potato plants (alteration of DNA sequence induced by irradiation) can be different between one somatic cell to another. This also happened to rodent tuber MV6 clones. Although they originated from one mother plant, they came from different somatic cells with different genetic make-up. Genetic variation between mutant clones might be due to the difference in DNA repair mechanism or random mutation induced by gamma irradiation (Pillay & Tenkouano, 2011). Genetic variations between each of the mutant clones can result in differences in the chemical contents.

Table 1 shows five mutant clones which had a higher dry weight than control, i.e. clone 6-1-2, 6-9-4, 6-2-6-3, 6-2-4-1, and 6-2-5-2. Four of them, i.e, clone 6-1-2, 6-9-4, 6-2-6-3, dan 6-2-5-2, contained higher amounts of anticancer compounds in their leaves and tubers compared with control (Tables 6 and 7). Those four clones have a potential to be developed into new superior varieties because they have a fast propagation rate and contain a higher amount of valuable anticancer compounds.

CONCLUSION

The chemical compounds in the leaves and tubers of gamma ray-irradiated rodent tubers were higher than the non-irradiated ones. A total of 11 anticancer compounds

were detected in the 6th generation of stable superior mutant clones (MV6) of rodent tuber. Of these, six new anticancer compounds were detected in the leaves and four were detected in tubers. Among the mutant clones, the mutant clone 6-1-1-2 produced the highest amount of new anticancer bioactive compounds. This is the first study of this nature to identify anticancer compound of the 6th generation stable superior mutant clones of rodent tuber using GC-MS method. Therefore, this study should be continued to develop purified bioactive compounds as anticancer drugs.

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Assessment of the Genetic Variation of Malaysian Durian Varieties using Inter-Simple Sequence Repeat Markers and Chloroplast DNA Sequences

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ABSTRACT

To date, 124 durian varieties have been registered with the Malaysian Department of Agriculture based on phenotypic characteristics. However, the levels and patterns of genetic variation among the varieties are still unknown. In this study, the leaves of 27 durian varieties were sampled from four durian orchards in Universiti Putra Malaysia, namely Bukit Ekspo, Putra Mart, Ladang Puchong and Ladang 5. Twenty five inter-simple sequence repeat (ISSR) primers were tested for PCR amplification on DNA samples. Twelve ISSR primers amplified 133 clear and reproducible DNA fragments and 122 (91.73%) were polymorphic, indicating a high level of genetic variation among these durian varieties. Primers flanking four chloroplast DNA (cpDNA) regions (*trn*L-*trn*F, *atp*B-*rbc*L and *trn*H-*psb*A intergenic spacers as well as the partial *mat*K gene) were tested for PCR amplification. Two cpDNA regions (*trn*L-*trn*F and *mat*K) were successfully amplified, but showed no variation in their DNA sequences, even when additional samples from Vietnam were included. The findings in this preliminary study lay a foundation for more comprehensive future studies on the genetic variation among durian varieties.

Keywords: Chloroplast DNA sequence, DNA barcoding, *Durio zibethinus*, genetic diversity, inter-simple sequence repeat

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INTRODUCTION

Durio is one of the genera in the family Malvaceae and is characterised by its most striking feature i.e. spiny fruit containing large seeds covered with fleshy or leathery arils (Nyffeler & Baum, 2001). A total of 34 species of *Durio* have been recorded (Idris, 2011; "The Plant List", 2013), and at least nine species of these produce edible fruit (Idris, 2011). Of the nine species, durian (*D. zibethinus*) is the most common and most widely cultivated. It is also one of the most popular tropical fruit in Southeast Asia.

In Malaysia, 124 durian varieties are registered with the Malaysian Department of Agriculture ("Varieties Registered for National Crop List", http://pvpbkkt.doa.gov.my/NationalList/Search.php) as of February 2017. It is noteworthy that the different 'types' of durian have always been termed differently; by the Malaysian Department of Agriculture as "varieties," and by the Malaysian Agricultural Research and Development Institute (MARDI) and Universiti Putra Malaysia (UPM) as "clones" (e.g. Abidin, 1991; Jawahir & Kasiran, 2008). For convenience, in this paper we shall use the terminology used by the Malaysian Department of Agriculture i.e. durian varieties. These varieties are registered solely based on their morphological character such as fruit shape, thorn size, aroma of the fruit and seed shape (Department of Agriculture, 2010). Morphological character in plants is easy to observe, but plants are subject to phenotypic plasticity as a direct result of environmental factors (e.g. climate, nutrient and moisture content, soil type etc.) and age, which may contribute to morphological variation (Ruwaida, Supriyadi, & Parjanto, 2009). To overcome the limitation of phenotypic plasticity, there is a need to carry out genetic characterisation on the registered durian varieties. Such data on genetic variation are important not only for the management of durian genetic resources, but also for exploring the possibility of developing genetic markers for future identification of durian varieties.

Inter-simple sequence repeats (ISSRs) and chloroplast DNA (cpDNA) sequences are two useful markers to study genetic variation in plants. ISSR is a PCR-based method which uses microsatellite sequences as primers to amplify regions in the genome that fall between two similar microsatellite sequences. The result is a series of amplified DNA fragments for each sample that can then be scored and compared to other samples to evaluate the amount of genetic variation present in the samples (Ng & Tan, 2015). CpDNA is maternally inherited, has a lower mutation rate compared to nuclear DNA and is widely used in genetic variation studies of plants at various taxonomic levels (Dong, Liu, Yu, Wang, & Zhou, 2012; Gielly & Taberlet, 1994).

ISSR markers do not require prior knowledge of genomic sequences and a high number of loci across genomes can be easily screened, while universal PCR primers have been developed for several cpDNA loci, making them suitable markers for genetic variation studies on durian, for which we have very little genetic information. Also,

although all commercial durian types are identified morphologically as *D. zibethinus*, it is unsure if all current varieties belong to the same species, as there has been no study done at the genetic level addressing this question. There is always a possibility of cross-breeding between different *Durio* species (i.e. interspecific hybridisation) to produce edible fruit leading to the array of varieties we see today. One way to determine if the durian types were mothered by *D. zibethinus* is through cpDNA sequencing.

In this study, we used ISSR and cpDNA markers to evaluate the levels and patterns of genetic variation present in a subset of Malaysian durian varieties. Specifically, we asked the questions: (1) What is the level of genetic variation present among Malaysian durian varieties? (2) What are the genetic relationships among the different durian varieties, and did they arise naturally in their assumed places of origin? (3) Are the commercial durian varieties we have today derived solely from *D. zibethinus*? Are there interspecific hybrids?

MATERIALS AND METHOD

Sampling of Durian Varieties, DNA Extraction, and Purification

Leaf samples of 27 durian varieties were collected for this study (Table 1). They were sampled from four orchards in UPM, namely Bukit Ekspo, Putra Mart, Ladang Puchong and Ladang 5. For DNA extraction, 100mg of fresh leaf material was ground in liquid nitrogen, and the total genomic DNA was extracted using the CTAB method (Doyle & Doyle, 1990). The crude DNA extract was further purified using the GF-1 Plant DNA Extraction Kit (Vivantis).

Table 1
Details of durian samples used in this study

No.	Variety	Common Name	Location of Sampling	Place of Origin*
1	D2	Dato' Nina	Putra Mart	Melaka
2	D7	-	Ladang Puchong	Selangor
3	D8	-	Ladang Puchong	Kuala Lumpur
4	D10	Durian Hijau	Putra Mart	Selangor
5	D16	-	Bukit Ekspo	-
6	D24	-	Putra Mart	Perak
7	D84	-	Ladang 5	Perak
8	D88	Bangkok 8	Ladang 5	Selangor
9	D96	Bangkok A	Ladang 5	Selangor
10	D99	Kop Kecil	Putra Mart	Thailand
11	D125	Kop Jantung	Ladang 5	Kedah
12	D145	Tuan Mek Hijau/Beserah	Ladang Puchong	Pahang
13	D148	Paduka	Ladang Puchong	Perak
14	D158	Kan Yau/Tangkai Panjang	Ladang Puchong	Kedah

Table 1 *(continue)*

No.	Variety	Common Name	Location of Sampling	Place of Origin*
15	D159	Mon Thong/Bantal Mas	Ladang Puchong	Kedah
16	D160	Buluh Bawah	Ladang Puchong	Selangor
17	D162	Tawa	Ladang Puchong	Selangor
18	D168	Durian Mas Hjh. Hasmah	Putra Mart	Johor
19	D169	Tok LiTok	Ladang Puchong	Kelantan
20	D172	Durian Botak	Ladang Puchong	Johor
21	D175	Udang Merah	Ladang Puchong	Pulau Pinang
22	D188	MDUR 78	Ladang Puchong	Terengganu
23	D189	MDUR 79	Ladang Puchong	Terengganu
24	D190	MDUR 88	Putra Mart	Terengganu
25	D197	Raja Kunyit/Musang King	Putra Mart	Kelantan
26	Durian Gergasi (DG)	-	Ladang Puchong	-
27	Durian Siam (DS)	-	Bukit Ekspo	-
28	Chanee	-	Vietnam	Thailand
29	Kanyao	-	Vietnam	Thailand
30	B31	-	Vietnam	Vietnam
31	Bi	-	Vietnam	Vietnam
32	Chuong Bo	-	Vietnam	Vietnam
33	Chin Hoa	-	Vietnam	Vietnam
34	HB11	-	Vietnam	Vietnam
35	Kho Qua	-	Vietnam	Vietnam
36	La Queo	-	Vietnam	Vietnam
37	Ri 6	-	Vietnam	Vietnam
38	Sau Huu	-	Vietnam	Vietnam
39	Tam Son	-	Vietnam	Vietnam

Place of origin of Malaysia samples is according to Department of Agriculture ('Recommended plant varieties in Malaysia'*, n. d.)

ISSR Genotyping

Twenty-five ISSR primers were initially tested on a subset of two durian DNA samples in two replicates, and only those that generated multiple, clear and reproducible bands were subsequently used to genotype all 27 durian samples featured in this study. The details of the ISSR primers are listed in Table 2. Single-primer PCR reactions were performed in 10 µL reaction mixtures, each containing 1× NEXpro™ e PCR Master Mix (Genes Laboratories, Korea), 1 µM ISSR primer and approximately 10 ng genomic DNA. A touch-down PCR profile was used, which comprised an initial denaturation of 3 min at 95°C, followed by 13 cycles of 30 s at 95°C, 30 s at 58-46°C (-1°C/cycle) and 1.5 min at 72°C, 25 cycles of 30 s at 95°C, 30 s at 45°C and 1.5 min at 72°C, and finally an extension step at 72°C for 7 min. The PCR amplicons were analysed by electrophoresis on 2% agarose gel, stained with ethidium

bromide and viewed under UV illumination. DNA bands between the range of 100 bp and 1500 bp were scored as 'present' (1) or 'absent' (0) for each individual to generate a binary ISSR data matrix before estimation of the basic parameters, including total number of bands, number of polymorphic bands and the percentage of polymorphic bands. To visualise the genetic relationship among the durian varieties, a Neighbour-Joining (NJ) tree was constructed based on the Dice similarity coefficient, using DARwin 6.0 (Perrier & Jacquemoud-Collet, 2006). The degree of confidence at each node of the NJ tree was evaluated through 1,000 bootstrap replicates.

Sequencing of cpDNA Loci

Table 2
ISSR Primers used in this study

No.	Primer name	Primer sequence (5'–3')	No. of bands	No. of polymorphic bands
1.	UBC 834	$(AG)_8$YT	-	-
2.	UBC 841	$(GA)_8$YC	9	9
3.	UBC 848	$(CA)_8$RG	12	10
4.	UBC 855	$(AC)_8$YT	12	10
5.	UBC 856	$(AC)_8$YA	-	-
6.	Ng2.01	$(AC)_8$B	7	7
7.	Ng2.02	$(AG)_8$B	-	-
8.	Ng2.03	$(TC)_8$V	-	-
9.	Ng2.04	$(TG)_8$V	-	-
10.	Ng2.05	$(CA)_8$D	-	-
11.	Ng2.06	$(CT)_8$D	-	-
12.	Ng2.07	$(GA)_8$H	-	-
13.	Ng2.08	$(GT)_8$H	-	-
14.	Ng2.09	$(AC)_8$SS	-	-
15.	Ng2.10	$(AG)_8$SS	13	13
16.	Ng3.01	$(ACA)_5$SS	13	11
17.	Ng3.02	$(AGA)_5$SS	12	10
18.	Ng3.03	$(TCA)_5$SS	16	15
19.	Ng3.04	$(TGA)_5$SS	-	-
20.	Ng3.05	$(ACT)_5$SS	-	-
21.	Ng3.06	$(AGT)_5$SS	11	11
22.	Ng3.07	$(TCT)_5$SS	-	-
23.	Ng3.08	$(TGT)_5$SS	4	3
24.	Ng3.09	$(ATC)_5$SS	9	9
25.	Ng3.10	$(ATG)_5$SS	15	14
		Total	133	122 (91.73%)

Note: Degenerate bases Y=C/T; R=A/G; B=C/G/T; V=A/C/G; D=A/G/T; H=A/C/T; S=C/G

Four sets of published primers (Table 3) were tested to amplify the partial matK gene, as well as the *trn*L-*trn*F, *atp*B-*rbc*L and *trn*H-*psb*A intergenic spacers. Primers that resulted in positive amplification were used to genotype all 27 samples from UPM. PCR amplicons were analysed by electrophoresis on 1% agarose gel, stained with ethidium bromide and viewed under UV illumination. PCR amplicons were then purified and sequenced on an ABI 3730 platform, through services provided by First Base Laboratories Sdn. Bhd. The nucleotide sequences were edited and assembled using the ATGC version 6.0 (Genetyx Corporation) software and finally aligned using Clustal W embedded in MEGA 7.0 (Kumar, Stecher, & Tamura, 2016). Sequences of both cpDNA loci were deposited in GenBank with the accession numbers KY860031–860084.

Table 3
cpDNA loci used in this study. Only the trnL-trnF and matK loci were successfully amplified in this study

Locus	Primer Name	Primer sequence (5'-3')	Amplification (+/-)	Approximate amplicon size (bp)	Source
*trn*L-*trn*F	*trn*L-c	CGAAATCGGTTAGACGTACG	+	1000	Taberlet et al., 1991
	*trn*L-f	ATTTGAACTGGTGACACGAG			
*atp*B-*rbc*L	*atp*B-1	ACATCKARTACKGGACCAATAA	-	-	Chiang, Schaal, & Peng, 1998
	*rbc*L-1	AACACCAGCTTTRAATCCAA			
*mat*K	*mat*K472F	CCCRTYCATCTGGAAATCTTGGTTC	+	800	Yu, Xue, & Zhou, 2011
	*mat*K1248R	GCTRTRATAATGAGAAAGATTTCTGC			
*trn*H-*psb*A	*trn*H-1	CGCGCATGGTGGATTCACAATCC	-	-	Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005

Note: Degenerate bases: Y=C/T; R=A/G; K=G/T

RESULTS

Analysis of ISSR Data

Of the 25 ISSR primers tested (Table 2), only 12 primers produced clear and reproducible bands, and these were subsequently used to genotype all the samples. The 12 primers generated a total of 133 bands that fell within the range of 100-1500 bp in molecular weight. An example of the generated banding pattern is shown in Figure 1. The number of bands amplified per primer ranged from 4 to 16 with an average of 11.08 bands per primer. Of the 133 amplified bands scored, 122 (91.73%) were polymorphic.

An NJ tree (Figure 2) was constructed to visualise the relationship among the different durian varieties sampled in this study. While general clustering of varieties was observed in the tree, support for the tree was low; only three nodes showed ≥50% bootstrap support.

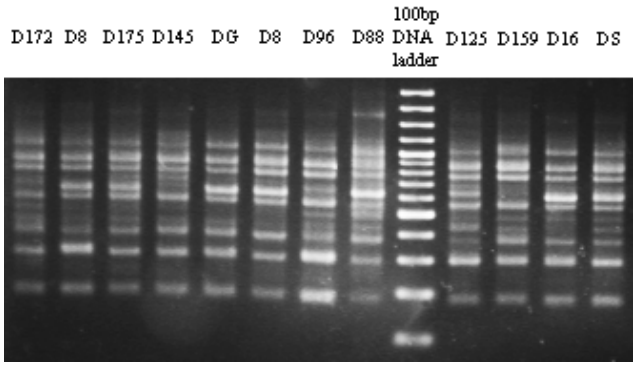

Figure 1. Example of ISSR amplification products of 12 varieties using primer Ng3.01, electrophoresed through 2% agarose gel

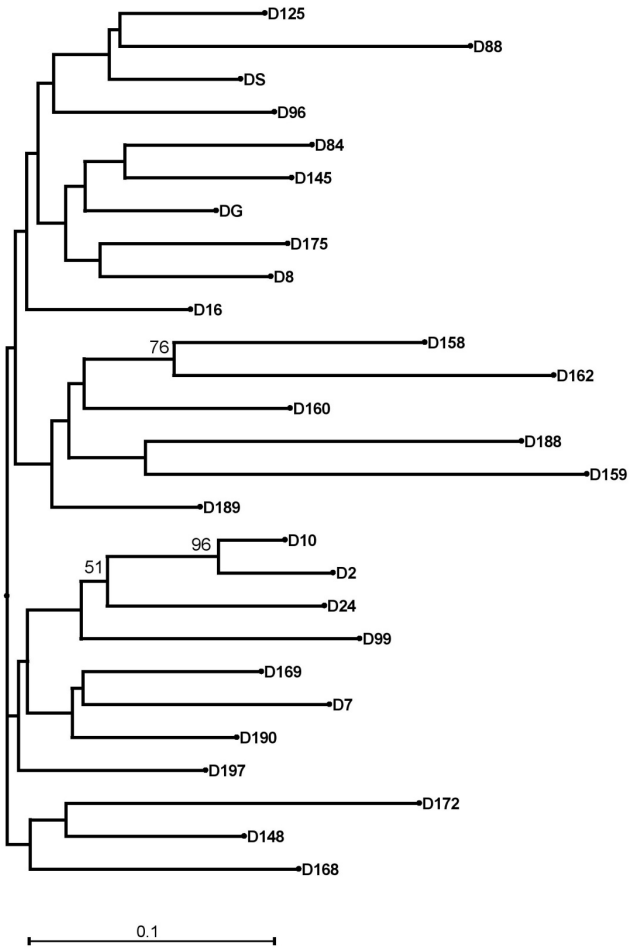

Figure 2. NJ tree of 27 durian varieties constructed on DARwin, with 1,000 bootstrap replicates. Only bootstrap values ≥50% are labelled on the nodes

Analysis of cpDNA Data

Two out of four cpDNA loci, *mat*K and *trn*L-*trn*F, were successfully PCR-amplified and sequenced. Sequencing of the cpDNA sequences across the 27 samples at the *mat*K and *trn*L-*trn*F loci revealed identical sequence lengths within each locus. The aligned lengths were 732 bp for matK and 870 bp for *trn*L-*trn*F. No variation was observed at both cpDNA loci.

DISCUSSION

Levels and Patterns of Genetic Variation

The level of genetic variation found in the durian varieties sampled in our study using ISSR markers was higher than what was found in the studies carried out by Vanijajiva (2012) using ISSR markers and Vanijajiva (2011) using RAPD markers, both on durian varieties from Thailand. The higher number of samples and loci used in this study could be the reason for the higher genetic diversity observed. Our results were comparable to the study done by Ruwaida et al. (2009), who used six RAPD markers to evaluate the genetic diversity in Indonesian durian varieties, which showed an average of 81.89% polymorphic bands. This shows that there is considerably high genetic variation among the different varieties of Malaysian durian.

CpDNA is known to be less polymorphic compared to nuclear DNA, within species (Banks & Birky, 1985). However, the observation of some degree of intraspecific cpDNA variation was not unexpected, as observed in some other cultivated species such as *Pisum sativum, Nicotiana debneyi, Quercus* and *Liriodendron* (Neale, Saghai-Maroof, Allard, Zhang, & Jorgensen, 1988; Okaura & Harada, 2002). The absence of genetic variation detected at the cpDNA loci may be due to the small sample size in this study. To further explore this possibility, we compared our cpDNA data to DNA sequences at the same loci across 12 Vietnamese commercial durian varieties (Giang, Tri, Ky, Muoi, & Hien, unpublished data; see Table 1). However, no variation was observed at both loci across the Malaysian and Vietnamese durian varieties. As cpDNA is maternally inherited, this raises the possibility that the various commercial durian varieties could have been derived from a small group of related mother trees through asexual propagation, among which cpDNA variation would have been very low.

From the aspect of biogeography, taxa that originated from geographically nearer areas would be more genetically related, assuming that these taxa were of natural origin (i.e. not human-mediated). The NJ tree that was constructed to shed light on the relationship among the various durian varieties used in this study however, did not display significant levels of confidence. This means that the varieties were essentially genetically closely related, and no significant relationship between a variety and its corresponding place/region of origin (see Table 1) was found. As durian has become a popular fruit crop in most of Southeast Asia, human activity (e.g. transplant of a variety from a source location to another location

followed by crossing with local varieties) seems to be the most possible cause for the non-conformity to biogeographical expectation in the derivation of these durian varieties.

Hybridisation in the Evolution of Malaysian Durian

Hybridisation between different durian varieties (intraspecific hybridisation) has been utilised to come up with superior varieties (e.g. D24 × D10 = D190, D10 × D24 = D188; (Sani, Abbas, Buniamin, Nordin, & Rashed, 2015). However, hybridisation between different species of *Durio* (interspecific hybridisation), to increase genetic diversity in cultivated durian, is not unheard of (e.g. *D. kutejensis* × *D. zibethinus* in Indonesia; [Hariyati, Kusnadi, & Arumingtyas, 2013]). While this study could not confirm if hybridisation between *D. zibethinus* and other *Durio* species took place in the evolution of popular Malaysian durian varieties, the durian varieties sampled in this study were most probably mothered only by *D. zibethinus*, as only a single genotype at each cpDNA locus was found. Future studies incorporating nuclear DNA loci would be useful to further explore the possibility of gene flow from other *Durio* species to the cultivated *D. zibethinus*.

CONCLUSION

Our results demonstrated the potential of using genetic markers to assess the genetic variability of durian varieties. The high level of genetic variation found in a subset of Malaysian durian varieties using ISSR markers provided a preliminary view for the potential development of strategies for germplasm conservation and genetic improvement of existing local durian varieties. However, such a result was not reflected in the cpDNA sequences used in this study. A higher number of cpDNA loci, as well as other genetic markers, should be included in future studies.

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Morphometric Sexing of Little Spiderhunter (*Arachnothera longirostra*) in Peninsular Malaysia

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ABSTRACT

Sexual dimorphism is often directly linked to sexual selection, mating systems and resource partitioning, which are crucial in species conservation and management. Many avian species, including pollinator birds, are sexually dimorphic with respect to size and colour, yet, such differences may be subtle for some species. In this study, molecular sexing was performed in addition to determining morphological parameters that can aid in future sex determination of a common forest pollinator, the little spiderhunter (*Arachnothera longirostra*), in Peninsular Malaysia. Based on 23 captures made in four forests, two out of seven body measurements (i.e. wing and tail lengths) were useful in predicting the sexes of the bird with 100% accuracy. In addition, significant differences were found in the head, bill, and total body lengths. Such findings will facilitate more effective sex identification in future field studies, particularly in the case of juveniles.

Keywords: *Arachnothera longirostra*, Discriminant function analysis, Morphometric sexing, Pollinators, Sexual dimorphism

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INTRODUCTION

Globally, three bird families, namely Trochilidae, Meliphagidae and Nectariniidae, are known as pollinators (Cronk & Ojeda, 2008). These families maintain key ecological functions and services whether in the natural or agricultural ecosystems (Ollerton, et al.,

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2011). Spiderhunters (Nectariniidae) are morphologically distinctive with their long decurved bill. Besides small arthropods, many spiderhunter species consume nectar and hence, also serve as pollinators (Yumoto et al., 1997; Momose et al., 1998; Sakai, et al., 1999; Phillipps & Phillipps 2011; Sakai, et al., 2013), which often exhibit trap-lining behaviour. Unlike the confamilial sunbirds that are often sexually dimorphic with males being relatively more colourful than females, it may be difficult to differentiate the sexes of spiderhunters, unless through careful examination of their body size and pectoral tufts (Cheke et al., 2001).

Malaysia has a total of 10 spiderhunter species with the majority of species living in wooded habitats (MNS-BCC 2015). This includes two Bornean endemics (Whitehead's spiderhunter *Arachnothera juliae* and Bornean spiderhunter *A. everetti*) as well as the recently included of the purple-naped spiderhunter (*A. hypogrammicum*; Moyle et al. 2011) which was once treated as a sunbird. The presence of a relatively high diversity of spiderhunter species, some of which are sympatric, poses more intricate questions with regard to interspecific segregation of niches (Collins, 2008), intersexual partitioning of resources (Paton & Collins, 1989; Temeles & Roberts, 1993) as well as mate choice of these species that are confined to the Oriental region. In addition, knowing the sexes of these pollinating birds may have implications on the understanding of key ecological functions (Ollerton, et al., 2011).

The little spiderhunter (*A. longirostra*) is abundant in disturbed and regenerating forests (Rahman et al., 2010; Wells 2010) and is frequently encountered during mist-netting surveys (Wells, 2010; Phillipps & Phillipps, 2011) compared with other Malaysian spiderhunter species. Male little spiderhunters can be differentiated from females by their larger sizes and the presence of pectoral tufts (Cheke, et al., 2001; Cheke & Mann, 2008; Wells, 2010, but see Jeyarajasingam, 2012), although Wells (2010) noted that the latter feature may develop later than other adult characteristics.

In this study, sexes of randomly caught little spiderhunters from Peninsular Malaysia were determined using molecular methods, before conducting discriminant function analysis on the morphometric measurements obtained from the field. The intention was to develop a way for morphometric sexing of the species for future field studies, eliminating the need for invasive sex determination.

MATERIALS AND METHODS

Sampling

Using mist-nets, we sampled little spiderhunters from three forest reserves located in Peninsular Malaysia, i.e. the Bintang Hijau (5°26'20"N, 100°55'27"E; Perak state), Sungai Lalang (3°1'29"N, 101°54'49"E; Selangor state), and Panti (1°51'38"N, 103°54'26"E; Johor state) Forest Reserves, as well as an isolated forest patch within the Shah Alam National

Botanical Garden (3°5'57"N, 101°30'12"E; Selangor), from January to August 2016.

Using a calliper and a ruler, morphological measurements were taken for the total body, head, wing chord, tarsus, tail, and bill (culmen) lengths, in mm (Figure 1). Weight was measured using a spring balance, in gram. Blood samples were collected by pricking the brachial vein with a sterile 27-gauge needle (Davis 2005), mixed with 100% ethanol and stored at 4°C. All the birds were released after their blood samples were taken.

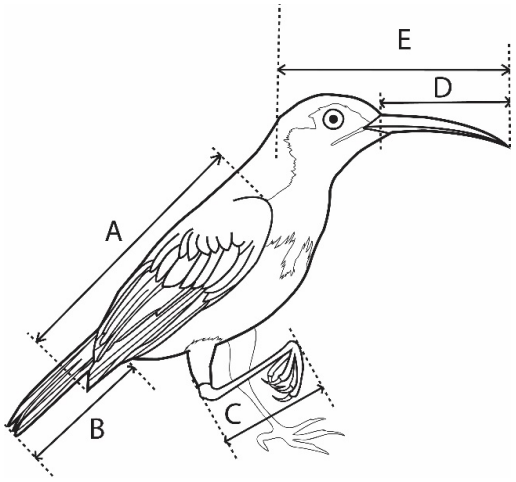

Figure 1. Measurement of (A) wing, (B) tail, (C) tarsus, (D) bill, and (E) head lengths

Molecular sexing

Total genomic DNA from blood samples was extracted from 20 µl of blood using the DNeasy Blood and Tissue Kit (Qiagen) based on the manufacturer's protocol. The extracted DNA samples were stored at -20°C until further analyses.

In birds, sex is determined using the ZW sex determination system, in which males are the homogametic sex (having two Z-chromosomes) and females are the heterogametic sex (having one each of the Z- and W-chromosomes). A primer pair (CHD1F: 5'-TATCGTCAGTTTCCTTTTCAGGT-3' and CHD1R: 5'-CCTTTTATTGATCCATCAAGCCT-3'; Lee et al. 2010) was used to amplify a section of the sex chromosome CHD gene that is present on both the avian W- and Z-chromosomes in differing fragment lengths. The PCR amplifications were performed in 10 µL reactions, each containing approximately 30 ng of genomic DNA as template, 1 µM of each primer, and 5 µL of NEXproTM ePCR 2× master mix (NEX Diagnostics). The PCR reaction profile comprised an initial denaturation of 3 minutes at 95°C; followed by 30 cycles of 30 sec at 95°C, 30 sec at 50°C, and 2 min at 72°C; and finally, an extension step at 72°C for 7 min. The PCR amplicons

were analysed by electrophoresis on 1.0% (weight/volume) agarose gel, stained with ethidium bromide and viewed under UV illumination. Males would present one DNA band (at ~500bp), while females would present two DNA bands (at ~300bp and ~500bp), on the gels.

Morphological data analyses

A Mann-Whitney U test was performed to compare differences in the measurements between male and female birds. All body measurements were reported in means and standard errors. Discriminant Function Analysis (DFA) with a stepwise procedure was applied on the measurements. All statistical tests were performed using SPSS Version 16.0 (SPSS, Inc., Chicago, Illinois).

RESULTS AND DISCUSSION

A total of 23 little spiderhunters were caught, including 14 from Sungai Lalang Forest Reserve, four from Bintang Hijau Forest Reserve, two from Panti Forest Reserve and three from Shah Alam National Botanical Garden. Eleven (47.8%) were males, as determined through DNA analysis (Figure 2). Visually, we successfully identified eight males (72.7% of the total males caught) through the presence of pectoral tufts. Of the three males which sex was difficult to identify in the field, there was only one juvenile with noticeable orange-yellow gape flange.

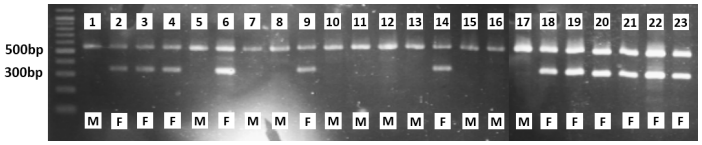

Figure 2. Molecular sexing results. Males show 1 band (ZZ) at ~500bp, and females show 2 bands (ZW) at ~300bp and 500bp

Significant differences were found in the head, bill, wing, tail, and total body lengths ($p \leq 0.01$; Table 1), but not in the tarsus length and weight. Based on DFA, a parsimonious model comprising wing and tail lengths as predictors provided the best possible prediction of the sex of a little spiderhunter with 100% accuracy (Figure 3). The discriminant function (D; Wilk's Lambda = 0.087, $\chi 2 = 48.785$, $P < 0.001$) was:

[D = 0.852*Wing length + 0.531*Tail length]

Based on centroids derived from DFA, a bird with a score on the DF closer to 3.580 would be a male whereas it was -2.983 for females.

Table 1
Morphometric measurements from little spiderhunter and results of Mann-Whitney U-test

Measurements	Male (n = 11)		Female (n = 12)		*Z*	*p*
	$\bar{X}$ ± SE	95% CI	$\bar{X}$ ± SE	95% CI		
Head length (mm)	60.09 ± 3.62	51.51-69.49	52.75 ± 0.54	51.57-53.93	-3.721	<0.001
Bill length (mm)	38.27 ± 0.88	35.92-40.08	34.25 ± 0.54	33.07-35.43	-3.382	0.001
Wing length (mm)	68.18 ± 0.33	67.33-68.67	60.17 ± 0.49	59.09-61.25	-4.097	<0.001
Tail length (mm)	45.18 ± 0.55	43.80-45.80	38.71 ± 0.59	37.42-40.00	-4.101	<0.001
Tarsus length (mm)	20.20 ± 0.13	19.90-20.50	19.25 ± 0.46	18.23-20.27	-2.458	0.014
Total body length (mm)	152.27 ± 2.49	145.80-158.20	142.50 ± 1.98	138.14-146.86	-2.994	0.003
Weight (g)	13.18 ± 0.52	11.90-14.50	12.75 ± 1.72	8.97-16.53	-1.686	0.092

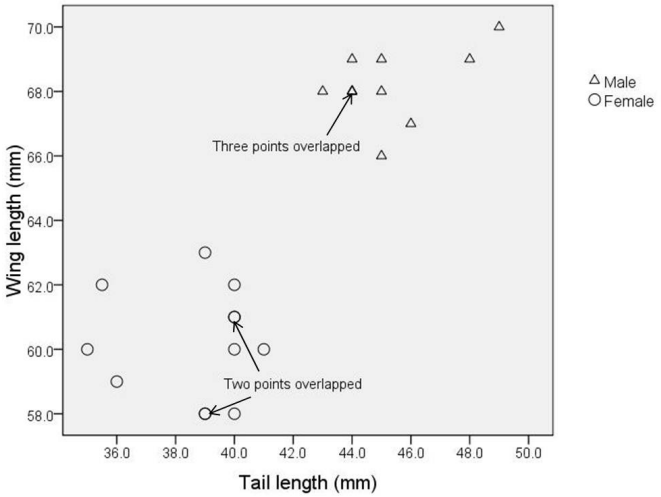

Figure 3. Scatterplot of wing length against tail length measured from the little spiderhunter showing a clear separation according to sexes (n = 23)

In this study, we obtained almost equal sample sizes for both sexes and all body measurements were similar to those reported in literature (Cheke et al., 2001; Cheke & Mann, 2008; Wells, 2010; Jeyarajasingam, 2012). Our results indicated that the little spiderhunter can be sexed based on five morphological parameters. Albeit not significantly heavier, male birds have longer bodies, wings, tails, bills, and heads. Despite the small sample size, the study has also demonstrated that pectoral tufts may be a good indication of sex i.e. adult male. However, such characteristics may not be observed in juveniles and subadults that do not have visible orange-yellow gape flange (Wells, 2010). Hence, for subadults and juveniles, wing and tail lengths may be useful to discriminate the sexes.

Being frequently encountered during mist-netting sessions, and being the most widespread among its congeneric counterparts, with specialised feeding structure, the little spiderhunter has great potential as a focal species for the study of complex ecological interactions in the tropics (Olsen et al., 2013). For example, since male little spiderhunters have longer bills than the females, it would be interesting to examine if differential foraging is present between the sexes, i.e. possible interactions between the birds, as pollinators, and floral anatomy (Paton & Collins, 1989; Sakai et al., 1999; Cronk & Ojeda, 2008; Sakai et. al., 2013). Such research is especially important in view of the increasing risk of losing avian pollinators around the world (Regan et al., 2015).

CONCLUSION

Using morphometric data collected in the field, and supported by molecular sex determination, we were able to evaluate the feasibility of morphometric sex determination in the little spiderhunter in Peninsular Malaysia. Since the sexing of birds is important in behavioural and ecological studies, this study provided an inexpensive, rapid, and accurate way for future sex determination of the little spiderhunter.

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Performance of Male Crossbred (Saanen×Local) Goats Fed Concentrate Diet

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ABSTRACT

The aim of this study was to assess the performance of concentrate feeding on intake and growth rate in goats. A total of 14 non-castrated male crossbred (Saanen×Local) goats of approximate age of 6 months and average initial live weight of 23.5±3.8 kg were used in a completely randomised design. The animals were divided into two treatment groups: control group and concentrate group. Both groups were fed their respective diets *ad libitum* throughout the experiment. The goats on the concentrate diet were also supplemented daily with 400 g fresh Napier grass variety for each animal. Chemical composition of the diets, intake and growth rate of the goats were evaluated. No differences ($p>0.05$) were observed in dry matter and organic matter intakes except for crude protein (CP) and neutral detergent fibre (NDF) ($p< 0.05$). The control group showed higher intakes of CP and NDF compared to the goats in the concentrate group. However, no differences were observed ($p>0.05$) in the growth performance and feed conversion ratio between the control and concentrate groups. There was no significant effect on daily weight gain of the goats fed the concentrate diets, suggesting that corn and soya waste with 400 g fresh Napier grass can support moderate weight gain in Saanen crossbred goats.

Keywords: Concentrate feeding, crossbred goat, feed intake, growth rate, soya waste

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INTRODUCTION

There is potential to exploit goat rearing in Malaysia to meet local demand for meat and milk. However, the performance of local goats is poor. Therefore, crossbreeding with high-yielding goats has been practised to improve the performance of local goats (Hirooka et al., 1997; Ariff et al., 2010). It is known that Saanen goats can enhance the productive performance of local goats through crossbreeding. Compared to local goats, Saanen crossbreds usually show better performance in milk production, birth weight of kids and daily weight gain (Sahni & Chawla, 1982).

For raising meat goats, one of the important factors in maintaining economic viability is how quickly and efficiently goats grow. High growth rate and efficiency decreases the time that it takes the kids to reach market weight, which in turn decreases the labour and feed cost associated with raising goats. Weaning kids receive their nutrition from two different sources: roughage and concentrate. In Malaysia, grass production is limited due to low soil fertility and lack of natural grassland (Chee, 1989). Farmers face shortage of grass especially during the dry season (Khaing et al., 2015). To overcome feed shortage during the dry season, more concentrates are usually offered to ruminants compared to the amount of roughage.

In this study, the treatment diet was composed of minimum ingredients such as corn and soya waste. Corn was characterised as energetic feed, while soya waste was characterised as inexpensive protein-rich feed (Rahman et al., 2014). It was hypothesised that a high-energy and high-protein diet would improve feed conversion efficiency by reducing feed intake. Therefore, the objective of this study was to evaluate the effect of concentrate feeding on intake and growth performance of male crossbred (Saanen × Local) goats.

MATERIALS AND METHOD

Experiment Site

The experiment was conducted from March to June 2015 at the Rumpun Asia Sdn. Bhd. (RASB) goat farm, Selangor, Malaysia. The latitude, longitude and average annual temperature were 3°28′ N, 101°38′ E and 28.3°C, respectively. The experiment procedure was entirely conducted according to the guidelines of the Institutional Animal Care and Use Committee of University of Malaya.

Experiment Design

A total of 14 non-castrated crossbred (Saanen×Local) males approximately six months old and with a mean initial liveweight (LW) of 23.5±3.8 kg were used in this study. The crossbred goat (Saanen × Local) was first crossed from a pure Saanen male goat and 'kacang' female goats. Before starting the experiment, the animals were dewormed. The animals were divided into two groups, with seven animals in each

group. The animals were individually kept in a single pen and fed for 98 days (14 days of adaptation and 84 days of evaluation) with two dietary treatment groups containing iso-caloric and iso-nitrogenous diets, namely: (i) control group (consisting of Napier grass, cracked corn and soybean) and (ii) concentrate group (consisting of cracked corn and soya waste). The animals in both groups were given the experiment feed twice a day, provided *ad libitum* to allow for 10% of refusal. To maintain rumen microbial activity and to prevent the risk of ruminal acidosis, the goats in the concentrate group were also given an additional amount of chopped Napier grass (400 g fresh/animal/d), which contained 22.1% dry matter (DM), 8.2% ash, 7.5% crude protein (CP) and 67.3% neutral detergent fibre (NDF) in DM form. The goats received an adequate supply of water and mineral blocks throughout the experiment. Soya waste (also known as okara) is an industrial by-product; in this study, it was supplied twice a week by a local supplier, stored anaerobically in containers. The corn and soybean were purchased locally.

The diet was formulated using the LUGRE programme (Langston University Goat Research Extension), which predicts the nutritional requirements of goats. It was estimated that the daily DM intake per goat would be 4% of the LW and the daily LW gain would be 100 g. The daily metabolic energy (ME) and CP requirements were calculated to be 7.9 MJ and 73 g, respectively. The daily feed intake was measured by weighing of daily offered feed and refusal of individual goats. To estimate intake, samples of offered feed and refusals were dried in an oven once a week for DM analysis. The goats in the concentrate group refused an average of 95±7.5 g DM feed/goat/d of their total diet, while the goats in the control group refused an average of 130±28 g DM feed/goat/d of their total diet. The goats in the concentrate group consumed all the offered Napier grass, while the goats in the control group refused about 68 g DM Napier grass/goat/d of their total refused diet, which included about 52% Napier grass, mostly the stem. The composition of feed and chemical composition of the experiment diets are presented in Table 1. The goats used in the experiment were weighed every 14 days using a weighing balance. The daily LW gain was calculated by difference between the initial LW and final LW of the individual goats and then divided by the experiment period.

Table 1
Composition and nutritive values of the experiment feeds

Items	Experiment group	
	Control group	Concentrate group*
Ingredients		
Napier grass (% DM)	28	-
Corn (% DM)	52	60
Soya waste (% DM)	-	40
Soybean (% DM)	20	-
Total	100	100
Nutrients		
Dry matter (%)	48.3	40.3
Organic matter (% DM)	96.1	97.8
Crude protein (% DM)	15.9	15.6
Neutral detergent fibre (% DM)	35.0	26.7
Total ash (% DM)	3.9	2.2
Metabolisable energy (ME) (MJ/kg DM)¶	11.8	12.3

Calculated ME value (MJ/kg DM) = 0.016 DOMD [g digestible organic matter/kg DM (AFRC 1998)]. Since DOMD was not determined in this study, the ME values are calculated from data published by several researchers who followed the methods developed by AFRC (1998) for estimating ME of Napier grass, corn, soybean and soya waste. DM, dry matter; MJ, mega joule.
*Goats received additional 400 g fresh Napier grass/animal/d, which contained 22.1% DM, 8.2% ash, 7.5% crude protein and 67.3% neutral detergent fibre on DM basis. These values were not reflected in the ration composition

Chemical Analysis

During the experiment period, samples of the feed were collected once a week and dried for 48 h at 70°C. Samples were ground to pass through a 1-mm sieve and then analysed for DM, ash and CP following AOAC methods (2005). The NDF was determined on ground samples following the methods of Van Soest et al. (1991). The results of the chemical analysis of the experiment feed ingredients are shown in Table 2.

Table 2
Chemical composition of experiment feed ingredients

Items	Dry Matter (%)	Organic Matter (%)	Crude Protein (%)	Neutral Detergent Fibre (%)	Ash (%)
Napier grass	22.1	91.8	7.5	67.3	8.2
Corn (cracked)	89.8	98.6	9.7	26.0	1.4
Soybean	90.0	95.1	44.0	13.0	4.9
Soya waste	22.1	96.6	23.5	27.8	3.4

Statistical Analysis

Measurements of feed intake and growth performance of the goats were subjected to repeated measures design using SPSS (version 12.0) based on the following model:

$$Y_{ijt} = \mu + T_i + \alpha_j + \beta_t + e_{ijt},$$

where Y_{ijt} = an observed value for measurement taken from animal j receiving treatment i at time t; μ = the overall mean; T_i = the mean effect of dietary treatment i; α_j = the fixed effect of initial body weight of animal j; β_t = the random effect of the measurement taken at time t; e_{ij} = the residual error.

RESULTS

Table 3 represents the effects of dietary groups on the feed and nutrient intake of the experiment goats. There was no significant difference ($p>0.05$) on total DM and OM intake between the diets evaluated. However, there were significant differences ($p<0.05$) in the intake of CP and NDF between the diets. The intake of CP was 177 vs. 162 g/d for the control group and the concentrate group, respectively, while the NDF was 378 vs. 326 g/d for the control group and the concentrate group, respectively. Clearly, the intake of CP and NF was higher ($p<0.05$) among goats in the control group than among those in the concentrate group.

Table 3
Dry matter and nutrient intakes of goats as affected by experiment feeds

Parameter	Experiment Group	
	Control Group (Mean ± SD)	Concentrate Group (Mean ± SD)
Intake		
Dry matter (g/head/d)	1113 ± 220.8[a]	1036 ± 205.5[a]
Dry matter (kg/d, % LW)	3.89 ± 0.5[a]	3.77 ± 0.5[a]
Organic matter (g/head/d)	1070 ± 211.3[a]	1006 ± 198.7[a]
Crude protein (g/head/d)	177 ± 33.5[a]	162 ± 30.7[b]
Neutral detergent fibre (g/head/d)	378 ± 81.2[a]	326 ± 70.0[b]

Means with the same superscript letters in the row are not significantly ($p>0.05$) different from each other. LW, liveweight; SD, standard deviation

Table 4 shows the effects of dietary treatment on the growth performance and feed conversion ratio of goats during the experiment period. No significant difference ($p>0.05$) was found on the initial LW (23.7 vs. 23.3 kg), final LW (33.6 vs. 31.6 kg) and daily LW gain (118 vs. 99 g/d) between the control group and the concentrate group, respectively. Similarly, no differences ($p>0.05$) were observed in the feed conversion ratio (9.6 vs. 10.5) between the control group and the concentrate group, respectively.

Table 4
Growth performance of goats affected by experiment feeds

Parameter	Experiment Group	
	Control Group (Mean ± SD)	Concentrate Group (Mean ± SD)
Initial LW (kg/head)	23.7 ± 3.8[a]	23.3 ± 3.8[a]
Final LW (kg/head)	33.6 ± 4.6[a]	31.6 ± 4.3[a]
Net gain of LW (kg/head)	9.9 ± 1.8[a]	8.3 ± 1.5[a]
Average daily LW gain (g/head)	118 ± 21.3[a]	99 ± 17.9[a]
Feed conversion (kg DM/kg gain)	9.6 ± 1.4[a]	10.5 ± 1.5[a]

Means with the same superscript letters in the row are not significantly ($p>0.05$) different from each other. LW, liveweight; SD, standard deviation; DM, dry matter

DISCUSSION

Slow growth rate is considered one of the major limiting factors in goat production. It may markedly improve by supplying the plane of nutrition. In this experiment, goats in the control group and the concentrate group gained LW at a rate of 118 and 99 g/d, respectively. Liveweight gain indicates that goats in both groups received a sufficient amount of energy and protein for maintenance and growth from their diet. The LW gain, however, was lower than the values obtained in the Saanen×Hair goat (140 g/d) by Gokdal (2013); this may have been due to not only diet but also genetics. Paengkoum et al. (2004) also observed LG gain that was similar to the results of this study.

Nutrition plays a vital role in improving LW; however, the degree of response varies with breed or type (Devendra & Burns, 1983). The range of DM intake was 3.77-3.89 kg/d (percentage of LW) for both groups, and this could be due to the chemical composition of the corn, soybean and soya waste, which had high amounts of energy and protein, and this attracted the goats to consuming more of this feed. This statement might be explained by the findings of Wiese et al. (2003), who suggested that higher DM intakes could be due to an availability of nutrients that are degraded by rumen microorganisms. The soya waste that partially replaced the Napier grass did not alter the intake of DM and OM, even with a lower intake of CP and NDF for a diet with soya waste. This probably occurred because of the slightly lower content of CP and NDF in the diet of the concentrate group, which may have contributed to intake without CP and NDF by the goats. In addition, the goats in the concentrate group received Napier grass (88.4 g DM/d/goat) to prevent acidosis, and this amount of Napier grass was not reflected in the table of ration composition (Table 1); the Napier grass contained only 7.5% CP, and this might have also contributed to lower CP intake by the goats in the concentrate group. However, higher NDF intake by the goats in the control group did not attribute to significant growth difference between

the groups; this is in line with the findings of Sheridan et al. (2000), who reported that Boer goats showed no significant difference in LW gain when fed diets of low (2.14 Mcal/kg DM) and high energy (2.60 Mcal/kg DM). Similarly, our finding is also in line with the previous results of Prieto et al. (2000), who reported that excess CP intake of the goats did not affect the growth rate of the kids.

On the other hand, it is known that increased NDF digestibility can exhibit in higher digestible energy and may subsequently lead to higher LW gain, but no digestibility trial was conducted in this study. Rahman et al. (2016) reported that goats fed a soya waste-supplemented diet showed improved NDF digestibility, which supported our present findings that there was no difference in BW gain between the two diets, even though the goats fed a concentrate diet (included soya waste) showed lower NDF intake. Soya waste contains 24.5% CP and 73% total digestible nutrients, and it is an inexpensive source of nutrients for animal consumption (Rahman et al., 2016). The results implied that both diets in the present study were sufficient to provide enough nutrients to increase the LW gain of the goats. Greater quantities of Napier grass can be replaced with soya waste in diets for Saanen crossbred goats without any adverse effects on their growth performance. The findings from this study provide useful information on the choice of diet depending on economic benefit, ease of use and forage availability.

CONCLUSION

The goats fed a concentrate diet had lower CP and NDF intake compared to the goats fed control diet, while no significant differences were observed in DM and OM intake. No significant differences were observed in the final LW, average daily LW gain and feed conversion ratio. These findings suggest that a concentrate diet may be useful for goat production, especially during the dry season when forage supply is limited.

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Antioxidative Activities in Coconut Cultivar against the Infestation of Red Palm Weevil (*Rhynchophorus ferrugineus* Olivier)

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ABSTRACT

The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* Olivier is a pest which targets coconut palms in Malaysia. The RPW-coconut interaction leads to a toxic reactive oxygen species (ROS), predominantly hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\cdot -}$), thus activating its protective antioxidant system. This study looks at the catalase; CAT, ascorbate peroxidase; APX and guaiacol peroxidase; g-POD specific activities as well as ascorbic acid, α-tocopherol, and carotenoids content in the most commonly planted coconut cultivar, MATAG. Fourteen months old MATAG plants were infested with RPW for 28 days. The antioxidant assays were carried out at 0, 7, 14, 21 and 28 days of infestation at upper and lower parts of the stem. The CAT activities were significantly higher ($p<0.05$) in the upper part of infested coconuts (11.72 ± 0.78 units/mg protein) compared with its control (1.68 ± 0.55 units/mg protein), especially at 7 days of treatment. G-POD specific activities were also significantly higher ($p<0.05$) in the upper part of infested coconuts (484.12 ± 31.30 units/mg protein) compared with its control (160.21 ± 47.58 units/mg protein). In contrast, APX specific activities were induced to 46.94 ± 2.26 units/mg protein, especially at 14 days of infestation at the lower part whereas the APX activities were slowly increased at the upper part of stem. The RPW infestation managed to significantly increase ($p<0.05$) the carotenoids content in infested MATAG whereas there were no significant changes in ascorbic acid and α-tocopherol in infested

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and control plants. This study revealed that different antioxidants have different role in combating the oxidative stress induced by RPW in MATAG cultivar.

Keywords: Coconut cultivar MATAG, Enzymatic antioxidants, Non-enzymatic antioxidants, Oxidative stress, Red Palm Weevil

INTRODUCTION

The coconut palm is grown abundantly in many tropical countries. It is a source of food, drink, fibre, vitamins, minerals and electrolytes associated with impressive health benefits. There are 12 different varieties of coconut in Malaysia, however, only 3 hybrids are recommended by Department of Agriculture (DOA), including Kelapa Wangi or Pandan (Aromatic Dwarf), MAWA, and MATAG (DOA, 2011). This study focused only on MATAG cultivars, a hybrid between seedlings from Malaysia and Philippines, Dwarf/Malayan Red Dwarf X Tagnanan Tall, as it is capable of producing more coconuts (about 10 to 22 coconuts per time) compared with other cultivars (Mohd. Taufik & Md. Akhir, 2014). This coconut hybrid begins to fruit on the third year of cultivation and can be harvested at 48 months (DOA, 2016). The MATAG shows high production of nuts as it can yield approximately 25,000-30,000 nuts per hectare per year which is more than the normal coconut or other hybrid coconut tree (PRESSREADER, 2016).

The coconut plantations in Malaysia are now under attack by the invasive red palm weevil, *R. ferrugineus* (DOA, 2011). Belonging to the order of Coleoptera, the adult RPW can grow more than 25 mm in length, and has red and black spots (Figure 1). This weevil can fly up to 1 km in distance uninterrupted. Completing one of the compulsory general characteristic of pest, this *R. ferrugineus* is said to be a fast breeder and able to breed in a wide range of climates (Rajamanickan, Kennedy & Christopher, 1995).

Figure 1. Adult red palm weevil

The RPW-coconut interaction has contributed to a great loss in the coconut industry. In 2011, an intensive three month-survey throughout Terengganu in over 800 ha of coconut plantations indicated that RPW attacked as many as 550,000 coconut trees, indicating a drastic increase and rapid spread of RPW population (Wahizatul, Zazali, Abdul Rahman & Nurul Izzah, 2013). El-Mergawy & Al-Ajlan (2011) reported that, *R. ferrugineus* spread slowly and attacked many palm species, especially in the Middle East and several countries of the Mediterranean Basin. In Malaysia, the first RPW infestation was detected in

2007 by the Department of Agriculture in all seven Terengganu districts. Currently, the infestation rate has drastically increased and RPW is found spread throughout the country. Wahizatul et al. (2013) reported that the RPW attack coconut palms in three ways: through the shoot and straight to the cabbage (edible palm pith) of the coconut, through the trunk and through the root system. Symptoms of RPW attacks are hard to detect as RPW is a concealed tissue borer. At severe infestation stage, the coconuts show signs of wilting, drooping of the leaves (like an umbrella or skirt-shaped leaves) (Figure 2).

Figure 2. Wilting and drooping of the infested leaves

In 2016, RPW attacked coconut plantations in 5 states of Peninsular Malaysia including Terengganu, Kelantan, Kedah, Penang and Perlis (DOA, 2016). Infestation of *R. ferrugineus* is believed to trigger the oxidative stress response in coconut plants, resulting in the overproduction of highly reactive oxygen species (ROS) (Gill & Tuteja, 2010). Infested coconut plants activate both enzymatic and non-enzymatic antioxidants as defence mechanisms against the infestation process. This study elucidates the defence mechanism in coconut-RPW interaction by measuring the enzymatic (catalase; CAT, ascorbate peroxidase; APX and guaiacol peroxidase; g-POD specific activities) and non-enzymatic antioxidants (ascorbic acid, α-tocopherol, and carotenoids contents) in MATAG stems. This study contributes to the understanding of the defence mechanism in coconut cultivars leading to the development of possible antioxidative markers in coconut-RPW infestation.

MATERIALS AND METHODS

Plant Materials

Thirty, 14-month-old plants of MATAG coconut cultivars were obtained from Kompleks Pertanian Negeri, Ajil, Hulu Terengganu, Malaysia. They were planted in Kampung Bukit Berangan, Tepuh, Kuala Nerus, Terengganu (Figure 3). The average temperature at the study site was between 30 to 32°C, humidity of 75-76%, sandy soil type and pH 5.8. For each infested and control treatment, three replicates of coconut plants were used. Each plant was infested with 10 red palm weevils per plant (Figure 4). Each control and treated plants was covered with a few layers of green mesh net (54cm X 54cm X 126cm) (Figure 5). The infestation was carried out for 28 days. The antioxidative defence mechanisms of the stem were evaluated by

measuring the enzymatic and non-enzymatic antioxidants in both control and infested plants at different distance. The upper part was measured 30cm above the soil line and lower part was 15cm above the soil line. The assays were carried out at 0, 7, 14 and 28 days of infestation. The experiments were repeated two times using Randomized Complete Block Design (RCBD).

Figure 4. Treated plant infested with 10 alive RPWs

Figure 3. Fourteen months old MATAG cultivars

Figure 5. Plants covered with green mesh net

Antioxidant Assays

Enzymatic antioxidants. The CAT specific activity was extracted based on Clairbone's method (1985). Stem tissues (0.15g) was ground with 1.0ml of 50mM phosphate buffer (pH 7.4) and clean sand at 0-4°C in pre-chilled mortar and pestle. The mixture was then centrifuged at 10000rpm (Eppendorf Centrifuge 5804R, Germany) at 4°C for 10 minutes. A total of 3.0ml reaction buffer containing 19mM H_2O_2 in 50mM phosphate buffer, pH 7.0 and 100µl of supernatant (enzyme extract) was added. The rate of changes in absorbance was measured at 240nm for 3 minutes using spectrophotometer (Shimadzu UV-1601A, Japan). The CAT specific activity was expressed in µmoles of H_2O_2 consumed per minutes per mg protein.

The APX specific activity was analysed following the method of Sairam, Shukla, & Sayena (1998) and Nakano & Asada (1981). Approximately 0.15g stem tissue was extracted with 1.0ml of 100mM phosphate buffer (pH 7.0) containing 1.0mM ascorbic acid in pre-chilled mortar and pestle at 0-4°C. Then, it was centrifuged at 10000rpm (Eppendorf Centrifuge 5804R, Germany) for 10 minutes at 4°C. Approximately 0.5ml 3mM ascorbic acid, 1.5ml 100mM phosphate buffer (pH 7.0), 0.1ml 3mM EDTA, 0.4ml enzyme extract and 0.3ml

distilled water were added. Finally, 0.2ml 1.5mM H_2O_2 was added into the above mixture to induce the reaction. The changes in absorbance were monitored at 290nm at 3 minutes and were expressed as moles ascorbate oxidised per hour per mg protein.

g-POD specific activity was estimated based on the method of Agrawal & Patwardhan (1993). Stem tissue (0.15g) was ground with 1.0ml of 100mM phosphate buffer (pH 7.0) in pre-chilled mortar and pestle at 0-4°C. The homogenate was then centrifuged at 10000rpm (Eppendorf Centrifuge 5804R, Germany) at 4˚C for 10 minutes. The reaction mixture consists of 3.0ml of solution containing 1.0ml 50mM phosphate buffer (pH 7.5), 1.0ml 20mM guaiacol, 1.0ml 30mM H_2O_2 and 100µl enzyme extract. The changes in absorbance were monitored at 470nm for 3 minutes and POD specific activity was expressed as µmoles of H_2O_2 consumed per minute per mg protein.

The total protein concentration was measured according to the method proposed by Bradford (1976). Coomassie Brilliant Blue G-250 (100mg) was dissolved in 50ml 95% ethanol. Then, 100ml concentrated phosphoric acid was added and the mixture diluted to 1.0L with distilled water. The solution was later filtered through a filter paper and stored at room temperature in light-proof bottles. A total of 100µl of enzyme extract was added to 3ml of Bradford's reagent and the absorbance measured at 595nm after 10 minutes. The protein standard curve was prepared with various concentrations (0 to 1.0mg/ml) of Bovine Serum Albumin (BSA).

Non-Enzymatic antioxidants. The procedure based on Jagota & Dani (1982) was followed to determine the amount of ascorbic acid. A total of 0.15g stem tissue was ground with pre-chilled mortar and pestle in 1.0ml of 10% trichloroacetic acid (TCA) and clean sand under low light intensity at 0-4°C. The ground sample was then centrifuged (Eppendorf Centrifuge 5804R, Germany) at 10,000 rpm for 10 minutes at 4°C. The supernatant obtained (300µl) was added into test tube containing 200µl 10% Folin reagent and 1700µl distilled water. After 10 minutes, the absorbance of the mixture was measured at 760nm. The amount of ascorbic acid in the sample was calculated based on the standard curve prepared using ascorbic acid at the range of 0 to 60µg/ml.

α-Tocopherol was extracted based on the method proposed Hodges, Andrews, Johnson and Hamilton (1996) and Kanno and Yamauchi (1977). A total of 0.15g stem tissue was ground with 1.5 ml acetone and clean sand in a mortar and pestle at 0-4°C. Then, 0.5 ml hexane was added. The mixture was vortexed for about 30 seconds followed by centrifuging at 10,000 rpm (Eppendorf Centrifuge 5804R, Germany) for 10 minutes. After the centrifugation, the top layer was discarded and the hexane extraction was repeated twice. The hexane-extract (0.5ml) was added into 0.4ml 0.1% (w/v) PDT (3-(2-pyridyl)-5,6-diphenyl-1,2,4 triazine),

0.4ml 0.1% (w/v) ferric chloride and 1.7ml absolute ethanol. The mixture was gently swirled and left for four minutes for colour development. Following this, 0.2 ml of 0.2 M orthophosphoric acid was added and the mixture was allowed to stand for 30 minutes at room temperature. The absorbance was measured at 554nm using spectrophotometer (Shimadzu UV-1601A, Japan). Amount of α-tocopherol was calculated based on the standard curve prepared using α-tocopherol (Sigma, type V) at various concentrations (0-1.4 µg/ml).

The carotenoid content was analysed based on the method proposed by Lichtenthaler (1987). Stem tissue (0.15g) was ground up with 3 ml of 80% (v/v) acetone and clean sand in a mortar and pestle. The mixture was centrifuged at 10,000 rpm (Eppendorf Centrifuge 5804R, Germany) for 10 minutes. Supernatant obtained was measured spectrophotometrically (Shimadzu UV-1601A, Japan) at three different wavelengths, i.e. 663.2, 646.8 and 470nm.

Statistical analysis. Data obtained were analysed using analysis of variance (TWO WAY ANOVA) of Statistical Package for Social Science software (SPSS) version 20. Multiple comparisons were performed using Duncan Multiple Range Test (DMRT) at $\alpha=0.05$ as significant level.

RESULTS AND DISCUSSION

MATAG hybrid was selected as it produces very high yield nuts per year with multifarious uses including coconut water, coconut milk production and also for grated coconut. Besides, one MATAG coconut tree can produce high quality copra eight times per year in the long term where it has thicker plump compared with other hybrid coconut as well as normal coconut (Sivapragasam, 2008).

Recently, RPW is reported as a pest of more than 40 palm species worldwide which not only destroy the coconut tree but also greatly affects its quality and quantity (Cangelosi, Clematis, Curir & Monroy, 2016). The activity of RPW must be monitored to prevent infestation (Vacas, Primo, & Navarro-Llopis, 2013). Infestation may alter the equilibrium between free radical production and defence mechanisms in favour of free radical production. The balance between the formation and detoxification of ROS is critical to plant cell survival. However, the degree of vulnerability and defence mechanisms of palm species against the RPW are still poorly known. Thus, the study of RPW-coconut interaction is very important to understand the responses of coconut against the RPW infestation.

Figure 6 shows the effect of coconut-RPW infestation on CAT specific activity in (A) upper part and (B) lower part of infested and non-infested MATAG stem at 28 days of infestation. The CAT specific activities of infested upper stem were significantly increased ($p<0.05$) from day 0 to 7 days of infestation compared with control (Figure 6A). Results of this study confirm the findings of Arutselvi, Balasaravanan, Ponmurugan & Muthu (2012). The authors reported that infested

turmeric leaves by *Udaspes folus* increased CAT activities in the leaves compared with control plants which could have been the result of higher production of ROS, particularly H_2O_2, as a response to the infestation. This is the earliest defence of the plant (Wojtaszek, 1997). Thus, the H_2O_2 may be removed by CAT and therefore, the formation of hydroxyl radical damage will be avoided. No significant difference ($p>0.05$) was observed in the infested and control plants after 21 days of treatment. The maximum activity was observed at 7 days of infestation (11.72 ± 0.78 units/mg protein). Higher activities in infested plants indicated that CAT enzyme involved in defence response and decreased the toxicity of ROS produced during the infestation process (Khorshidi & Sherafatmandjour, 2013). However, CAT specific activities in control dropped significantly ($p<0.05$) after day 7 in addition to reduced infestation of the lower stem (Figure 6B). This might be due to the inactivation and degradation of CAT (Feireabend, Schaan, & Hertwig, 1992). Lower CAT specific activities in lower stem might also be related to low concentration of H_2O_2 produced. Zamocky, Janecek and Koller (2002) stated that CAT has been proven to be inefficient in converting low concentrations of H_2O_2 compared with APX. Figure 6 shows CAT specific activities were significantly higher in upper stem compared with the lower stem. It may be suggested that the infestation site might influence the antioxidative responses in this plant. Wahizatul et al. (2013) suggested that the infestation in coconut trees could occur through the shoot, trunk and root system. Since young MATAG plants were used in this study, the infestation started at the root of the plants. The distance between the infestation sites to the lower part of the stem was only about 12cm, so excess production of H_2O_2 may be produced, thus enhancing the CAT activities at an early phase of infestation. The increase of H_2O_2 could act as signalling molecules to trigger the CAT activities in the upper part of the infested stem.

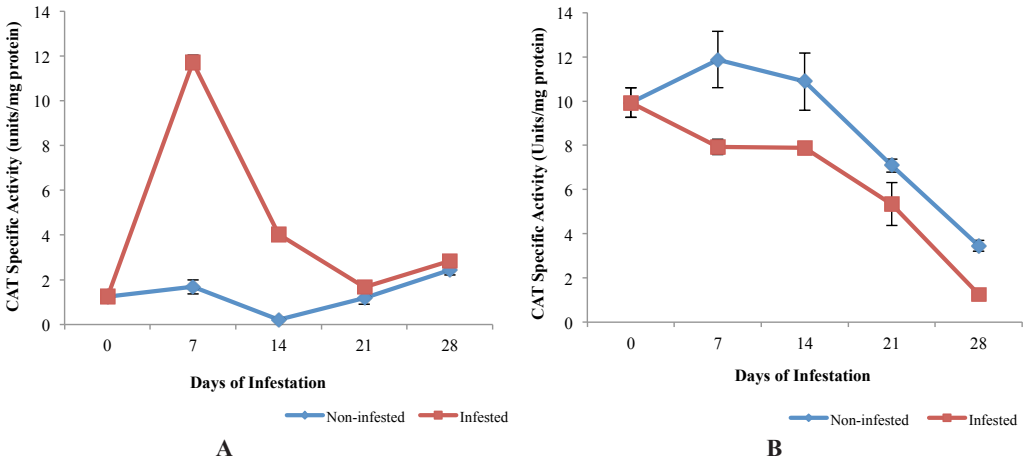

Figure 6. The effect of coconut-RPW infestation on CAT specific activity in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

The APX is a key enzyme that plays an important role in ascorbate-glutathione cycle, main H_2O_2-detoxification system in plant chloroplasts (Asada, 1992). In particular, APX has higher affinity for H_2O_2 and utilises ascorbate as specific electron donor to reduce H_2O_2 to water in chloroplasts, cytosol, mitochondria and peroxisomes, as well as in the apoplastic space (Sofo, Scopa, Nuzzaci & Vitti, 2015). The APX-specific activities in infested upper stem slowly increased throughout the experiment, whereas the non-infested stem showed significantly ($p<0.05$) higher APX specific activities at 21 days of experiment (27.45 ± 0.28 units/mg protein) (Figure 7A). Higher APX specific activities were observed in the infested upper stem of coconuts compared to with control, especially at initial and later stages of experiments (Figure 7A). The infested lower stem showed the same pattern at 14 days of infestation (Figure 7B). The considerable increase in APX activity observed can protect plants, which, under stress conditions, present sustained electron flows and are the main producers and targets of ROS action (Foyer & Shigeoka, 2011). Enhanced APX specific activities in the lower part at 14 days of infestation might be also related to decrease the toxicity of ROS, particularly H_2O_2 as APX play a secondary role in H_2O_2 scavenging as observed by Gondim, Filho, Costa, Alencar & Prisco (2012) in salt stress maize and Khorshidi & Sherafatmandjour (2013) in fennel. In addition, continuous increases in the antioxidant enzyme activities may protect the cell structure by eliminating the ROS produced (Ahn, Oke, Schofield & Paliyath, 2005).

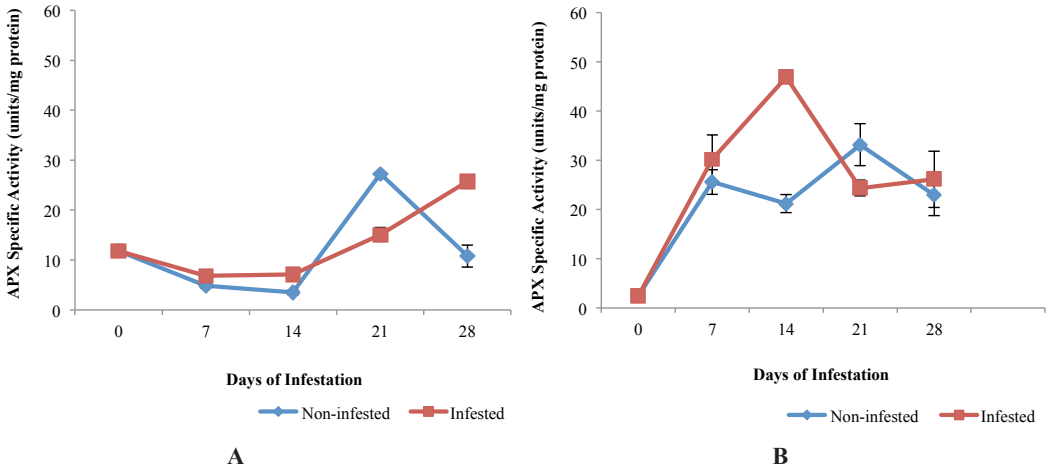

Figure 7. The effect of coconut-RPW infestation on APX specific activity in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

The PODs are major enzymes associated with defence related pathways in plants and pathogens (Van Loon, Rep & Pieterse, 2006). Almagro et al. (2009) reported the importance of PODs in auxin metabolism, cross-linking of cell wall components, lignin, suberin and phytoalexin synthesis, as well as in metabolism of ROS. The PODs decompose H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants. In this study, infested upper stem significantly enhanced ($p<0.05$) the g-POD specific activity to a maximum activity at day 7 (484.12 ± 31.30 units/mg protein) compared with non-infested stem (160.20 ± 47.58 units/mg protein) (Figure 8A). Similarly, infected lower part of stem induced higher g-POD specific activities after 7 to 21 days of infestation compared with its control (Figure 8B). Thus, the increase in g-POD specific activity in this study may help to reduce the oxidative stress generated by the imbalanced production of ROS in coconut due to RPW infestation. Previous study on the induced POD activities also noted infested cabbages of PANDAN, MATAG and MAWA coconut cultivars as a response to RPW attacked (Norhayati, Afzan, Jannah, & Nurul, 2016). Similar elevated POD specific activities were also observed in infestation of *Coccus hesperidum* on its host plant *Nephrolepis biserrata* (Golan, Rubinowska, & Gorska-drabik, 2013) and of *Spodoptera litura, Aphis craccivora* and *Bemisia tabaci* on cowpea, cotton and tomato (Singh, Dixit, Singh, & Verma, 2013).

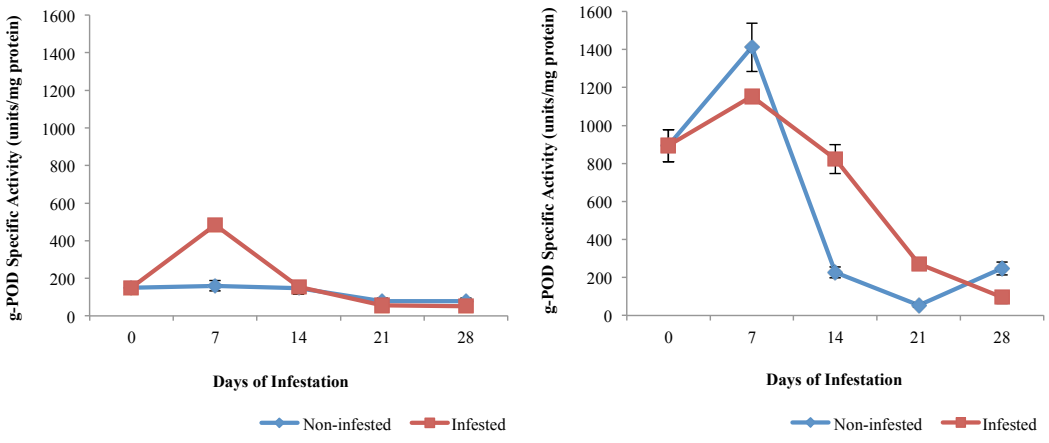

Figure 8. The effect of coconut-RPW infestation on g-POD specific activity in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

Ascorbic acid is the most important vitamin in fruits and vegetables. This substance helps to protect organism from cell membrane damage and other structures by neutralising free radicals that occur as a result of oxidative stress (Rekha et al., 2012). Amount of ascorbic acid in infested and non-infested upper stem remained unchanged throughout

the experiments except at day 21, where the amount of ascorbic acid in infested plants were lower (388.60±4.87 µg/g fwt) compared with the non-infested plants (Figure 9A). In this study, the antioxidant activity of ascorbic acid might be associated with resistance to oxidative stress in coconut plants. Infested lower stem has significantly ($p<0.05$) higher amount of ascorbic acid at day 14 of infestation compared with non-infested stem (Figure 9B). Niki (1987) suggested that the ascorbic acid increment is to help the scavenging of free radicals in plants. This also indicated that ascorbate acid as the hydrogen donor had eliminated the generation of H_2O_2 in MATAG plants. Sarkar, Srivastava & Dubey (2009) reported that ascorbate acts as a specific antioxidant by donating an electron and convert the free radicals to more stable products and thus, terminating free radical initiated chain reactions.

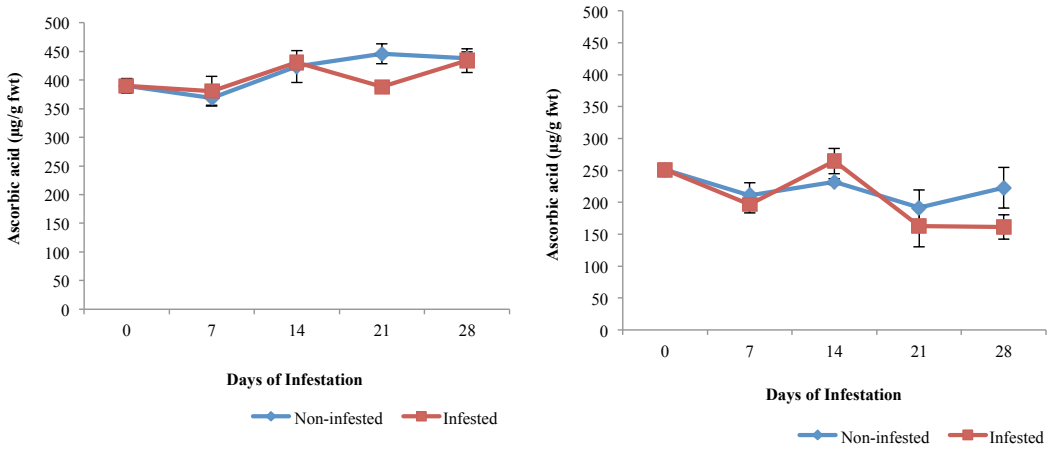

Figure 9. The effect of coconut-RPW infestation on ascorbic acid content in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

Tocopherol is a well-known nature's major lipid soluble chain-breaking antioxidant that helps to protect biological membranes and lipoproteins from oxidative stress (Serbinova, Kagan, Han, & Packer, 1991). The main biological function of α-tocopherol is its direct inducing of cellular responses to oxidative stress through modulation of signal transduction pathways (Azzi, Boscoboinik, & Hensey, 1992). Based on Figure 10, infested upper and lower MATAG stem shows no significant differences ($p>0.05$) in the amount of α-tocopherol along the experiments. However, the α-tocopherol drastically reduced to 14.52 ± 3.17 µg/g fwt and 12.78 ± 0.35 µg/g fwt in control of upper and lower stem respectively. After seven days, the α-tocopherol level was comparable with infested plants. This might be due to the adaptation of the plants towards the environmental conditions on the site at early stages of experiment. Munne-

Bosch (2005) reported that amount of α-tocopherol also changed during plant growth and development as well as in response to oxidative stress. These changes were due to the altered expression of pathway related genes, degradation and recycling. This also indicated that α-tocopherol level and its composition vary during cell development and in response to biotic stress (Collakova & Dellapenna, 2003).

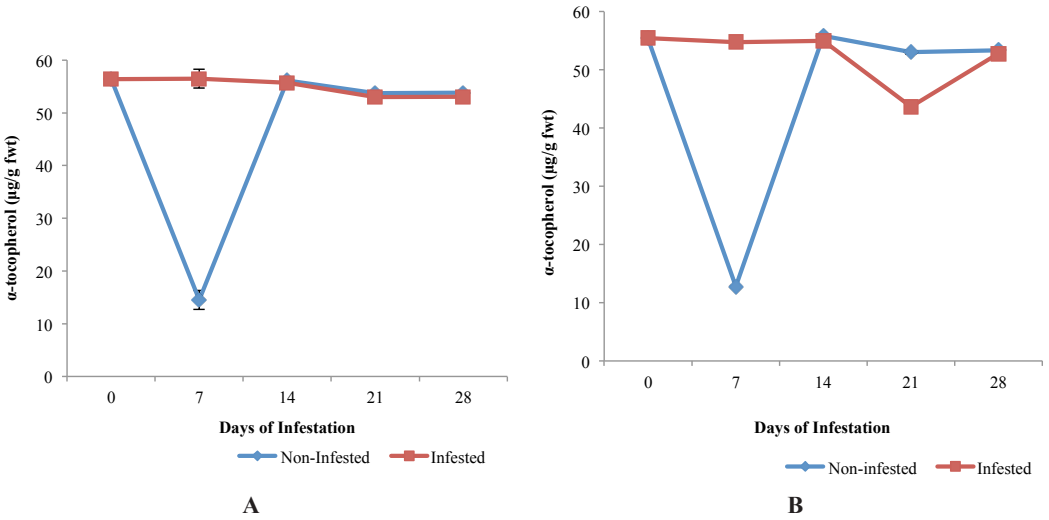

Figure 10. The effect of coconut-RPW infestation on amount of α-tocopherol in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

Carotenoids play an important role in both photobiological and non-photobiological systems. The fact that carotenoids can be readily oxidised, and thus inhibit other oxidation reactions has been known for many years (Krinsky, 1979). Burton & Ingold (1984) proved that carotenoids can function directly as antioxidants by reacting with active oxygen species. Generally, the carotenoids content fluctuated in both control and infested upper stem of MATAG cultivar. However, significantly higher ($p<0.05$) carotenoids content were produced in infested upper stem compared with the non-infested plants (Figure 11A). Lower carotenoids content was observed at the infested lower stem during the later stages of infestation (Figure 11B). Carotenoids protect photosynthetic organisms against potentially harmful photooxidative processes (Bartley & Scolnik, 1995). Thus, this helps the cell to encounter the over production of ROS resulting in damage to the photosynthetic apparatus that can causes photoinhibition (Breusegem, Vranova, Dat, & Inze, 2001).

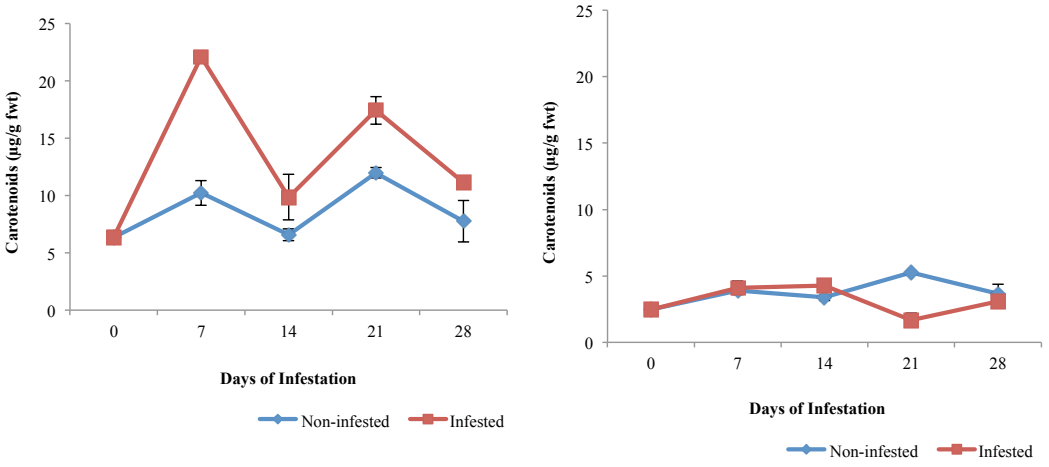

Figure 11. The effect of coconut-RPW infestation on carotenoids content in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

CONCLUSION

Results indicated that most of the antioxidants studied i.e. CAT, APX and g-POD specific activities as well as ascorbic acid, α-tocopherol and carotenoids content showed significant increase as a response to the oxidative stress induced by the RPW infestation. Most of the antioxidants were higher at the lower part of infested stem at early stages of experiment but then signalled the infested upper stem after 14 days of infestation. The activation of antioxidants may enhance the resistance of MATAG cultivar toward RPW infestation. Further studies need to be undertaken to better understand the tolerance level of MATAG plant against RPW infestation.

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Effect of Residue Management and N and S Fertilisation on Cane and Sugar Yield of Plant and Ratoon Cane

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ABSTRACT

Residue management in sugar-cane cultivation is crucial for improving soil health, as it positively impacts the increase of sugar cane productivity. The study aimed to describe the effect of sugar-cane residue management using ammonium sulfate fertiliser and its substitute on cane and sugar yield in plant and ratoon cane. A pot experiment was conducted using a factorial block randomised design. The first factor is N and S fertilisation, consisting of ammonium sulfate (AS), urea, gypsum and bio-compost. The second factor is the residue management consisting of four levels, namely burnt residue, residue incorporated into the soil, residue put on the soil surface and composted residue. These treatments were tested on the first and second cane. The results showed that the composted residue gave the highest increase in cane and sugar yield by 83.7% and 81.2%, respectively on the ratoon cane when compared with the plant cane. Fertilisation using urea, bio-compost and gypsum showed the highest cane yield. The results suggested that composted residue can be applied in sugar-cane cultivation in dry land to increase nutrient uptake and cane and sugar yield in plant and ratoon cane.

Keywords: Cane and sugar yield, fertilisation, plant cane, ratoon cane, residue management

INTRODUCTION

In the sugar-cane cropping system, the burning of residue after harvest is a customary practice worldwide including in Indonesia, which, in 2015, had 461,732 hectares of sugar-cane plantation (Directorate General of Estate Crops, 2016). The most crucial factor that must be considered before removing crop residue is its impact on soil

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organic matter. Nurhidayati (2013) reported that the average sugar-cane residue left on the field is 10-15% of the total biomass of sugar cane. Sugar-cane trash can provide 40 to 120 kg N ha^{-1} $year^{-1}$ (Franco et al., 2010; Oliveira et al., 2002; Robertson & Thorburn, 2007). The C-to-N ratio of sugar-cane trash is 100:1, meaning that sugar-cane trash has a high organic-C content. Thus, on average, 1 kg sugar-cane trash contains 450 g C and 4.5 g N. In one study, the recovery rates of N from residue incorporated into the soil varied from 2% to 15% of the total N contained in sugar-cane residue (Ambrosano et al., 2005; Meier et al., 2016; Vitti et al., 2010).

In addition to the problem of residue burning, sugar-cane farmers in Indonesia often face the problem of soil acidification, which results in low nutrient availability. Hartemink (1998) reported that soil acidification occurred because of the application of ammonium sulfate fertiliser in the long-term. Nurhidayati et al. (2011) reported that soil pH of sugar-cane land in East Java, Indonesia ranged from 4.5-6.5. This condition impacted the decline of soil fertility and the increase of the fertiliser application rate. Azman et al. (2014) stated that soil acidity is a major agronomic problem due to the presence of Al, decrease of P availability and nutrient deficiencies. Application of soil amendment materials such as lime is needed to overcome soil acidity and improve soil fertility. Nurhidayati and Basit (2015) reported that application of bio-char of sugar-cane trash gave better results on soil derived from sugar-cane land than other soil amendment like lime-calcite and boiler ash. Application of bio-char of sugar-cane trash can increase N uptake by sugar cane. Thus, utilisation of sugar-cane residue as soil amendment provides the best management practice, including the application of bio-char from sugar-cane residue to improve soil pH and composted residue to enhance soil organic matter content.

Organic soil amendment contributes directly to nutrient availability as well as nutrient and water-holding capacity of soil. It also plays a key role in both soil health and the formation of water stable aggregates in the soil that affect infiltration, aeration and drainage (Bot & Benites, 2005). It provides carbon and energy for soil microorganisms that are essential for the nutrient cycle in the soil. Some microorganisms form mutually beneficial relationships with plant roots and provide nutrients for plants in exchange for energy through the formation of simple sugar (Cooperband, 2002).

Sugar-cane residue management practices have been widely applied in sugar-producing countries, including Australia. Over the last few years, sugar-cane growers have increasingly adopted a system of green cane trash blanketing (GCTB), where trash is retained as an undisturbed layer on the soil surface and cultivation is greatly reduced (Kingston & Norris, 2001). However, in Indonesia, residue management is not common. Indonesian sugarcane growers always burn sugar-

cane residue after harvest. This practice decreases soil quality, as indicated by low soil C-organic content, ranging from 1.04-1.85% (Nurhidayati et al., 2011). Management of sugar-cane residue has been extensively studied, especially its effect on runoff and soil erosion (Prove et al., 1995), soil organic carbon on the upper 10-20 cm of soil (Franzluebbers, 2010), soil organic carbon in deeper soil layers (Jobbagy & Jackson, 2000), soil respiration and crop productivity of sugar cane (Kennedy & Arceneaux, 2006), soil C and fertility of cane lands (Robertson & Thorburn, 2007) and the contribution of N (Fortes et al., 2013), but little is known about the effects of sugar-cane trash management combined with chemical fertilisation on nutrient uptake and cane and sugar yield.

Nurhidayati and Basit (2014) reported that the application of compost of sugar-cane trash increases the rate of N mineralisation due to increasing earthworm activity. When crop residue was incorporated into soil in the process of soil tillage, it not only improved soil biology related to the availability of plant nutrients; it also involved soil aggregation (Holland, 2004). According to Malhi et al. (2006), residue management simultaneously improves soil and increases crop yield in order to maintain high crop production and minimise adverse impact on the environment. However, it did not significantly affect the availability of N in the topsoil, although it can reduce the aggregate amount that is sensitive to erosion. Agricen (2014) reported that valuable nutrients for the next season's crops come from previous crop residue. The objective of this study was to describe how sugar-cane trash and fertilisation management affect nutrient uptake and cane and sugar yields. This study was conducted over two planting seasons of sugar cane (plant and ratoon cane).

MATERIALS AND METHOD

Study Site and Soil Characteristics

A pot experiment was conducted at the experimental field of the Agriculture Faculty, University of Islam Malang from December 2014 to March 2016. The experimental field is 505 m above sea level and has an average temperature ranging from 20-28°C, while rainfall is 1,750 mm per year. The ratoon cane was grown after harvesting plant cane in August 2015 until March 2016. Soil samples were collected from sugar-cane land in Karangploso district, Malang regency, East Java. The soils were chosen to be representative of the group of soils from sugar-cane land with low pH. The samples (0-10 cm) were taken from areas with more than 10 years of sugar-cane monoculture. The soil consisted of 18.1% clay, 61.4% silt and 20.5% sand, and is classified as silty loam in texture. It was analysed for its chemical properties. The results are presented in Table 1. These samples were used in a pot experiment in which the sugarcane was grown.

Table 1
Chemical properties of the different types of soil used in this study

Soil Type	pH 1:1		C-organic (%)	N total	C/N	OM content %	P-Bray1	SO_4	K	CEC
	H_2O	KCl					(mg kg^{-1})		(mg/100 g)	
Incep-tisols	4.9	4.5	1.0	0.12	8.3	1.73	60.56	6.20	0.16	18.65

Experiment Design and Treatments

The experiment was laid out in a randomised factorial block design. The first factor was N and S fertiliser from several fertilisers consisting of ammonium sulfate, urea and gypsum and had six levels as presented in Table 2. The second factor was the residue management consisting of four levels: the residue was burned (M1); the residue was incorporated into the soil (M2); the residue was placed on the soil surface (M3); and the residue was composted (M4). The combination of the two factors made 24 kinds of treatments and one control (no fertiliser and residues) and they were repeated three times to obtain 75 experiment pots. In addition to residue management treatments, sugar-cane residue bio-char was added as a soil amendment to increase soil pH except for the control. Nurhidayati and Basit (2015) reported that the bio-char of sugar-cane trash is the best soil amendment to improve soil chemical properties of sugar-cane land. The residue application rate was 5 t ha^{-1}. The percentage of the components of the composted sugar-cane trash was: organic-C, 28.1%; total N, 0.81%; C/N ratio, 34.7; lignin, 13.3 %; ash, 10.2%; cellulose, 40.1%; polyphenol, 2.01%; and gross energy, 3,028 kcal/kg.

Table 2
The treatment combinations used in this study

Treatments	N Doses (kg ha^{-1})	S Doses (kg ha^{-1})	AS (kg ha^{-1})	Urea (kg ha^{-1})	Bio-Compost (kg ha^{-1})	Gypsum (kg ha^{-1})
Control (No Fertilisation)	-	-	-	-	-	-
N1+S1 (AS)	100	120	500	-	-	-
N2+S2 (AS)	140	168	700	-	-	-
N1+S1 (U+G)	100	120	-	223	-	522
N2+S2 (U+G)	140	168	-	312	-	730
N1+S1 (U+B+G)	100	120	-	110	1950	730
N2+S2 (U+B+G)	140	168	-	155	2750	938

Note: N=Nitrogen; S=Sulfur; AS=Ammonium Sulfate; G=Gypsum; B=Bio-compost; U=Urea; N and S content in AS=20% and 24%; S content in gypsum=19%; N content in urea=45%; N content in bio-compost=2.57%; gypsum was used as S fertiliser source and Ca content in gypsum was not calculated in the dose of the treatments

Experiment Procedure

The soil sample used in the medium was air-dried and ground. An amount of 40 kg dry soil was put into 75 plastic pots (top diameter=47 cm, bottom diameter=40 cm and height=32 cm). Each plastic pot had 20 holes for perforation. The residue was added to the soil one week before planting at 5 t ha^{-1}, equivalent to 100 g per 40 kg soil on dry weight basis. The composted residue was prepared by grinding sugar-cane trash and composting it using EM4 (Effective Microorganism) for 30 days. The residue was applied in accordance with the treatment in the second factor. Sugar-cane bud chips of BL-red are the most widely planted sugar-cane cultivar in East Java, planted after seedling for one month. One bud chip was placed in each pot, 10 cm into the soil. Basal fertiliser of P and K (15:15) at dose of 400 kg ha^{-1} was applied to each pot. The chemical fertiliser was applied after two weeks of planting. Its dose was adjusted to pre-determined treatments. The plant cane was harvested after seven months and cut, while the ratoon cane was continued for seven months. The bio-compost and gypsum were applied three weeks after cutting. In addition to the treatments, P and K fertilisers were applied four weeks after cutting at 400 kg ha^{-1} for all the treatments. Half of the urea and ammonium sulfate were applied four weeks after cutting the stalk of sugar cane. The remaining urea and ammonium sulfate were applied eight weeks after cutting.

Measurement of Observation Variables

Leaf samples from top of the plants were collected for analysis of leaf N and S content at four months of plant age. The leaf samples were chopped, homogenised and dried at 70°C in a hot-air oven. The dried samples were ground in a stainless steel mill. The wet-acid oxidation of the leaf samples was based on Kjeldahl oxidation in concentrated H_2SO_4 for determination of total N and turbidimetry methods using $BaCl_2$ reagent for determination of S-SO_4 (Okalebo et al., 2002). The N and S uptake was calculated from the nutrient content; it was multiplied by the dry weight of the total biomass. The variables of sugar-cane yield consisted of the fresh weight of cane and total biomass and the dry weight of the total biomass measured by yield per pot (kg) and then converted in t ha^{-1}. The sugar content (%) was determined using a refractometer to measure its brix value and a polarimeter to measure its pol value (Bokhtiar & Sakurai, 2007). The sugar yield (t ha^{-1}) was calculated from the sugar content multiplied by the cane yield (t ha^{-1}).

Statistical Analysis

The analysis of variance (ANOVA) for various crop characteristics was performed following F test. When F was significant at the $p \leq 0.05$ level, treatment means were separated using the Tukey test (Version 14.12).

RESULTS AND DISCUSSION

The Effect of Sugar-Cane Residue Management and N and S Fertilisation on Cane Yield

The N and S fertilisation and the type of residue management had a significant influence on sugar-cane yield (Figure 1). The yield of ratoon cane was higher than that of the plant cane. This was caused by higher N and S nutrient uptake of ratoon cane than in plant cane (Table 3). The highest increase of cane ratoon yield (61%) was in the treatment using a mixture of urea, bio-compost and gypsum. The treatment of N2+S2 using AS fertiliser showed the highest yield (99.7 t ha^{-1}), which was statistically identical to N1+S1 and N2+S2 using a mixture of urea, bio-compost and gypsum by 96.7 and 98.6 t ha^{-1} for ratoon cane (Figure 1). The maximum cane yields might have been due to high N uptake. The highest N uptake was found in N2+S2 using AS fertiliser (Table 3). However, N uptake is not the only factor determining cane yield. A high S uptake can increase cane yield as well as N1+S1 treatment using a mixture of U+B+G (Table 3).

Table 3
N and S uptake by sugar cane influenced by fertilisation and residue management

Treatments	N uptake (kg ha^{-1})		S uptake (kg ha^{-1})	
	PC	RC	PC	RC
Fert. management				
N1+S1 (AS)	426,70 a	630,83 a	12,09 a	52,23 a
N2+S2 (AS)	591,38 c	834,22 c	32,17 d	54,40 ab
N1+S1 (U+G)	439,88 ab	680,68 ab	21,11 c	50,48 a
N2+S2 (U+G)	456,66 ab	703,39 b	22,55 c	48,35 a
N1+S1 (U+B+G)	435,56 ab	690,45 ab	16,69 b	84,50 c
N2+S2 (U+B+G)	479,44 b	714,90 b	21,11 c	64,10 b
HSD 5%	45.34	67.41	1.92	9.73
Residue management				
M1	447,62 a	545,44 a	21,63 bc	42,36 a
M3	460,50 a	727,35 c	19,61 a	62,23 c
M4	535,23 b	934,59 d	22,23 c	79,04 d
HSD 5%	33.22	49.39	1.41	7.13

Means followed by different letters for each factor in the same column are statistically significantly different as shown in the Tukey test at p=0.05
Note: HSD=Honest Significant Difference

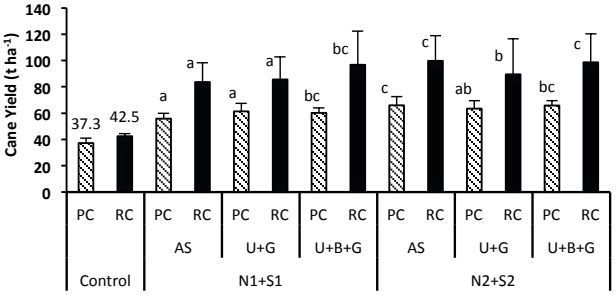

Figure 1. Effect of N and S fertilisation on cane yield of plant cane (PC) and ratoon cane (RC). (The figures accompanied by the same letters for each sugar-cane plant are not significantly different at HSD 5%
Note: AS=Ammonium sulfate, U=Urea, B=Bio-compost, G=Gypsum)

The N and S fertiliser from the mixture U+B+G in low dose gave an equal yield as the AS treatment in the higher dose of N and S fertiliser. This treatment also showed a higher yield than did the mixture U+G in the higher dose of N and S fertiliser (Figure 1). Overall, the residue management in the first planting increased the yield and ratoon cane. The composted residue management increased the yield of ratoon cane as high as 83.7% (116.3 t ha^{-1}) compared to plant cane (63.3 t ha^{-1}) (Figure 2). The results of this study also indicated that burning the residue can increase the yield of plant cane, as occurred in this experiment when compared with the control, but it did not significantly increase the yield of ratoon cane (Figure 2). Hesammi et al. (2014) reported that burning residue can release nutrients rapidly and increase nutrient uptake and crop production in a brief time, but it increases the loss of soil moisture in future plantings. Sugar-cane residue has a very high C/N ratio. Residue made from sugar cane compost can reduce the C/N ratio, and this helps soil microorganisms to degrade the compost of sugarcane residue for release of plant nutrients into the soil.

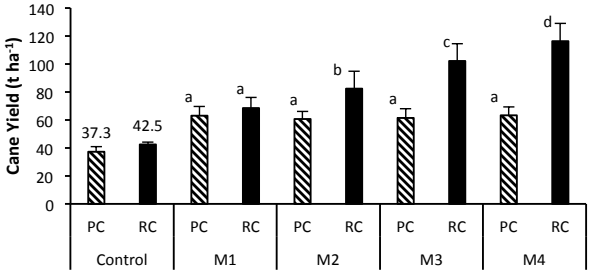

Figure 2. Effect of residue management on cane yield of plant cane (PC) and ratoon cane (RC). The figures accompanied by the same letters for each sugarcane plant are not significantly different at HSD 5%
Notes: M1=Residue burnt; M2=Residue incorporated into the soil; M3=Residue put on the soil surface; M4=Residue composted)

The interaction between fertiliser and residue management influenced significantly on sugarcane yield. The treatment using a high dose (N2+S2) of U+B+G mixture with composted residue management tended to give the highest cane yield on the PC, but the value was not significantly different from other treatments. The treatment using a low dose (N1+S1) of U+B+G mixture showed the highest cane yield on the RC (Table 4). This showed that the treatment was more efficient for sugar-cane cultivation.

Table 4
Cane yield as influenced by interaction between fertilisation and residue management

Treatments		Cane Yield (t ha^{-1})			
		PC		RC	
Fert. Management	Residue Management				
N1+S1 (AS)	M1	52.00	* a	67.83	* ab
	M2	57.08	* abc	80.44	* abcd
	M3	54.33	* ab	82.92	* bcde
	M4	59.83	* abcde	103.33	* efgh
N2+S2 (AS)	M1	59.08	* abcde	78.40	* abcd
	M2	66.83	* def	88.17	* cdef
	M3	68.33	* ef	110.97	* ghi
	M4	69.58	* ef	121.42	* hi
N1+S1 (U+G)	M1	67.75	* def	68.17	* abc
	M2	60.67	* bcdef	72.25	* abc
	M3	56.25	* abc	96.33	* defg
	M4	60.67	* bcdef	105.58	* efgh
N2+S2 (U+G)	M1	69.25	* ef	60.71	* a
	M2	55.58	* abc	71.50	* abc
	M3	67.83	* def	109.52	* ghi
	M4	61.08	* bcdef	116.19	* ghi
N1+S1 (U+B+G)	M1	65.50	* cdef	67.17	* Ab
	M2	60.58	* abcde	83.72	* bcde
	M3	57.25	* abc	106.25	* fgh
	M4	57.42	* abcd	129.83	* i
N2+S2 (U+B+G)	M1	64.50	* bcdef	68.58	* abc
	M2	63.17	* bcdef	98.00	* defg
	M3	64.42	* bcdef	106.67	* fgh
	M4	71.17	* f	121.25	* hi
Control (No Treatment)		37.25		42.46	
HSD 5%		10.53		20.64	
Dunnet 5 %		5.97		13.78	

Means followed by different letters in the same column are statistically significantly different using the Tukey test at p=0.05
Note: HSD=Honest Significant Difference; *: significantly different from control using the Dunnet test at p=0.05

Singh et al. (2005) reported that the residue management of the previous crop either placed on the soil surface or put into the soil played an essential role in the nutrient cycle. It altered the soil environment, which in turn influenced the microbial population and activity in the soil and influenced subsequent nutrient transformation. Thus, crop residue management is beneficial for soil quality as it adds organic substance to the soil, increasing water infiltration and retention capacity of the soil. It also supports the pH and facilitates the availability of nutrients for soil biology and plant absorption activity (Bot & Benites, 2005).

Bot and Benites (2005) added that crop residue management can capture the rainfall volume on the surface, increasing infiltration and soil moisture, while reducing evaporation and preventing soil surface desiccation. This condition can provide a favourable growing medium for plants. The improvement of the soil's physical qualities due to crop residue management influences crop performance (Verhulst et al., 2009).

The Effect of Sugar-Cane Residue Management and N and S Fertilisation on Sugar Content and Yield

The results of this study showed that sugar content and sugar yield in plant cane ($p<0.05$) was not significantly influenced by N and S application (Table 5). The findings of this study were consistent with the results of Bologna-Campbell et al. (2013), who reported that parameters of brix, fibre content and sucrose in sugar-cane juice were

Table 5
Sugar content and sugar yield as influenced by fertilisation and residue management

Treatments	Sugar Content (%)		Sugar Yield (t ha^{-1})	
	PC	RC	PC	RC
Fert. management				
N1+S1 (AS)	10.63	10.00 a	5.96	8.46 a
N2+S2 (AS)	9.98	10.24 a	6.60	10.34 c
N1+S1 (U+G)	10.49	10.82 c	6.41	9.31 ab
N2+S2 (U+G)	10.32	10.80 c	6.54	9.62 bc
N1+S1 (U+B+G)	10.60	11.03 c	6.37	10.39 c
N2+S2 (U+B+G)	10.21	10.45 b	6.73	10.26 c
HSD 5%	NS	0.36	NS	1.08
Residue management				
M1	9.58 a	10.55 ab	6.04 a	7.21 a
M2	10.24 b	10.67 b	6.21 a	8.76 b
M3	10.85 c	10.36 a	6.69 b	10.61 c
M4	10.77 bc	10.64 b	6.81 b	12.34 d
HSD 5%	0.58	0.26	0.54	0.79

Means followed by different letters for each factor in the same column are statistically significantly different using the Tukey test at p=0.05.
Note: HSD=Honest Significant Difference; NS=Not significant

not influenced by N and S in the plant cane for 16 months. However, the results of the calculation of sugar production potency increased along with the higher N doses as the result of increased weight of sugarcane yield. Residue management significantly influenced sugar content and sugar yield on the plant cane, where M3 and M4 showed the highest sugar content and sugar yield (Table 5).

The sugar content of sugarcane is influenced by a complex combination of various factors such as climatic conditions, genetic factors (sugar-cane varieties) and the crop management in the ripening phase, when sugar accumulation in sugar-cane crop happens (Keating et al., 1999). Soil moisture and temperature are the main variables involved in the ripening process, and the combination of both factors can stimulate the intensity of the sugar-cane ripening process (Cardozo & Sentelhas, 2013). In the ripening phase, ratoon cane had a higher soil temperature than plant cane. The growth of ratoon cane lasted during the dry season (Table 6). Therefore, the soil temperature was higher while the soil moisture was lower than in the plant cane. This condition can increase sugar content. Residue management affects the soil's physical conditions, especially soil moisture and temperature. Lingle and Irvine (1994) reported that environmental conditions affected active enzyme (enzyme invertase) in the sugar-cane growth and ripening process.

Table 6
Soil temperature and moisture during ripening process as influenced by the fertilisation and residue management

Treatments	Soil Temperature (°C)		Soil Moisture (%)	
	PC	RC	PC	RC
Fert. management				
N1+S1 (AS)	24.88	25.58	44.33 bc	32.18 a
N2+S2 (AS)	25.31	25.33	44.17 bc	36.21 b
N1+S1 (U+G)	24.96	25.49	45.41 c	38.88 c
N2+S2 (U+G)	25.06	25.72	44.28 bc	38.88 c
N1+S1 (U+B+G)	25.20	25.73	42.09 b	38.80 c
N2+S2 (U+B+G)	24.79	25.71	39.42 a	34.85 ab
HSD 5%	NS	NS	2.53	3.77
Residue management				
M1	25.38 c	26.09 b	39.47 a	34.23 a
M2	25.19 b	25.33 a	42.94 b	36.50 ab
M3	24.73 a	25.49 a	45.45 c	37.26 b
M4	24.83 ab	25.49 a	45.28 c	38.55 b
HSD 5%	0.37	0.62	1.85	2,76

Note: Means followed by different letters for each factor in the same column are statistically significantly different using the Tukey test at p=0.05
HSD=Honest Significant Difference; NS=Not significant

Concerning the ratoon cane, N and S fertilisation were significantly different ($p<0.05$) and influenced the sugar content and sugar yield. The highest sugar content and sugar yield were found in the treatments using low doses of a mixture of U+G and U+B+G and the treatment using a high dose of a mixture of U+G (Table 5). Bologna-Campbell et al. (2013) reported that sugar production was increased after the implementation of N doses that led to an increased production of stalks. The sugar yield of the N1+S1 treatment using a mixture of U+B+G was not significantly different from the treatment of N2+S2 using the AS fertiliser and the mixture of U+G and U+B+G for ratoon cane (Table 5). This meant that the treatment of N1+S1 using a mixture of U+B+G was more efficient than the treatments using other fertilisers due to the low rates of application that produced a high yield of sugar cane. Residue management using compost showed the highest sugar yield for plant and ratoon cane (Table 5). These results showed that residue management influenced the quality of ratoon cane positively. This condition according to Chan et al. (2002) is caused by the retention of crop residue on the soil surface, preventing surface crust formation by enhancing the water stable aggregate; this is not the case when burnt residue is used, even by using zero tillage.

CONCLUSION

The treatment of urea+bio-compost+gypsum produced the highest sugar-cane yield in ratoon cane. The treatment of a fertiliser mixture combination of 110 kg ha^{-1} urea + 1950 kg ha^{-1} bio-compost + 522 kg ha^{-1} gypsum and the composted residue management was the most efficient treatment with the highest sugar yield for ratoon cane. These results suggest that composted residue can be applied in the sugar-cane field to increase nutrient uptake and produce a higher cane and sugar yield in plant and ratoon cane.

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Partial Purification and Characterisation of Cellulase from Sugarcane as affected by postharvest storage of Sugarcane (*Saccharum officinarum* L) stem

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ABSTRACT

This study was aimed at evaluating the effect of storing sugar cane stem at room temperature on the activity of its cellulase enzyme. Cellulase was partially purified and characterised from freshly harvested sugarcane (FHS) and stored sugarcane (SS) (*Saccharum officinarum* L) using 80% ammonium sulphate precipitation and dialysed; the FHS had 136.52 units/mg protein while the SS 184.53 units/mg proteins. The K_m value of cellulase of SS was 0.09 mg/ml while that of FHS was 0.540 mg/ml. The substrate specificity on different cellulose materials (orange peel, banana peel, maize starch, sugarcane bagasse, maize cob and apple pomace) showed varying results for the two enzyme sources. The enzyme from FHS showed 100% activity with banana peel and sugar cane bagasse while the enzyme from SS showed 100% activity with the peels of orange and banana and sugar cane bagasse. Maize cob and apple pomace as carbon source showed very little cellulase activities, 17.4% for FHS and 26.6% for SS. The optimum pH value of partially purified cellulase of FHS was 4.0 while that of SS was 7.0 and the enzyme was optimally active at 40°C for both sources. At the concentrations of 1.0 mM and 10.0 mM, Ca^{2+} and Na^{2+} caused the enzyme activity to increase by 100% residual activity in both FHS and SS. Cellulase of stored sugarcane have increased activity compared with cellulase of freshly harvested sugarcane since it exhibited a very low K_m.

Keywords: Cellulase, purification, pH, sugarcane, specific activity, temperature

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INTRODUCTION

Cellulase is important and essential in the conversion of cellulose into fermentable sugar (Li et al., 2009). This is a complex enzyme consisting of endo-*β*-glucanase (EC 3.2.1.4), exo-*β*-glucanase (EC 3.2.1.91) and *β*-glucosidase (EC 3.2.1.21) the combined action of these three enzymes components is necessary when cellulose is to be hydrolysed (Gielkens et al., 1999; Lin et al., 2009; Balasaravanan et al., 2013).

Cellulase enzymes are important in industrial applications, especially in alcoholic fermentation, malting and brewing, in the textile industry for softening of cotton and denim finishing, in the laundry industry for the production of detergents, fibre modification, in the pulp and paper industry and extraction of fruit and vegetable juices (Hanif et al., 2004; Zhou et al., 2008).

Cellulose is considered the most abundant biomass on the earth; it is made up of long polymers of glucose units with β 1-4, linkage (Shallom & Shoham, 2003; Venkata et al., 2013). Cellulose accounts for half of the dry weight of plant biomass and secondary sources of agricultural wastes (Haruta et al., 2003). There are huge quantities of agricultural and industrial cellulosic waste which has attracted global attention for its use as a renewable resource for conversion to bio-based products and bioenergy (Li et al., 2009). Cellulose degradation by microorganisms converts cellulose through acid or enzymatic hydrolysis into soluble sugars. This shows microbial cellulose utilisation is very important in the largest material flows of the biosphere. However, there are still large quantities of cellulosic materials that are yet to be exploited (Lynd et al., 2002; Sethi et al., 2013).

The sugarcane bagasse is one of the most abundant agricultural wastes and a possible energy source when considering the production of second-generation ethanol since sugarcane bagasse is rich in hydrolysable polysaccharides (UNICA, 2012). Sugarcane bagasse contains 45–55% cellulose, 20–25% hemicellulose, 18–24% lignin, 1–4% ash and less than 1% wax (Thomas, 2009). The cellulosic and lignocellulosic residue is an attractive feedstock for ethanol production as it is available in large amounts and at a low cost. The conversion of waste materials to fuels and chemicals has an economic value while reducing environmental impacts (Pereira et al., 2008).

To the best of the present authors' knowledge, this is the first study to examine the effect of post-harvest storage of sugar cane stem on the cellulase activity of sugar cane. This study was designed to evaluate the cellulase activity of sugar cane affected by postharvest storage of its stem at room temperature.

MATERIALS AND METHODS

Material

Mature sugarcanes *Saccharum officinarum* with no blemish were freshly harvested from a local farm in Ile-Ife, Osun state, Nigeria.

Sample Preparation

The freshly harvested sugar cane stem was transported to the laboratory in an ice container where they were washed in cold saline and divided into two lots, one was stored at room temperature 28±2°C for five days termed stored sample (SS) while the other was used immediately and termed freshly harvested (FHS).

Preparation of crude enzyme

The sugar canes were scrapped gently and then cut into bits. One hundred grams (100 g) of sugar cane was homogenised in a Warring Blender using three volumes of 0.05 M citrate buffer of pH 4.8. The homogenate was filtered using a double layer cheese cloth then centrifuged using Centurion cold centrifuge (R-1880) at 4000 rpm for 30 minutes at room temperature. The aliquot of the supernatant was assayed for its cellulase activity and to determine its protein concentration. The supernatant was then precipitated with 80% ammonium sulphate.

Enzyme Assay

Cellulase activity was measured based on Zhang et al. (2006). One millilitre (1.0 ml) of enzyme extract, 1.5 ml of 1 % viscous carboxymethyl cellulose were mixed in 0.05 M citrate buffer of pH 4.8. The mixture was incubated in a water bath at 50°C for 1 hour. The experimental and control tubes underwent incubation at the same temperature and time. 2 ml of 3,5- dinitrosalicylic acid (DNSA) reagent was added to terminate the reaction. The mixture was boiled for 30 minutes, cooled and optical density was measured at 540 nm. One unit of cellulase activity is the amount of enzyme that released a reducing sugar equivalent to 1 µmol glucose per minute under the specified assay conditions. A standard calibration curve of glucose was made and used for the estimation.

Determination of Protein Concentration

The protein concentration was measured Bradford (1976) and Bovine Serum Albumin (BSA) was used as the standard.

Ammonium Sulphate Precipitation

Ammonium sulphate $(NH_4)_2SO_4$ salt was added to the crude enzyme, stirred gently until the whole salt had completely dissolved in the supernatant; this brought the supernatant to 80% $(NH_4)_2SO_4$ saturation (560 g/L). The mixture was kept in the fridge overnight at 4°C; it was then centrifuged at 4,000 x g for 30 minutes at room temperature. The precipitate was collected and re-suspended in 0.1 M phosphate buffer of pH 7.2. The ammonium sulphate precipitate was then desalted using sephadex G 25 to remove the ammonium sulphate salt.

Determining Kinetic Parameters

The desalted fraction from $(NH_4)_2SO_4$ precipitation was then used for the kinetic studies. The kinetic parameters (K_m and V_{max}) of the enzyme were determined by

varying concentrations of 1% carboxymethyl cellulose and measuring the initial reaction velocities (µmol of glucose/min) at 50°C for 1 hour. The reaction mixture contained varying concentration between 0.1 ml and 1.0 ml of CMC solution and cellulase activity was determined as earlier described. Plots of (1/V) versus 1/[S] were made according to Lineweaver and Burk (1934).

Effect on some Cellulosic Substrates

The substrate specificity of the enzyme was determined using different compounds that include: orange peel, banana peel, maize starch, sugarcane bagasse, maize cob and apple pomace in a typical cellulase assay mixture. The percentage activity of the enzyme was measured using CMC as the control.

Effect of pH on the Enzyme Activity

The effect of pH on the enzyme activity was measured using the method proposed by Agboola and Okonji (2004). Fifty mili molar (50 mM) of citrate buffer with pH 3-5; 50 mM of phosphate buffer with pH 6-8 and 50 mM of borate buffer with pH 9-10 were used. The cellulase activity was assayed as described in the enzyme assay section.

Effect of Temperature

The varying temperatures between 40°C and 100°C were used to investigate the effect of temperature on the enzyme activity and to measure its optimum temperature. The assay mixture was initially incubated at the temperature above for 10 minutes; the reaction was then initiated with the addition of an aliquot of the enzyme which had been equilibrated at the above temperature. The cellulase activity was assayed as previously described (Zhang et al., 2006).

Effects of Cations on the Enzyme Activity

The effects of various cations on the activity of cellulase were carried out. The cations tested were Ca^{2+}, Na^{2+}, Ba^{2+}, Mn^{2+} and K^{+} at 1.0 mM and 10 mM in a typical cellulase assay mixture. The chlorides of the metals were dissolved in distilled water. The control has no cations and it has 100% activity.

RESULTS

Tables 1 and 2 show the results of partial purification of cellulase from both freshly harvested sugarcane (FHS) and stored sugarcane (SS) respectively. The specific activity of enzyme after partial purification using ammonium sulphate precipitation and dialysis were found to be 136.52U/mg and 184.53U/mg for FHS and SS respectively.

Table 1
Purification table for cellulase of freshly harvested sugarcane FHS

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification fold
Crude extract	36.1	4160	115.24	100	1
80% $(NH_4)_2SO_4$ precipitation	10.76	1469	136.52	35	1.19

Table 2
Purification table for cellulase of stored sugarcane SS

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification fold
Crude extract	46.8	6689	142.9	100	1
80% $(NH_4)_2SO_4$ precipitation	17.9	3303	184.53	49.4	1.19

Kinetic Parameters

The Lineweaver-Burk plot to determine the kinetic parameters (K_m and V_{max}) of cellulase from freshly harvested and stored sugarcane for carboxymethyl cellulose (CMC) are presented in Figures 1 and 2 while the values obtained from the figures are presented in Table 3. It showed that FHS has Km of 0.540 and V_{max} of 38.02 while SS has Km of 0.09 and V_{max} of 40.98

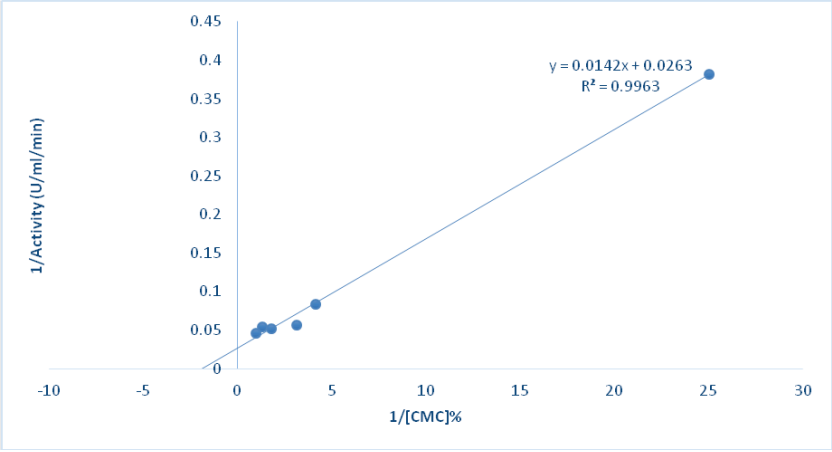

Figure 1. Lineweaver-Burk plot of 1/V against 1/[S] at varying concentration of CMC for FHS

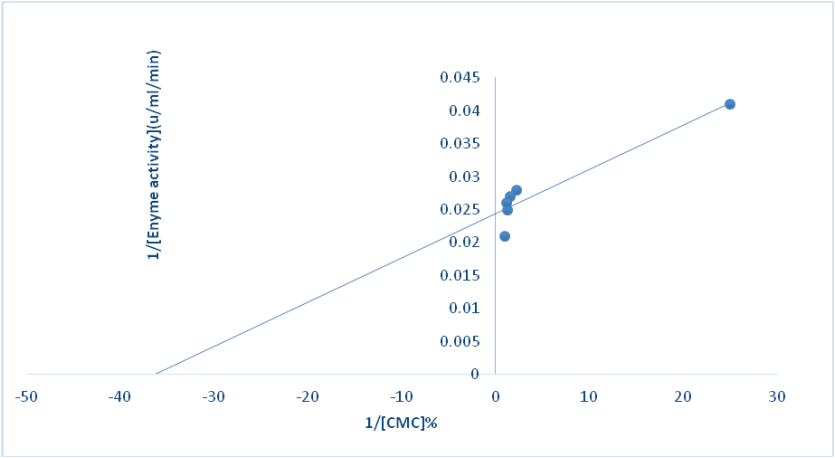

Figure 2. Lineweaver-Burk plot of 1/V against 1/[S] at varying concentration of CMC for SS

Table 3
Summary for the kinetic parameter of cellulase for FHS and SS

Substrates	Km	Vmax
CMC(FHS)	0.540	38.02
CMC (SS)	0.09	40.98

Effect on other Cellulosic Substrates

The results of enzyme specificity are shown in Figure 3 and Figure 4 for FHS and SS respectively. The enzyme from FHS showed 100% activity with banana peel and sugar cane bagasse while the enzyme from SS showed 100% activity with orange peel, banana peel and sugar cane bagasse.

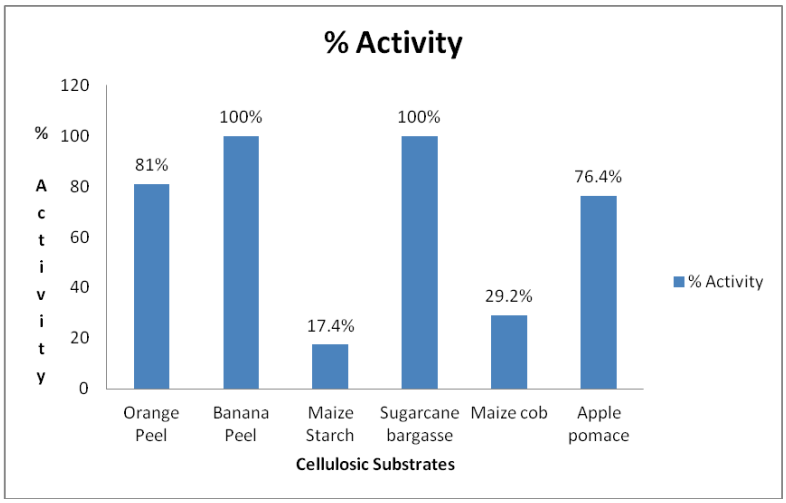

Figure 3. % substrate specificity of cellulase of freshly harvested sugarcane FHS

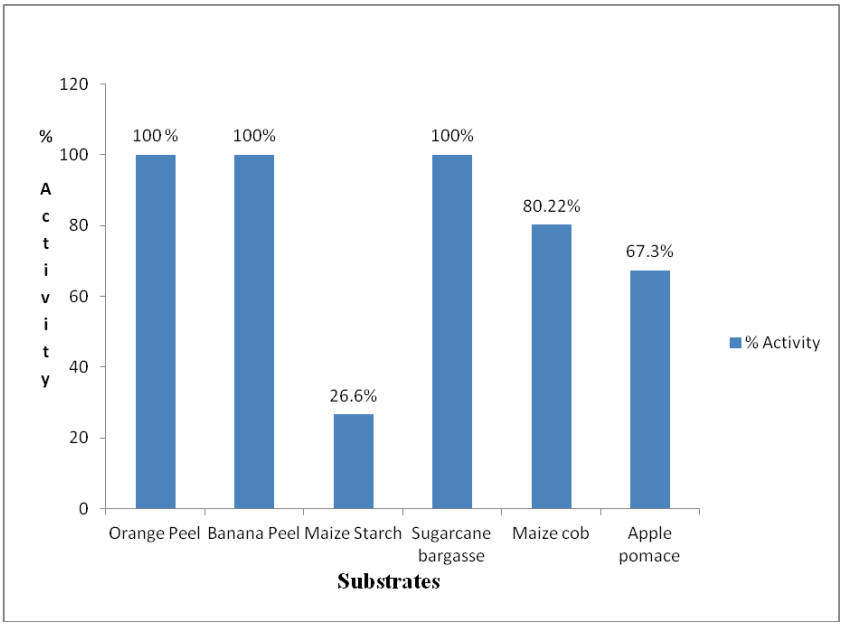

Figure 4. % substrate specificity of cellulase of stored sugarcane SS

Effect of Temperature

Partially purified cellulase of both freshly harvested FHS and stored sugarcane SS exhibited a maximum activity at 40°C as shown in Figures 5 and 6.

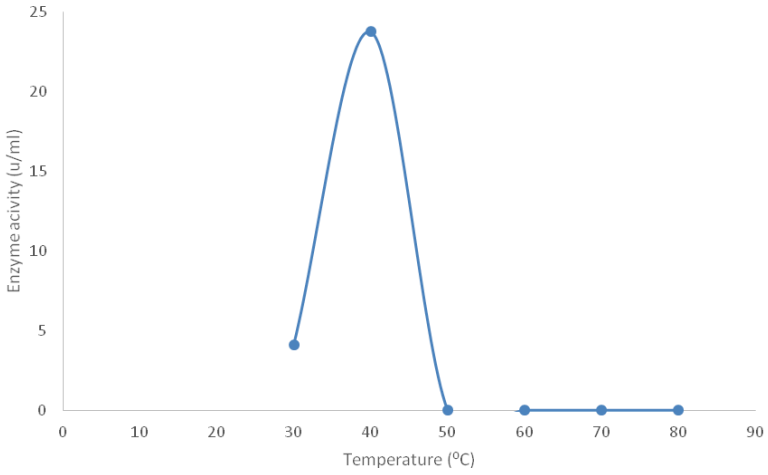

Figure 5. Effect of temperature on freshly harvested sugarcane FHS cellulase

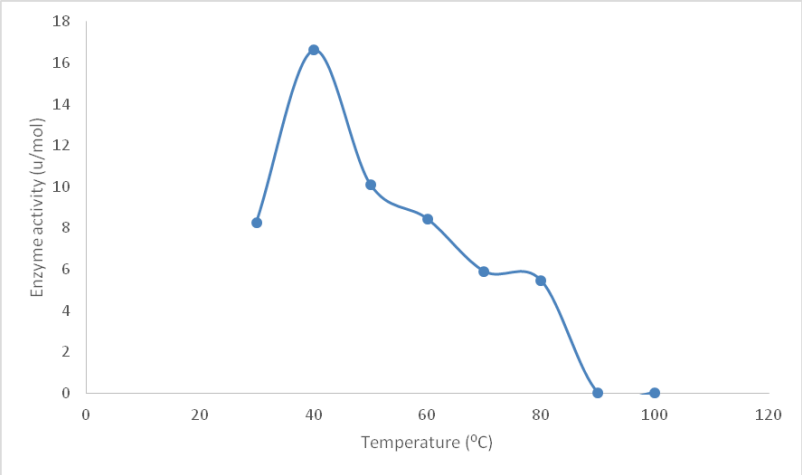

Figure 6. Effect of temperature on stored sugarcane SS cellulase

Effect of pH

The cellulase activity of freshly harvested sugarcane (FHS) exhibited an optimum pH of 4.0 while that stored sugarcane (SS) showed an optimum pH of 7.0 as seen in Figures 7 and 8 respectively.

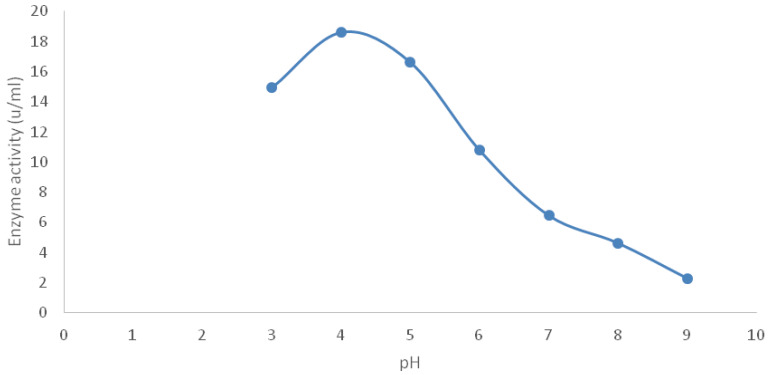

Figure 7. Effect of pH on cellulase of freshly harvested sugarcane FHS
Enzyme assay at different buffers and pH: 50 mM citrate buffer (pH 3-5); 50 mM phosphate buffer (pH 6-8) and 50 mM borate buffer (pH 9-10)

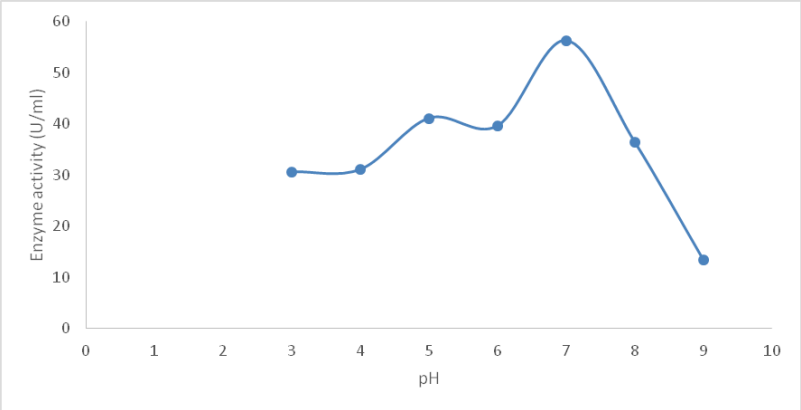

Figure 8. Effect of pH on cellulase of stored sugarcane SS
Enzyme assay at different buffers and pH: 50 mM citrate buffer (pH 3-5); 50 mM phosphate buffer (pH 6-8) and 50 mM borate buffer (pH 9-10)

Effects of Metallic Salts on the Enzyme Activity

The result of the effect of chloride salts shown in Table 4, indicated the activity of cellulase from FHS and SS were not inhibited by chloride salts.

Table 4
Effect of chloride salts on activity of cellulase of freshly harvested sugarcane FHS and stored sugarcane SS

Chloride salts	FHS % Residual activity		SS % Residual activity	
	1mM	10mM	1mM	10mM
$CaCl_2$	100	100	100	100
NaCl	98.79	100	100	100
$BaCl_2$	67.67	78.43	70.54	76.32
$MnCl_2$	62.45	76.04	69.45	74.86
KCl	76.76	85.43	76.32	83.96

DISCUSSION

In postharvest, enzymes can be active and their activity can positively or negatively influence organoleptic characteristics of fruits. It is imperative to understand the different reactions the enzymes catalyse in plant tissues, so as to exploit their advantages and avoid their undesirable effects (Tomás-Barberan & Espin, 2001). Sugarcane suffers postharvest losses in recoverable sugar as a result of deterioration of stale cane (Solomon, 2009).

The specific activity of partially purified cellulase using 80% ammonium sulphate precipitate obtained from FHS and SS samples were 136.52 units/mg protein and 184.53 units/mg protein respectively. The SS had a higher specific activity of cellulase

than the FHS. Some enzyme activities have been reported to increase when fruits are stored at ambient temperature. Barrelt and Gonzalez (1994) reported increase in Polygalacturonase activity in stored cherry, also acid invertase activity has been reported to have 1.5 - 7.0-fold increase in SS (Solomon et al., 1990; Batta & Singh 1991; Saxena et al., 2010). The increase in the activity of cellulase enzyme in SS may be due to physiological activities that continue in fruit after harvesting, which is the breakdown of cellulose to glucose for the sustenance of the sugarcane and this required cellulase enzyme in the conversion of cellulose to glucose. Physiological activities continue in all plant crops following harvesting (Rhodes, 1980). The increase in cellulase activity can also be attributed to stalling and senescence, also exogenous cellulase of bacteria origin as a result of infections of the sugarcane stem by microorganism that breakdown cellulose with the help of cellulase enzyme to supply their carbon source.

The K_m of the partially purified cellulase from freshly harvested sugarcane was 0.540 mg/ml which showed that the cellulase from sugarcane has affinity for carboxymethyl cellulose (CMC) as substrate. The K_m values of cellulase from different sources using CMC as substrate have been reported, Enokibara et al., (1991) 0.28% for cellulase from *Favouls arcularicas*, Busto et al., (1996) 1.32% from T. reesei, Begum & Absar (2009) 0.83% from *Aspergillus oryzae* and Bakare et al., (2005) 3.1mg/ml from *Pseudomonas fluorescens*.

The difference in K_m value of cellulase enzyme may be due to the different sources of its isolation. The K_m value of cellulase in this work for SS was found to be 0.09 mg/ml which showed that the cellulase from SS has a higher affinity for carboxymethylcellulose (CMC); this is expected as the cellulase enzymes in SS are more active metabolically in breaking down cellulose than in FHS. Since the cellulase of the SS exhibited the highest enzyme activity.

The optimum temperature for the partially purified cellulase of FHS and SS was at 40°C. The optimum temperature for the activity of cellulase enzyme varies; Bakare et al., (2005) reported 35°C for cellulase from *Pseudomonas fluorescens*. Fagbohunka et al. (2012) reported 30°C for cellulase from the haemolymph of giant African snail (*archachatina marginata*) while 60°C was the optimum temperature of cellulase of the peel and corm of *Amorphophallus paeoniifolius* (Singh et al., 2014, 2015). The temperature at which enzymes performed optimally depends on the temperature of the environment at which the source of the enzyme thrives best. When the temperature is higher than the optimum temperature, the enzyme gets denatured and the activity decreases (Bakare et al., 2005).

Partially purified cellulase from freshly harvested sugarcane exhibits an optimum pH of 4 while cellulase from SS has an optimum pH of 7. The enzyme functions within the pH range of 3 to 8. The optimum pH of 4 obtained for cellulase of FHS may be due to the physiological state of a fresh sugar cane. The optimum pH of 7 for

cellulase of stored sugarcane may be due to the physiological activities in SS which has resulted in deterioration of the cane. Lionnet (1986) observed that during deterioration of sugarcane, sucrose is lost, lactic acid and ethanol production increases - which could make the pH tends towards neutral.

The substrate specificity of the partially purified cellulase enzyme from FHS and SS was tested using various carbon sources such as orange peel, banana peel, maize starch, sugarcane bagasse, maize cob and apple pomace. In this study, it was found out that when maize starch was used as a carbon source, very little cellulase activities were detected, 17.4% for FHS and 26.6% for SS, whereas higher activities i.e. 100% were detected when orange peel, banana peel and sugarcane bagasse were used as a carbon source in both FHS and SS. Cellulase production is a big factor in the degradation of cellulosic material and it is essential to ensure cellulase production economically viable. The cost of the substrate is very important in the economics of an enzyme production; therefore, different substrates can be utilised for cellulase production for comparison (Ahmed et al., 2009). The differences in the activities of cellulase in these cellulosic materials may be due to the physicochemical constituents of these carbon sources. Physicochemical composition of agro-residues such as cellulose, hemicellulose, lignin, nitrogen, and minerals could influence enzyme activities, so also is the presence of an activator or an inhibitor in the agro-residues and the diffusion of the catabolite (Patagundi et al., 2014)

Chloride salts of calcium, sodium, barium, manganese and potassium improved the residual activity of the enzyme. This improvement in the residual activity of cellulase has been reported by Fagbounka et al. (2012) on the cellulase from the haemolymph of giant African snail (*Archachatina marginata*).

CONCLUSION

In summary, this study showed the presence of cellulase activity in FHS and SS. Cellulase of SS showed an increased activity compared with cellulase of FHS), this shows that storing sugarcane at room temperature 28±2°C causes increase in the activity of the cellulase enzyme. The present study thus shows that cellulase from SS is suitable for commercial applications.

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On-farm Diversity of Indigenous Rice (*Oryza Sativa* L.) Landraces in Border of Eastern Himalaya

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ABSTRACT

Eastern Himalaya is still unexplored in terms of the traditional rice, a reservoir of qualitative traits. The traditional rice is in serious threats from the social diversion and reduction in agricultural practices. The study was conducted to evaluate the status of the genetic resource of indigenous Rice (*Oryza sativa* L.) landraces and its diversity. Forty-one rice varieties were reported from diverse elevation exposures. Both univariate and multivariate statistical analysis had provided plenty of evidence on existence of polymorphism. Pearson's correlation of traits revealed1.8 % of the trait combinations correlated strongly (r = 0.68–1.00), 2.23 % correlated weakly ($r \leq 0.35$), while 5.69% correlated moderately (r = 0.36–0.67). The dendrogram obtained from Euclidian distance and UPGMA (Unweighted Pair Group Method with Arithmetic Mean), revealed three distinct clusters. The cluster analysis using the UPGMA and Euclidean distance revealed the range of genetic distance to be 10 to 757 and obtained three different clusters based on hierarchical clustering. The similarity was observed to be maximum between ACC47 and ACC48 and minimum between ACC46 and ACC49. Out of thirty independent principal components (PCs), top five PCs cumulatively account for 51.74% of the variance. Individual analysis of the factor loadings of the characters in the retained PCs showed that grain related traits have highest positive factor loadings in both PC1 (15.30% of the total variation) and PC2 (16.30% of the variance). While, the first two principal components (PC1 and PC2) cumulatively explained 27.61% of the total variance. The region has a potential for rice genetic resources, which can be a source of impending qualitative traits that can be useful for breeding purposes.

Keywords: Rice, agronomy, plant genetic resource, agrobiodiversity

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INTRODUCTION

The indigenous upland rice landraces of Arunachal Pradesh, withstanding wide range of agro-ecological constraints represents an incredible genetic inconsistency. It is extensively cultivated in slash-and-burn agriculture (locally called as *jhum kheti*) of this remote region (Wangpan et al., 2012). As the future productivity depends on the conservation of indigenous landraces, the importance of these germplasm cannot be ruled out *as a whole*.

The only way to ensure food security for future generations is to exploit the present day genetic diversity of different crop species and to identify the promising one for future breeding programs. Most importantly, a trait possessing high heritability along with high genetic advance would be valuable assets in such selection and breeding programs (Parikh et al., 2012). Even though India is one of the major centres of rice diversity, modern hybrid varieties of crops have caused a rapid erosion of the indigenous rice diversity, (Deb, 2006). Moreover, with the introduction of homogenous inbred lines, the indigenous landraces are disappearing fast and our information about them is still partial (Ray et al., 2013).

Conservation of rice still needs fundamental information about the reliable morphology. While, the qualitative characters are important for plant description and are mainly influenced by the consumer's preference, socioeconomic scenario and natural selection (Hien et al., 2007). Thus, characterization of crop germplasm through different morphological traits is critical for the assessment of its genetic potential. Phenotyping is an important activity to evaluate the first-hand information on the utilization of germplasm fundamental in order to provide necessary information for plant breeding programs (Rabara et al., 2014).

As most of the indigenous rice thrive naturally in uplands, the evidence about them is still inadequate. Therefore, to fill this gap, the untapped upland rice cultivars of this region were characterized and evaluated using morphometric markers. This technique is a low-level yet powerful taxonomic tool and thus is useful for the preliminary depiction of cultivars prior to their characterization via more vigorous marker technologies (Zapico et al., 2010).

Owing to its rich and diversified distribution, the traditional rice landraces of this region has tremendous potential in the context of research and allied activities. The characterization and conservation of these rice accessions are inevitable not only for posterity, but also for the deployment in searching for improved genes with improved characters, including glutinous or aromatic grains, high yielding in stress condition, tolerant to drought and resistance to pest etc. Thus, there is an urgent need to explore the available germplasm and conserve these indigenous landraces before it is lost in time. Besides, the present study will also be useful for germplasm managers in planning for future germplasm acquisitions of the region.

MATERIALS AND METHODS

Study Area

Tirap and Longding districts of Arunachal Himalaya lies between the 26º 38' N and 27º 47' N latitudes and 96º 16' E and 95º 40' E longitudes. Tirap district is bounded by Myanmar towards the South, Changlang District of Arunachal Pradesh towards the East, Dibrugarh District of Assam in the North and Longding district towards the West. The district derived its name from the "Tirap River" and is covered with high hills and deep gorges. The Longding district was carved out from Tirap district is inhabited by Nocte and Tutsa tribes with many sub-tribes; whereas, Longding district is the land of the lone Wancho tribe. Occupying a distinct geographical area, each of these tribes has their own rich social norms, customs, beliefs and practices. These tribes are mostly agrarian, earning their livelihood through traditional farming where rice form the principal food crop.

Field Survey and Data Accumulation

The field visit was carried out during year 2013 to 2015 in 24 rural hamlets for the collection of rice samples and ethnobotanic field data with the help of standard questionnaires (Jain & Mudgal, 1999, Tangjang et al., 2011). The experimental data were based on measurement of the 10 rice plant randomly chosen in the fields for each landraces. The characteristics of the mature rice plant were recorded shortly after the anthesis prior to harvest. The rice landraces were subjected to characterization using both quantitative and qualitative morphogenetic traits of different rice varieties (Zapico et al., 2010). Data were compiled for twelve qualitative and twenty quantitative traits following NBPGR descriptor (Mahajan et al., 2000). The mean values of the data obtained from the survey of three consecutive years were used for the analysis. The size of the rice grains was classified following Cruz and Khush (2000). The yield component, however, was estimated following the equation of Yoshida (1981).

Data Analysis

As described by Hutchenson (1970), using the phenotypic frequencies, the Shannon-Weaver diversity index was calculated. To keep Shannon-Weaver diversity index between 0 and 1 the formula suggested by Hennink and Zeven (1991) was followed. An arbitrary scale was also adapted using PAST software, to classify the computed indices as maximum (H'= 1.00), high (H'= 0.76–0.99), moderate (H' = 0.46–0.75) and low diversity (0.01–0.45) (Jamago and Cortes 2012). A correlation (Pearson) heat map was constructed to visualize correlation between the traits that had weak ($r \leq 0.35$), moderate ($r = 0.36$–0.67) and strong ($r = 0.68$–1.00) correlations (Taylor, 1990). Multivariate statistical analysis was performed using Principal Component Analysis (PCA) and Cluster Analysis (CA). PCA and CA were assessed using XLSTAT (version-2014) and STATISTICA (version-8.0) software respectively.

RESULTS AND DISCUSSION

The present study recorded a total of 41 indigenous upland rice varieties with accession codes ranged from ACC01-ACC54 (Table 1) from the jhum fields. These varieties were subjected to score and a measurement of 12 qualitative and 20 quantitative morpho-agronomic traits.

Table 1
Vernacular name and accessions code of indigenous upland rice varieties of Eastern Himalayas

Sl No.	Local Name	Accessions Code	Sl No.	Local Name	Accessions Code
1	Aratlisa	ACC01	22	Patam	ACC32
2	Aratratnu	ACC02	23	Phanu	ACC33
3	Chahchia	ACC04	24	Phihsa	ACC34
4	Chahchiang	ACC05	25	Sahtho	ACC35
5	Chhaggo	ACC06	26	Sahkhee	ACC37
6	Chahlo	ACC07	27	Sahzaan	ACC38
7	Chahmai	ACC08	28	Saulingnu	ACC39
8	Chahnu	ACC09	29	Semoi-K	ACC40
9	Chahsa	ACC10	30	Semoi-L	ACC41
10	Chahyong	ACC13	31	Senai	ACC42
11	Chahzaa	ACC14	32	Taigo	ACC43
12	Champo	ACC17	33	Toinu	ACC45
13	Honai	ACC18	34	Toisa	ACC46
14	Lailo	ACC22	35	Zaamkhee	ACC47
15	Longri	ACC23	36	Zaamlo	ACC48
16	Lozon	ACC24	37	Zaamnu	ACC49
17	Maichong	ACC25	38	Zaamsa	ACC50
18	Maijah	ACC26	39	Zaamzan	ACC51
19	Maujah	ACC27	40	Zungnu	ACC53
20	Aaosah	ACC30	41	Langmai	ACC54
21	Osusah	ACC31			

Quantitative Trait Evaluation

Significant ranges of variations for quantitative agro-morphological traits were recorded among the indigenous landraces (Table 2). Maximum plant height was observed in ACC41 (191 cm); whereas, minimum plant height was recorded in ACC04 (134.098 cm). Plant height is a complex character, controlled by the internodes (Cheema et al., 1987). Interestingly, the accessions having a short plant height may improve their resistance against lodging; thus, reducing the losses of yield (Ookawa et al., 2010).

Accession ACC23 displayed maximum flag leaf length with an average of 75.7 cm; whereas, ACC06 displayed a minimum of 43.0 cm. ACC49 with 2.82 cm and ACC04 with 1.78 cm displayed maximum and minimum leaf width respectively. Flag leaf plays an important role in grain filling, while top two leaves produce 80% of the stored carbohydrate in the grains (Gladun and Karpov 1993). Additionally, it is the major source of phloem-delivered photo-assimilates during the grain-filling stage and important in cereal breeding for improved lodging resistance (Biswal and Kohli 2013).

Culm length, ranged from 24.2 cm (ACC41) to 41.9 cm (ACC27); while, the culm diameter among the accessions ranged from minimum 0.36 cm (ACC47) to maximum 0.8 cm (ACC38). The score of culm diameter was observed to be higher as reported by Rabra et al., (2014). On the other hand, culm numberranged from 4.00 (ACC04) to 6.00 (ACC25, ACC27, ACC28, ACC32, ACC35, ACC39, ACC41). Most of the accessions with maximum number of culms were observed with the maximum height in comparison to their counterparts. Likewise, ACC41 with maximum number of culms was observed to be the tallest of all. The rice varieties' having large culm have long spikes and contains highest number of grains per panicle (Wu et al., 2011). The rice accession ACC27 with larger culm (culm length and diameter) in comparison to ACC41 was scored higher for yield attributes (spikelet per panicle and filled spikelet per panicles).

ACC08 had maximum number of tillers (13.6) as well as total number of filled tillers (10.6); whereas, ACC06 was recorded with minimum number of total tiller (5.2) as well as filled tiller (4.8). Number of spikelet per panicle ranged from 192.20 (ACC45) to 349.60 (ACC34). ACC26 with an average score of 327.2 and ACC45 with 174.6 were observed with maximum and minimum number of filled spikelet per panicle respectively. The score of tillers per plant was comparatively low, which was one of the criteria used by IRRI rice breeders in selecting donor parents to be used in developing new plant types (NPTs) of rice (Peng et al., 1994). The range of the grain length among the rice genotypes varied from 0.561 cm (ACC10) to 0.899 cm (ACC02). Grain width among the rice genotypes varied from 0.257 cm (ACC39) to 0.368 cm (ACC49). The sterile lemma length among the rice genotypes ranged from 0.132 cm (ACC17) to 0.281 cm (ACC23). All the accessions were recorded with sterile lemma of maximum length, which contributes to the total photosynthates stored in the grains (Chakravorty et al., 2013). Accession ACC51 was scored with maximum 1000 grain weight of 28.69 grams, while ACC04 was scored a minimum of 13.63 grams. Li et al., (2010) proposed the grain weight to be one of the most important characters enhancing polymorphism in rice genotypes. Accession ACC46 was recorded with minimum grain yield of 346.06 kg ha^{-1}, while accession ACC49 was recorded with a maximum grain yield of 1102.55

kg ha^{-1}. Peng et al., (1993) attributed the heightening in grain yield to the favorable effects of improved leaf N concentration, photosynthetic rate of flag leaves and increased filled grain percentage.

Plant height, leaf width, culm number, grain width and kernel thickness were the only character with CV values less than 10 % (Table 2). Thus, a high CV score of total tiller (20.44), productive tiller (20.30), kernel length and breadth ratio (22.71 %) and grain yield (31.34 %) per plant, indicates that the selection based on these characters is expected to be more effective.

Table 2
Descriptive statistics for 20 quantitative agro-morphological traits of 41 indigenous rice of Eastern Himalayas

Variables	Range	Mean	Std. deviation	CV (%)
PH	13.498 to 19.100 cm	164.28	12.38	7.54
LL	4.30 to 7.570 cm	61.42	8.09	13.17
LW	0.178 to 0.2.82 cm	2.28	0.17	7.56
CL	2.420 to 4.190 cm	31.39	3.22	10.26
CN	4.20 to 6.00	5.19	0.47	9.02
CD	0.036 to 0.08 cm	0.49	0.08	17.23
TT	5.20 to 13.60	7.94	1.62	20.44
FT	4.80 to 10.60	6.75	1.37	20.30
TFS	174.60 to 327.20	271.43	30.99	11.42
TUS	14.00 to 29.00	21.17	3.62	17.11
SN/P	192.20 to 349.60	292.60	31.92	10.91
GL	0.561 to 0.899 cm	6.82	0.84	12.35
GW	0.257 to 0.368 cm	3.12	0.29	9.38
SLL	0.132 to 0.281 cm	2.12	0.30	13.91
KL	0.334 to 0.638 cm	4.69	0.66	14.13
KW	0.208 to 0.488 cm	2.71	0.45	16.61
KT	0.142 to 0.216 cm	1.82	0.17	9.42
KL/KW	0.100 to 0.279	1.78	0.40	22.71
1000 GW	13.63 to 28.69 g	19.96	3.70	18.53
Y/H	346.06 to 1102.55 kg	642.51	201.35	31.34

Note: PH= Plant height; LL= Leaf length; LW= Leaf width; CL= Culm length; CN=Culm number; CD= Culm diameter; TT= Total tiller; FT= Filled Tiller; TFS= Total filled spikelet; TUS= Total unfilled spikelet; SN/P= Spikelet number per panicle; GL= Grain length; GW= Grain width; SLL= Sterile lemma length; KL= Kernel length; KW= Kernel width; KT= Kernel thickness; KL/KW= ratio of kernel length and kernel width; 1000 GW= 1000 Grain weight; Y/H= Yield per hectare

Qualitative Trait Evaluation

Rice genotypes were characterized for important leaf traits at late vegetative and flowering stages. Of the total, 70.73% (29 accessions) were recorded with light green, 2.44% (2 accessions) with medium green and 26.83% (11 accessions) with the dark green intensity of green color. The divergence was also observed among the accessions for basal leaf sheath color; thirty-four (82.93%) rice accessions showed green color, six (14.63%) accessions were purple lines, whereas only one (2.44%) with purple color. The leaf pubescence of leaf blade were scored as strong (36.59%), medium (53.66%) and weak (9.76%). Interestingly, the divergence was recorded nil for the flag leaf angle. The intensity of leaf color correlates with the nitrogen concentration (Nachimuthu et al., 2007), which finally affect the yield.

Two types of panicle curvature of main axis were observed among varieties, i.e. straight (12.20%) and semi-straight (87.80%). A total of 80.49% was recorded with well exerted panicle, whereas 19.51% of them were recorded with mostly exerted panicles. The panicle secondary branching was observed as strong (87.80%) or clustered (12.20%). Moreover, the accessions were observed with erect (12.20%) or semi erect (63.41%) attitudes of panicle branching.

Accessions ACC09 and ACC47 were recorded with dark brown decorticated grain color, while remaining accessions have white pericarp. The grains, with red and black pericarp colors have a higher concentration of phenolic compounds (Zhou et al., 2004). The maximum level of variation was recorded against lemma and palea color of rice grains, such as straw (75.61%), brown (17.07%), reddish to light purple (2.44%), purple furrow on straw (2.44%) and black color (2.44%).

Presence of awn was reported only from four rice accessions (ACC8, ACC9 ACC22 and ACC30). Three different awn colors were observed viz., ACC22 and ACC30 with black (25%), ACC8 with brown (50%) and ACC9 with yellowish white (25%). The long awn protects the grains from pilfering by birds and animal (Rabara et al 2014). Besides, short awns allow easier harvesting (Hu et al., 2011). Variations were also found among the studied rice accessions for grain (kernel) size and shape. Grains were characterized as very long (17%), medium (49%) and long (34%). Except accessions ACC01, ACC02, ACC23 and ACC37, which were observed with medium-slender shape, remaining accessions, were recorded to be short-bold in shape

Qualitative and Quantitative Traits Diversity

Diversity index (H'), which accounts for the abundance and evenness of the agro-morphological traits revealed a high degree of diversity among the landraces (Table 3). The existence of high variability, as shown by diversity values recorded indicates that the diversity among the populations is due to variation in traits.

Table 3
Shannon-Weaver indices (H') for 32 agro-morphological traits of 41 indigenous rice of Eastern Himalayas.

Agronomic traits	Code	Evenness_e^H/S	H'	Remark
Leaf pubescence	PB	0.9812	0.99	High
Basal leaf sheath colour	BS	0.869	0.96	High
Leaf intensity of green colour	LG	0.8669	0.96	High
Panicle curvature	PC	0.9823	1.00	Maximum
Awning	AW	1	0.37	Low
Awn colour	AC	0.7844	0.31	Low
Flag leaf angle	FA	1	1.00	Maximum
Panicle exertion	PE	0.9892	1.00	Maximum
Panicle secondary branching	PS	0.9004	0.97	High
Panicle attitude of branching	PA	0.9645	0.99	High
Decorticated grain colour	DG	0.948	0.99	High
Grain lemma and palea	LP	0.7917	0.94	High
Plant height	PH	0.9972	0.999	High
Leaf length	LL	0.9914	0.998	High
Leaf width	LW	0.9972	0.999	High
Culm length	CL	0.9949	0.998	High
Culm number	CN	0.9961	0.999	High
Culm diameter	CD	0.9865	0.996	High
Total tiller	TT	0.9805	0.995	High
Filled tiller	FT	0.9806	0.995	High
Total filled spikelet	TFS	0.9935	0.998	High
Total unfilled spikelet	TUS	0.9857	0.996	High
Spikelet number per panicle	SN/P	0.994	0.998	High
Grain length	GL	0.9928	0.998	High
Grain width	GW	0.9957	0.999	High
Sterile lemma length	SLL	0.9902	0.997	High
Kernel length	KL	0.9904	0.997	High
Total tiller	TT	0.9805	0.995	High
Filled tiller	FT	0.9806	0.995	High
Total filled spikelet	TFS	0.9935	0.998	High
Total unfilled spikelet	TUS	0.9857	0.996	High
Spikelet number per panicle	SN/P	0.994	0.998	High
Grain length	GL	0.9928	0.998	High
Grain width	GW	0.9957	0.999	High
Sterile lemma length	SLL	0.9902	0.997	High
Kernel length	KL	0.9904	0.997	High
Kernel width	KW	0.988	0.997	High
Kernel thickness	KT	0.9957	0.999	High

Table 3 *(continue)*

Agronomic traits	Code	Evenness_e^H/S	H'	Remark
Kernel length and width ratio	KL/KW	0.9761	0.993	High
1000 grain weight	1000 GW	0.9836	0.996	High
Yield per hectare	Y/H	0.9539	0.987	High

Both qualitative and quantitative traits have a diversity index by an average of 0.873 and 0.997 respectively, displaying the quantitative agronomic traits to be comparatively more abundant and dispersed. The traits such as flag leaf angle, panicle related descriptors such as curvature and exertion with maximum diversity were dispersed equitably as compare to other characters. Whereas, the awn related trait had the lowest H' value (0.31 and 0.37). The flag leaf angle trait was observed invariants and have displayed a maximum diversity as well as the evenness (E=1 and H'=1). Ranging between 0.94-0.99, seven (58.33%) qualitative traits scored high diversity with an average index of 0.97. Two of these traits were panicle-related, three were leaf related and the rest were grain related. Most of the quantitative traits, however, had moderate (5 traits) to high (12 traits) diversity indices. Ranged from 0.993 to 0.999, all the quantitative traits showed a high diversity with an average H' score of 0.997. Minimum diversity and evenness, however, was recorded with kernel length and width ratio, while the plant height displayed highest diversity and evenness.

Correlations among the Agro-Morphological Traits

Only 1.8 % of the trait combinations were correlated strongly (r = 0.68–1.00), 2.23% were weakly ($r \leq 0.35$) correlated, while 5.69% were moderately (r = 0.36–0.67) correlated (Figure 1). The analyses showed a moderate correlation between the plant height and culm number. This indicates the importance of culm number in the heightening of the plant. Similarly, Chakravorty et al. (2013) observed correlation of plant height with the culm diameter and culm number. Rabara et al. (2014) also observed a significant amount of correlation of culm number with the panicle number; while, Moukoumbi et al. (2011) reported that the tall rice landraces had long and wide leaves. Filled or productive tiller, on the other hand, was correlated (moderately) with the leaf width, but strongly correlated with the total tiller. Thus, leaf width may have contributed to the productivity of the tiller.

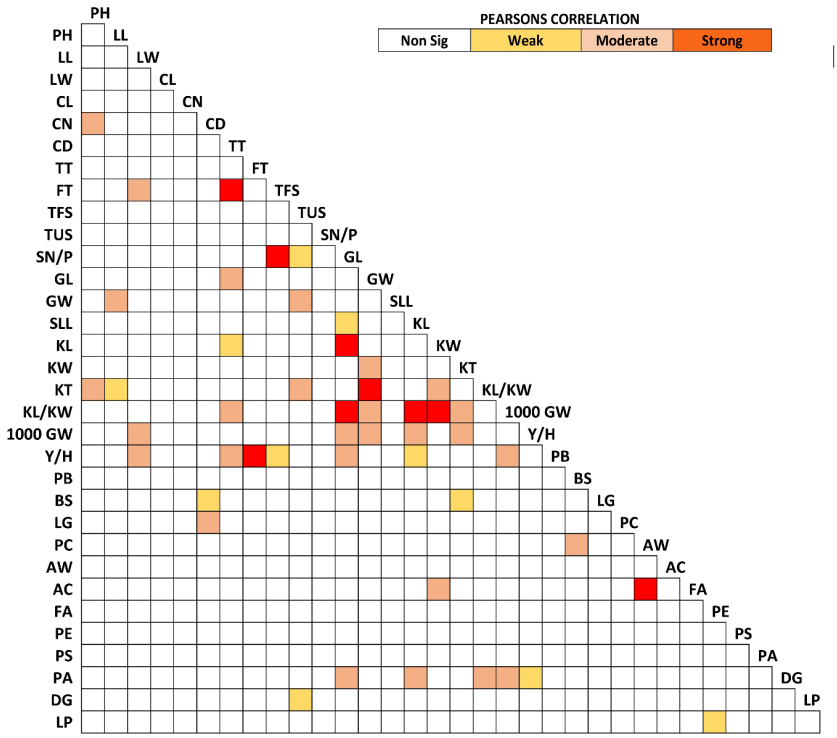

Figure 1. Heat Map of Correlation: The heat map of Pearsons correlation at alpha=0.05, displaying strong (1.8 %), weak (2.23%) and moderate (5.69%) correlation for 32 agro-morphological traits of indigenous rice in Eastern Himalaya

Note: PH= Plant height; LL= Leaf length; LW= Leaf width; CL= Culm length; CN=Culm number; CD= Culm diameter; TT= Total tiller; FT= Filled Tiller; TFS= Total filled spikelet; TUS= Total unfilled spikelet; SN/P= Spikelet number per panicle; GL= Grain length; GW= Grain width; SLL= Sterile lemma length; KL= Kernel length; KW= Kernel width; KT= Kernel thickness; KL/KW= ratio of kernel length and kernel width; 1000 GW= 1000 Grain weight; Y/H= Yield per hectare; PB=Leaf pubescence BS= Basal leaf sheath color; LG= Leaf intensity of green color; PC= Panicle curvature; AW= Awning; AC=Awn color; FA= Flag leaf angle; PE=Panicle exertion; PS= Panicle secondary branching; PA= Panicle attitude of branching; DG= Decorticated grain color; LP= Grain lemma and palea

Spikelet number per panicle was correlated strongly with total filled spikelet, but weakly with the total unfilled spikelet. The ratio of kernel length and width was moderately correlated with total tiller, grain width and kernel thickness; while, strongly correlated with grain length, kernel length and kernel width. Moderate correlation was also observed for grain width with leaf length and total unfilled spikelet. Moreover, the kernel thickness was moderately correlated with plant height and weakly correlated with the leaf length, which shows the role of plant height and leaf length in thickening of kernels.

Weak correlation was observed between sterile lemma length and grain length. Kernel length was weakly correlated with total tiller, but strongly with the grain length. Kernel width, on the other hand,

was strongly correlated with grain weight. One thousand grain weight was moderately correlated with leaf width and all the grains related traits (grain length and width; kernel length and thickness). The correlation analyses of grain yield with other agronomic traits revealed the importance of productive tillers, leaf width, total tiller, grain length and decorticated grain thickness in the heightening of grain yield.

Qualitative characters such as basal leaf-sheath color were observed to be correlated (weak) with culm diameter and kernel thickness. The correlation was also detected between leaf intensity of green color and culm diameter. Panicle curvature was moderately correlated with the basal leaf sheath color. Awn color was also correlated with kernel width and awning property. The panicle attitude of branching was moderately correlated with the grain related traits (grain length, kernel length, kernel length and width ratio and 1000 grain weight); while, weakly correlated with grain yield. Decorticated grain color was weakly correlated with the total unfilled spikelet. Leaf pubescence was moderately correlated with panicle exertion.

The traits with strong positive correlations are the heritable and genetically controlled traits which could be transmitted into desired genotypes (Kisua et al., 2015). The traits that had moderate to high correlations, however, could be further used as a base for the utilization for the breeding purposes as well as for planning future germplasm collection targeting the specific traits.

Multivariate Cluster Analysis and Principal Component Analysis

The variation among the rice genotypes was revealed by cluster analysis using the UPGMA and Euclidean distance. A wide range of genetic distance ranged from 10 to 757, indicates the existence of high genetic diversity. Three different clusters based on hierarchical clustering was obtained (Figure 2), irrespective of their inhabiting geographical locations. In the truncated tree, cluster-I had 15, cluster-II had 20 and cluster-III had 6 accessions. Duplicates were not reported among the accessions in the cluster analysis. The minimum distance score of 10 was recorded between the population of ACC47 and ACC48, indicating the high genetic similarity. While, the maximum distance of 757 was observed between ACC46 and ACC49.

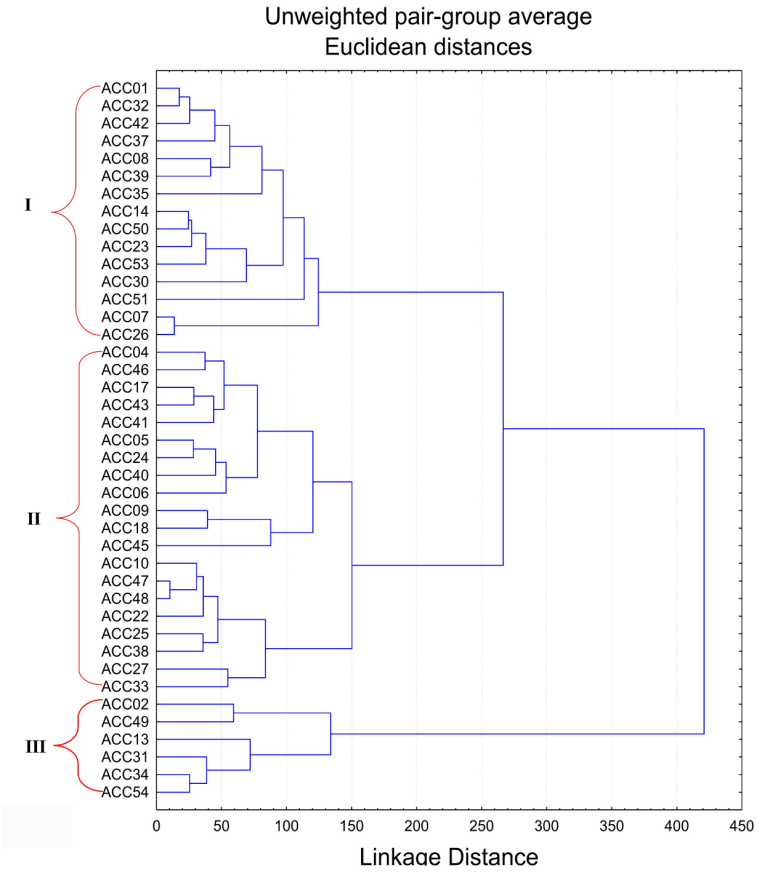

Figure 2. Multivariate Analysis of 32 agronomic traits of indigenous rice of Eastern Himalaya: Dendrogram showing 3 clusters, generated using Euclidean distance and UPGMA clustering for 41 indigenous rice accessions

Based on the morphological data, three distinct clusters were observed (Table 4). The members of Cluster-I have average plant height (ranges from 149.00 to 173.50 cm) and leaf width (ranges from 2.12 to 2.46 cm). The cluster was observed with a maximum total number of tillers (ranges from 7.80 to 13.60) and culm diameter. The grain yield is very high and thousand grain weights were scored maximum in this group (16.84 to 28.69 g). The cluster also has a maximum number of productive tillers (except ACC30). They also share qualitative characters such as, green basal leaf-sheath color, light green colored leaves, semi straight panicle curvature of main axis, well exerted panicles, strong secondary branching, white decorticated grain color and straw colored grain lemma and palea. Accessions ACC08, ACC04 and ACC30 have brown, while ACC42 have purple furrow on straw colored lemma and palea.

Table 4
List of rice accessions arranged in 3 groups according to the cluster analysis

Cluster Groups	Rice Accessions
Group 1	ACC01, ACC32, ACC42, ACC37, ACC08, ACC39, ACC35, ACC14, ACC50, ACC23, ACC53, ACC30, ACC51, ACC07 and ACC26
Group 2	ACC04, ACC46, ACC17, ACC43, ACC41, ACC05, ACC24, ACC40, ACC06, ACC09, ACC18, ACC45, ACC10, ACC47, ACC48 ACC223, ACC25, ACC38, ACC27 and ACC33.
Group 3	ACC02, ACC49, ACC13, ACC31, ACC34 and ACC54.

In case of cluster-II, the members were observed with a maximum culm number. The group displayed least number of total tillers as well as productive tillers, less thousand grain weight and average grain yield. Maximum individuals were observed with weak leaf pubescence, green colored basal leaf sheath, light green leaves, straight panicle curvature, no awns (ACC09 and ACC22), well exerted panicles, strong panicle secondary branching, semi erect branching of panicles, white decorticated grain color and lemma and palea of varied colors. Interestingly, most of the members scored highest in all the phenotypic traits.

Cluster-III accessions were recorded with the maximum score for all the grain related traits. The accessions are tall, and have a maximum total number of tillers and productive tillers. The score for the spikelet number per panicle and grain length was very low. They also shared qualitative characters such as, medium and strong leaf pubescence, green leaf sheath color (except ACC04 with purple), light green leaves (except ACC54 with dark green), semi-straight curvature of panicles, awnless, well exerted panicles, strong panicle secondary branching, semi-straight branching of panicles, white decorticated panicles and straw colored lemma and palea (except ACC31 having red color).

To supplement the cluster analysis, PCA was engaged to reduce the complexity of the dataset. Out of thirty independent principal components (PCs), top five PCs cumulatively account for 51.74% of the variance. According to Clifford and Stephenson (1975), the first three PCs play imminent role in reflection of varying patterns, thus, only first three PCs was used for the analysis. Individual analysis of the factor loadings of the characters in the retained PCs showed that grain related traits have highest positive factor loadings in PC1. These traits were grain length, kernel length and yield per hectare score with factor loadings of 0.8052, 0.7575 and 0.7618 respectively. These three morphological characters could have contributed to the maximum variability in PC1 which explained 15.30% of the total variation in the dataset. Similarly, in PC2 the grain related traits were observed with maximum positive factor loading. Thus, grain width, kernel width and kernel thickness were the major morphological characters that have contributed to the variation in PC2, which

explained 16.30% of the variance. In case of PC3, morphological traits such as total filled spikelet, spikelet number per panicle and awning showed a high loading of 0.7998, 0.8049 and 0.4647 respectively, which accounts for 8.90% of the total variation.

The first two principal components (PC1 and PC2) cumulatively explained 27.61% of the total variance (Figure 3). In this combination, accessions ACC49, ACC35, ACC13, ACC34, ACC31, ACC54, ACC26, and ACC50 were observed with extremely high positive scores reflecting the highest contribution from 1000 grain weight, kernel thickness, grain yield, leaf width, filled tillers, leaf pubescence, awning, leaf intensity of green color, panicle curvature and plant height. On the other hand, accessions ACC24, ACC40, ACC42, ACC10, ACC46, ACC05, ACC09 and ACC04 showed low scores contributed from basal leaf-sheath color, total unfilled spikelet, leaf length, panicle secondary branching and decorticated grain color. Additionally, the results of this study also corroborate with those of Moukoumbi et al., (2011) in several aspects. Some exceptions were, however, also pointed out in terms of variations in cluster formation and grouping behaviors, which may be the result of the variations in environmental and soil edaphic factors.

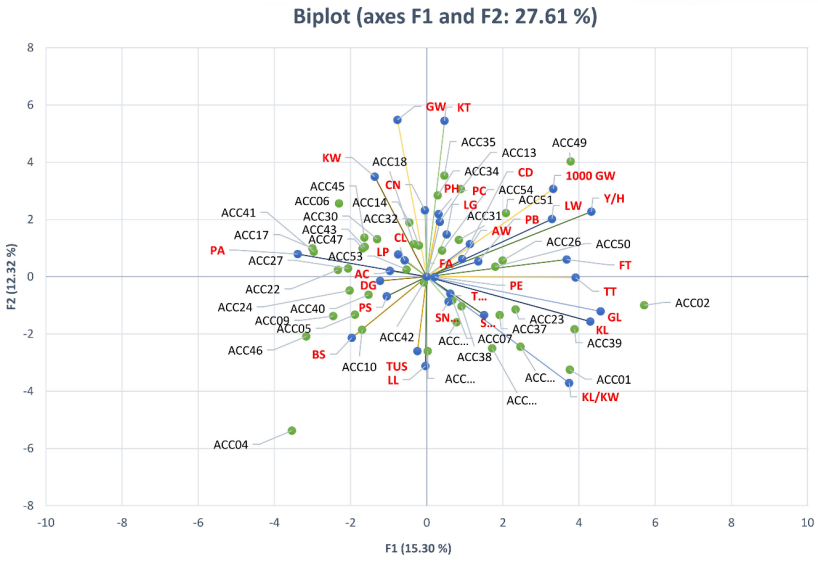

Figure 3. Multivariate Analysis of 32 agronomic traits of indigenous rice of Eastern Himalaya: Score biplot of F1 (PC1) and F2 (PC 2) based on 32 agro-morphological traits explaining 27.61% of total variance. Agro-morphological traits represented by blue colored dots, whereas rice accessions represented by green

CONCLUSIONS

The local inhabitants of this region are still maintaining their indigenous rice with its wide range of phenotypic traits in their jhum fields for many decades; and have optimized their entire practices corresponding to their diverse cultural and local ecological needs. The univariate as well as multivariate analysis of the agro-morphological traits had unveiled the existence of the ample polymorphism among the rice accessions. The results of the present experiment have noticeably specified the significance of the measured agro-morphological traits to identify naturally existing divergent clusters. It will be more suitable to make the selection of the traits based on plant height, leaf area, culm length, culm diameter, culm number, number of spikelet per panicle, productive tillers, grain length/breadth ratio, apparent grain yield and 1000 grain weight. The promising landraces including ACC01, ACC02, ACC04, ACC08, ACC23, ACC26, ACC27, ACC34, ACC38, ACC41, ACC49 and ACC51 could be used as a reservoir of valuable gene pool of indigenous rice. Multivariate analysis, including clustering pattern and PCA could also suggest the breeders about the appropriateness of different landraces of rice future endeavors. The above results also suggested that, the agro-morphological traits could be used efficiently to characterize the rice cultivars prior to documentation and subsequently on farms conservation of indigenous rice farms as well as in the seed banks.

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Salinity Stress and its impact on Morpho-Physiological Characteristics of *Aloe Vera*

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ABSTRACT

Aloe Vera is a valuable medicinal plant. Its leaf and gel in particular are widely used as skin care and in medical applications. Salinity however, is an abiotic stress, and can negatively affect the plant's morphological characteristics as well as quality and quantity of its phyto-chemical compounds of, including total phenol, total soluble sugars and its components, namely sucrose, glucose, and fructose. In order to investigate the impact of salinity stress on morphological and physiological traits of plant, different levels of NaCl, namely 0 (control), 50, 100, 150, 200 and 250 mM were applied in a complete randomised design with three replications under greenhouse conditions. The results indicated that salinity stress has significant negative effect on the plant's morphological traits, such as its weight, leaf length, leaf weight, gel weight, root length; and biochemical traits such as total phenol, total soluble sucrose, glucose and fructose. The results of this study indicate that salinity stress has significant negative effect on *Aloe Vera*'s morphological traits which results in yield loss. Moreover, biochemical traits such as photosynthetic and defences of plants are also affected. It is thus, clear that *Aloe Vera* is susceptible to salinity stress.

Keywords: Medicinal Plant, Morphological traits, Phenol compounds, Phyto-Chemical, Soluble sugars

INTRODUCTION

Aloe Vera L., a valuable medicinal plant, is a perennial liliaceous which grows in tropical and sub-tropical regions. It has thick lace-shaped green leaves with jagged edges and sharp points, joined at the stem in a whirled pattern. Although more than 250 species of *Aloe* genus have been

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identified worldwide, only two species, *Aloe barebadensis* and *Aloe aborescens*, are commercially important. *Aloe Vera* contains different nutritional contents such as vitamins, minerals, enzymes, sugars, phenol compounds, lignin, saponine, sterol as well as amino acids. It is widely used in healthcare and cosmetic products. Carbohydrate contents, which account for about 25% of its dry weight, are known to improve the immunity response of the human body (Green, 1996; Kahlon, 1991; Sheets, 1991). In recent years, studies have been conducted to identify the characteristics of the colourless gel available inside the leaves as well as substance produced by the outer layer of the leaves (Liu et al., 2011; Ni et al., 2004). The plant gel, which contains glucomannan as a type of emulsion polysaccharide, is a common moisturising ingredient in cosmetics (Chen et al., 2012; Zapata et al., 2013).

There are some phenol compounds such as anthraquinone found in the juice of *Aleo Vera*. This substance is commonly used as a laxative and it is known to have a strong antibacterial as well as sedative quality (Thu et al., 2013). The gel contains 99% water and a pH value about 4.5, and it is a common over-the-counter medicine for skin diseases. The extracts can be used to relieve cancer pain, digestive disorders and even AIDS. Due to its huge utilisation in pharmaceutical, cosmetic and food industries, the demand for quality planting material of *A. Vera* is increasing. (Bedini et al., 2009; Botes et al., 2008; Eshun & He, 2004; Grace et al., 2008; Lad & Murthy, 2013; Rodríguez et al., 2010). The clinical trials of *Aloe Vera* have been conducted for skin conditions, management of burn and wound healing, constipation, tumours, and gastrointestinal disorders (Rajasekaran et al., 2006). Mass propagation of uniform and healthy plants through tissue culture is the only available technique for large scale production of clonal plants in a short time. Several attempts have been made over the last few decades to develop tissue culture systems of *Aloe* spp., but still efficient regeneration protocols are requisite for large scale production of true-to-type plants of this commercially important species (Amoo et al., 2012; Das et al., 2010; de Oliveira & Crocomo 2009; Gantait et al., 2011; Haque & Ghosh, 2013; Rathore et al., 2011; Singh et al., 2009).

Water deficiency, salinity and temperature differences can cause a lot of damage to plants. Although, this plant is tolerant to unfavourable conditions such as poor soil, the adverse effects of salinity and drought on this plant is considerable (Tubabicer et al., 2004).

Salt stress retards plant growth and yield, and has become a a serious problem in the world (Horvath et al., 2007; Kirdmanee, 2009; Moghbeli et al., 2012). This stress is one of the most important abiotic stresses in arid and semiarid regions (Olfati et al., 2012; Sahu et al., 2011; Talebi et al., 2015; Zhang et al., 2010; Zheng et al., 2004). Better understanding of the mechanisms that enable plants to adapt to salt stress and maintain growth, would help in the selection of stress tolerant cultivars (Jin et al., 2007).

According to a survey, more than 800 million hectares of land throughout the world are salt affected (Anonymous, 2008). The impact of salt stress has been correlated with some morphological and physiological traits such as reduction in fresh and dry weight (Chartzoulakis & Klapaki, 2000). In fact, salinity affects plant metabolism by disturbing their physiological and biochemical processes of plants due to ionic and osmotic imbalances which slows down plant growth and productivity (Munns, 2005). The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution, nutritional imbalance, specific ion effect, or a combination of these factors (Ashraf & Harris, 2004). Studies on plant tolerance to salt stress cover many aspects of the influences of salinity on plant behaviour, including alterations at the morphological, physiological and molecular levels. Recently, investigations have focused on biotechnology, transgenic plants, improvement of breeding, screening methodologies and modification of the genetic structure of existing crops aiming at enhanced adaptation to salinity conditions (Mahdava et al., 2006). Zan et al. (2007), studying physiological and ecological characteristics of plants with seawater irrigation, reported that salinity stress results in decrease in tissue water, total soluble sugars and glucose. Mustafa (1995) suggested that in *A. Vera*, 0.1% salinity has resulted in an increase in growth parameters while 0.4% salinity reduced growth parameters. Additionally, he reported the highest amount of carbohydrate compounds was obtained with 0.4% salinity. The aim of this study was to examine the effect of salinity and traits related to growth, and phytochemical compounds in gel and leaf of *A. Vera* L. plant.

MATERIALS AND METHODS

The experiment was conducted in the greenhouse facilities of Imam-Khomeini Higher Education Center in Karaj, Iran in spring of 2015.

In-vitro plantlets of *Aloe Vera* were transplanted into 30 cm diameter pots containing cocopeat and perlite under greenhouse conditions. The experimental plants were irrigated with a nutrient solution containing different levels of NaCl including 0 (as control), 50, 100, 150, 200 and 250 mM, with consequent EC including 7.7, 13.9, 18.7, 22.8, 25.7 and 1.3 µs, in a Randomized Complete Design (RCD) with three replications. After the trial period of six months, the treated plants in each replication were assessed for morphological and physiological characteristics as follow:

Morphological measurements

The measurements include bush height, number of leaves, leaf length, leaf weight, leaf gel weight, root length and weight.

Determination of total soluble sugars

The amount of total soluble sugars was estimated using Anthrone reagents as discussed by Thimmaiah (2004). One hundred mg sample was placed in a boiling

tube and hydrolysed with 5 ml 2.5 N HCl in a water bath for three hours before it was neutralised with solid sodium carbonate. The volume was made to 100 ml followed by centrifuge at 5000 rpm for 10 min. The supernatant was collected and one ml sample was taken for analysis. Four ml Anthrone reagent was added to aliquot and heated for one minute in the water bath (70°C). The sample was then rapidly cooled and the change of green to dark green colour was read at 630 nm compared to the blank.

Determination of total phenol

Total phenols content was measured using the Folin- Ciocalteau reagent (McDonald et al. 2001). The extract sample (0.5 ml) was mixed with 0.5 ml Folin-Ciocltеau reagent, and then 4 ml 1 M aqueous Na_2CO_3 was added to the mixture. The mixture was allowed to stand for 15 minutes, and phenols were measured using colorimetric method at 765 nm using UV visible spectrophotometer. Total values were expressed in terms of Gallic acid equivalent and total phenol contents were calculated as Gallic acid from a calibration curve (Shui & Leong, 2002).

Statistical Analysis

Data for each parameter was subjected to one-way analysis of variance (ANOVA) and significant differences between treatment means and simple correlation coefficient of traits were determined by Duncan's multiple range test (DMRT) in a RCD, using the SPSS software package (version 16). Microsoft Office Excel (version, 2007) was applied to draw the diagrams.

RESULTS AND DISCUSSION

Morphological traits

The results indicate that some morphological characteristics including bush weight, leaf weight, leaf gel weight, root length and root weight ($p<0.05$) as well as leaf length ($p<0.01$) were significantly affected by salinity stress, while bush height and number of leaves were not affected (Table 1).

Table 1
Analysis of variance for the effect of salinity stress on morphological traits in Aloe Vera

Changes sources	Df	EC	M.EC	Busch weight (g)	Busch hight (cm)	Leaf number	Leaf length (cm)	Leaf weight (g)	Leaf gel weight (g)	Root length(cm)	Root weight (g)
Treatment	5	433.12**	228.79**	4495.91**	3.99ns	1.17ns	25.76*	214.79**	65.85**	162.14**	39.49**
Error	24	1.81	1.61	765.45	18.03	1.83	9.35	11.61	0.65	18.20	3.24
CV		7.1	7.4	19.3	14.1	16.7	12.8	19.7	14.3	17.8	15.1

Table 2
Mean comparison of the effect of salinity stress on morphological traits in Aloe Vera

Treatment NaClmMolart	Busch weight(g)	Busch hight(cm)	Leaf number	Leaf length (cm)	Leaf weight (g)	Leaf gel weight (g)	Root length (cm)	Root weight (g)
50	152.6ab	30.2a	8.0a	25.9a	21.7b	5.8b	23.2ab	16.8a
100	144.1b	31.4a	8.4a	22.4ab	12.8c	4.7c	21.0ab	12.0b
150	155.9ab	30.1a	8.6a	23.6ab	14.9c	5.8b	17.9bc	12.2b
200	118.0bc	28.9a	8.0a	20.5c	11.2c	2.4d	10.1d	10.8bc
250	102.3c	29.6a	7.2a	24.4a	14.6c	2.4d	13.0cd	8.1c
Control	186.9a	30.8a	8.2a	26.6a	28.5a	12.3a	24.4a	12.2b

Table 2 shows that the bush weight appeared highest in control plants and lowest when irrigated with 250 mM salt. Except root weight, other traits appeared to be also the highest in control. The highest root weight was obtained in 50 mg/lit salt treatment. In fact, salinity negatively affected most traits except number of leaves and bush height, but they did not correspond to the same pattern. As had been expected, the higher the salinity stress, the less the value of these traits, so that the lowest value referred to conditions between 200 and 250 mM.

Effect of salinity stress on biochemical traits

The results showed that salinity stress affect biochemical traits of *Aloe Vera* gel and leaf, such as total phenol compounds, total soluble sugars and their components including sucrose, glucose, and fructose, significantly ($p<0.01$) (Table 3). The phenol compounds were lower in leaves produced under stress conditions, so that the lowest content referred to leaves of plants exposed to 250 mM of NaCl and untreated plants, control plants, induced the highest content. However, the phenol content in gel was lower as the lower concentration of salt was applied and in high level of stress, minor changes were observed during treatments. The effect of salinity in soluble sugars of leaf and gel are shown in Table 4. Soluble sugars showed different pattern in plants, so that 150 and 250 mM of salt caused highest and lowest values respectively, and the value of control treatment appeared to be less than the value caused with 150 mM NaCl. Glucose content also followed approximately the same pattern as soluble sugars. In addition, sucrose content in gel was the highest in 150 mM salt treatment, but in leaf, the stress resulted in decreasing the content compared with control treatment. On the other hand, salinity stress led to an increase in fructose content in gel which was highest in plants treated with 100 mM salt, while in leaf, it decreased (see Figure 8). Although there is no similar pattern for the effect of salinity stress on carbohydrate compounds, as Table 4 indicate, total soluble sugars and their components appeared to have an increase with a certain level of the stress, particularly in gel, in which 150 mM NaCl was mostly

found to be the level so that the higher and lower levels of salt resulted in negative effect on the carbohydrate compounds production in the plant. In terms of sucrose and fructose contents of leaves, stress at any level caused lower values compared with *r* control. In terms of total phenol compounds in the leaf, a decrease in the content caused by the stress was observed. However, this effect did not follow any certain pattern. In fact, some times, the content in low level of stress turned out to be lower compared with severe stress conditions. Accordingly, the highest decreasing effect on yield of total phenol in gel appeared by the lowest level of stress. In addition, a descending trend of phenol content in gel occurred as the level of stress reduced. For both leaf and gel, control plants contained the highest amount of phenol. The increase in total soluble sugars and their components under certain levels of stress, particularly in gel, indicates that stress on its own does not necessarily determine the content of such compounds, but the level of stress can be regarded as the main determinant of that. Moreover, content of soluble sugar components can vary, especially at150 mM NaCl stress level.

Table 3
Analysis of variance for the effect of salinity stress on biochemical traits in Aloe Vera

Changes sources	Df	Phenols leaf gel (mg/ gDW)	Phenols leaf (mg/gDW	Total sugar leaf gel (mg/ gDW)	Total sugar Leaf (mg/gDW)	Sucrose leaf gel (mg/ gDW)	Sucrose leaf (mg/ gDW)	Glucose leaf gel (mg/gDW)	Glucose leaf(mg/ gDW)	Frouctose leaf gel(mg/ gDW)	Frouctose leaf (mg/gDW)
Treatment	5	2386.9*	121305.1**	1.022**	1.036**	1272.1**	3673.3**	1012255.5**	28199.4**	205104.4**	142908.5**
Error	24	625.4	3421.8	0.027	0.013	3.7	8.6	1638.8	227.7	2986.6	1119.7
CV		10.3	7.2	17.1	7.6	6.5	3.4	6.6	7.1	15.1	7.1

Table 4
Mean comparison of the effect of salinity stress on biochemical traits in Aloe Vera

Treatment NaCl (mMolar)	Phenols leaf gel (mg/ gDW)	Phenols leaf (mg/ gDW)	Total sugar leaf gel (mg/ gDW)	Total sugar leaf (mg/ gDW)	Sucrose leaf gel (mg/ gDW)	Sucrose leaf (mg/ gDW)	Glucose leaf gel (mg/ gDW)	Glucose leaf (mg/ gDW)	Frouctose leaf gel (mg/ gDW)	Frouctose leaf (mg/ gDW)
50	219.2c	825.8c	0.76c	1.53c	22.4c	68.3d	423.6c	179.3c	541.2b	505.6bc
100	236.0bc	918.8b	0.84c	1.39c	23.2c	95.3b	720.7b	184.1c	652.0a	532.0b
150	233.8bc	791.2c	1.74a	2.19a	52.2a	94.5b	1475.6a	340.6a	477.6b	467.5c
200	243.8b	783.6c	1.09b	1.21d	32.5b	74.2c	475.8c	164.4cd	216.5c	372.2d
250	253.0ab	540.5d	0.38d	0.88e	23.1c	66.3d	269.3e	152.1d	215.3c	235.8e
Control	265.9a	998.5a	0.93bc	1.78b	24.2c	138.4a	323.6d	275.7b	177.4d	741.4a

As discussed earlier, salinity stress negatively affects morphological traits, for which there is no similar pattern. In fact, higher level of stress does not necessarily result in greater decreasing effect and less severe stress does not necessarily have less impact on the traits. However, the more severe the stress, the less rate of growth is observed. As to total phenol compounds, the stress has resulted in decreased content, but no certain association was observed. It could be concluded that only soluble sugars increase with certain levels of stress.

There are several research studies indicating the impact of salinity stress, as an environmental stressing factor, on plant growth (Ashraf & Harris, 2004; Mahdava et al., 2006; Zan et al., 2007). The results of some studies show that morphological and physiological traits such as fresh and dry plant weights are adversely affected by salinity stress (Abdollahi et al, 2011). In fact, deleterious effects of salinity on plant metabolism are due to disorder in physiological and biochemical process caused by ionic and osmotic imbalances, resulting in the reduction of growth and yield (Cha-um & Kirdmanee, 2009). Salinity is reported to affect number of leaves, plant height, root weight, total gel weight, dry root weight of *Aloe Vera* (Moghbeli et al., 2012). The results of similar studies showed that low water potential of soil reduces fresh leaf weight, plant growth rate and leaf yield (Rodriguez-Garcia et al., 2007). Based on the reports, although *Aleo Vera* is relatively tolerant to dry condition, salinity can have deleterious morphological and physiological impacts; the impact of increased salinity is more prominent on the leaf length (Fuentes, 1988). Increased level of salinity stress can alter the content of chlorophyll, soluble carbohydrate, proline and total soluble solid (TSS). Shams et al. (2015) reported that salt could reduce growth and gel yield as well as chlorophyll content of *Aloe Vera*. In addition, higher concentration of sugars, particularly sucrose accumulation, as a consequence of salinity stress, has been broadly reported (Murthy et al., 2013; Zan et al., 2007) but a reduction in glucose content has been reported as well. The reduction of soluble sugars and starch is more common in leaves of trees exposed to long-term salinity stress, thus, no sugar accumulation is observed in such plants (De Oliveira et al., 2009).

CONCLUSION

This study revealed that salinity stress negatively affected a number of growth-related characteristics of *Aloe Vera* plant such as bush weight, leaf length, leaf weight, gel weight as well as root length while traits including bush height and number of leaves were not significantly affected. Although there are different impacts at different levels of salinity, the leaf length showed a significant reduction, but in some levels of salinity, no significant difference was observed compared with control. Furthermore, root length appeared to decrease significantly in salinity stress, but the value was higher than the control in 50 mM NaCl. It could be concluded that

the level of stress can be considered as the determining factor in growth traits.

In addition, the results of study show a significant impact of salinity stress on phyto-biochemical traits. However, the patterns vary according to the type of biochemical compounds as well as extraction sources. As was mentioned above, reduction of total phenol compounds is associated with the level of stress, but the reduction rate depends on the salt concentration as well as the extraction source (leaf or gel), and it cannot be concluded that stress has increasing or decreasing effect on the content. Based on the level of salinity stress, carbohydrate compounds including total soluble sugars, sucrose, glucose and fructose contents were also affected. The highest amount of soluble sugars in leaf and gel, sucrose in gel, glucose in leaf and gel with 150 mM NaCl and fructose in gel with 100 mM NaCl was recorded, although the control produced the highest amount of sucrose and fructose contents in leaf. Furthermore, there were different patterns for the changes of the traits by salinity stress, so that for some traits, high or low level of stress resulted in reduction as the same effect, while for some other traits there was somewhat various changes. In addition, salinity stress appeared to increase soluble sugar content in the plant, particularly in gel.

In terms of measuring biochemical characteristics which play a considerable role in food and pharmaceutical qualities of the plant, salinity stress is not known the main factor in decreased plant yield, but what is more important is the level of stress. It is noteworthy that the ratio of carbohydrate constituents appears to be different with various levels of stress and this is true of other biochemical traits affected by salinity stress. In other words, salinity stress in different levels negatively affect plant growth characteristics, while its effects on phyto-chemical traits as well as trend of changes are different according to the composition, part of plant used for extraction and stress level. Complying with other research reports on the influence of salinity stress on morphological and biochemical traits of *Aloe Vera*, the result of present study shows level of stress is an important rather than stress on its own, to assess its effects on plant growth.

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Field evaluation of tomato varieties/breeding lines against tomato yellow leaf curl virus disease (TYLCV)

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ABSTRACT

Tomato Yellow Leaf Curl Virus (TYLCV) is currently the most devastating virus of cultivated tomatoes in tropical and subtropical regions, accounting for significant yield losses in cultivated tomato in Ghana. Severe population outbreaks of the whitefly vector (*Bemisia tabaci*), are usually associated with high incidence of the disease. Resistance breeding is the surest solution to TYLCV in developing viable seeds for increased tomato production in Ghana. The Wild tomato (*Solanum pimpinellifolium* L.) is a recognised crop Wild species (CWS) with resistance genes to different diseases including the TYLCV disease and possesses good fruit quality traits in Ghana. Three (3) cultivated tomato varieties and seven breeding lines developed from crosses between the Wild tomato and three hybrids, three backcrossed lines and the Wild tomato were evaluated with their parents against TYLCV disease under local field conditions. Field appraisal of whitefly populations, disease incidence and severity, agronomic and yield characteristics of the tomato varieties/breeding lines were undertaken to hasten selection of tolerant/resistant varieties or breeding lines in the breeding programme. Wild tomato (ISS_{AP} = 0.31 and ISS_{DP} = 0.76) and Woso (ISS_{AP}

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= 1.90 and ISS_{DP} = 2.27) recorded the least and highest average symptom severity on all plants (ISS_{AP}) and diseased plants only (ISS_{DP}); while the least and highest disease incidence was recorded by the Wild tomato (11.10%) and Roma (43.05%). Roma which recorded the highest population of whiteflies in the dry season also exhibited the highest symptom severity on all plants as well as diseased plants during the study period. There was a significant symptom relapse in Wild tomato and Woso x Wild in 6-8 WAT for both ISS_{AP} and $ISS_{DP.}$ Number of fruits per plant, ISS_{AP} and ISS_{DP} were positively and/or inversely correlated (r = 0.98, 0.93, -0.83) with average whitefly count, percent disease incidence and yield (t/ha).

Keywords: Backcross, Geminiviruses, varieties/ breeding lines, Ghana, Tomato, TYLCVD, resistance breeding

INTRODUCTION

Tomato yellow leaf curl virus (TYLCV) disease, caused by geminiviruses and transmitted by whitefly (*Bemisia tabaci* Genn.), has become a major problem in tomato cultivation globally, particularly in the tropics and subtropics (Czosnek & Laterrot, 1990, pp.1-6; Moriones & Navas-Castillos, 2000, pp.123-124; Moriones, Amo, Accotto, Noms, & Cavallarin, 1993, p. 953; Bellotti & Arias, 2001, pp.813-824). In Ghana, TYLCV disease is reported to be widespread, accounting for severe yield losses (Horna, Smale, & Falck-Zepeda, 2006, p. 23; Osei, Akromah, Shih, & Green, 2010, pp. 315-323). Three new distinct TYLCV-causing Begomoviruses were detected and reported from Akumadan and Kumasi, the major tomato producing communities in the country (Osei, Akromah, Shilh, & Green, 2008, p. 1585). Production losses vary from very dramatic to mild with devastating yields loss in the rainy season (Osei et al., 2010, pp. 315-323; Horna et al., 2006, p. 27). Whitefly populations and diseases in the dry season are usually severe during the dry season, especially with relatively higher incidence of *Bemisia tabaci* from cassava fields in the dry season (Appiah et al., 2012, pp.31-37). This variation in whitefly population has been attributed to differences in temperature and relative humidity (Triparthi & Varma, 2002, p.476).

Most commercial tomato varieties have been found to be completely susceptible to TYLCV, compelling breeders to screen Wild tomato accessions and some commercial varieties for potential resistance genes (Pilowsky & Cohen, 2000, pp. 351-353). Breeding for resistance to TYLCV in cultivated tomato varieties appears to be the most ideal control measure for the virus (Pico, Diez, & Nuez, 1999, p.1008; Osei et al., 2008, p.1585; Horna et al., 2006, p.31). Efforts have been initiated to introgress resistance genes from Solanum pimpinellifolium into some commercial varieties/breeding lines in Ghana (Quartey, 2010, p.173; Nunoo, 2010, pp.87-105). Field evaluation is essential to identify resistant plants after introgression of resistance gene from resistant plants. Field evaluation of resistance in some

tomato varieties/breeding lines has been widely used for primary appraisal of resistant lines (Osei et al., 2010, pp. 315-323; Lapidot & Friedmann, 2002, p.127). Under natural field conditions, spontaneous whitefly inoculation occurs, inducing severe TYLCV symptoms, especially during high whiteflies populations in the field. A number of breeding lines including three newly generated backcross lines (BC1) were developed and three of these breeding lines and their parents, together with their respective first backcross (BC1) generations and a local accession of the Wild tomato (*S. pimpinellifolium*) were evaluated in the field for their resistance to TYLCV disease. The objective of this study was to identify TYLCV resistant/tolerant varieties/breeding lines among 10 breeding lines of tomato.

MATERIALS AND METHODS

Study Area

The experiment was conducted at the research farm of the Biotechnology and Nuclear Agriculture Research Institute (BNARI) of the Ghana Atomic Energy Commission (GAEC), Kwabenya, Accra. The experimental field is located at latitude 05°40'N and longitude 0°13'W, at an elevation of 76 m above sea level within the Coastal Savannah Agro-Ecological Zone. The soil at the site is the Nyigbenya-Haatso series, which is a typically well-drained savannah Ochrosol (Ferric Acrisol) derived from quartzite schist (FAO/UNESCO, 1994, p.146). The maximum and minimum average temperatures for the study period were 30.7 and 23.2 oC respectively with a mean annual rainfall of 220 mm (Local Weather Station, 2013).

Experimental Material

Seeds of three tomato varieties, six breeding lines and a landrace (Wild) were raised in a nursery and seedlings transplanted to the field for evaluation in the field against tomato yellow leaf curl virus disease (TYLCVD) (Table 1).

Table 1
Identities and characteristics of tomato varieties/breeding lines used in the study

Varieties/Breeding Lines	Status	Pedigree	Growth habit
S. pimpinellifolium	Wild	-	Indeterminate
Wosowoso	Local	-	Determinate
Cherry Red	Exotic	-	Determinate
Roma	Exotic	-	Determinate
Hyb-1	Hybrid	Woso x Wild	Semi-indeterminate
Hyb-2	Hybrid	Roma x Wild	Semi-indeterminate
Hyb-3	Hybrid	C-Red x Wild	Semi-indeterminate
BC-1	Backcross	Woso x (Woso x Wild)	Semi-indeterminate
BC-2	Backcross	Roma x (Roma x Wild)	Semi-indeterminate
BC-3	Backcross	C-Red x (C-red x Wild)	Semi-indeterminate

Experimental Design and Field Management Practices

Seedlings were raised in trays filled with a mixture of topsoil, cow dung and coconut husk in the ratio 3:1:1 in a screen house. At 28 days after sowing (DAS) when 3-4 leaves were fully expanded, the seedlings were transplanted to the field. The Randomised Complete Block Design (RCBD) was used with four replications. A plot size of 3.2 m x 3.6 m with a planting distance of 80 cm x 60 cm was used for all varieties/breeding lines. Each plot contained 24 plants out of which ten (10) inner-rowed plants were randomly selected and tagged for data collection on all parameters studied. Agronomic practices such as watering, mulching and fertilisation were undertaken. Watering was done twice daily for the first two weeks after transplanting (2 WAT) and subsequently reduced to once daily using a watering can. The NPK (15-15-15) fertiliser was applied two weeks after transplanting at 250 kg ha-1 (Osei et al., 2010, pp. 315-323). Hand weeding was done frequently to control weeds. To avoid bias in data collection, pests and diseases were not controlled.

Data Collection

Whitefly Population Survey. A weekly count of whitefly populations was done on five broadly expanded leaves that were randomly selected on each of the 1 sample plants. Counting was started 2 WAT (after transplanting) and continued weekly for seven weeks. Leaves were gently turned with little or no disturbance of the whiteflies and their number on the adaxial side of each leaf were counted. This was done early morning (6:00am - 7:30am) before sunrise to avoid whiteflies from being too active when the sun rises.

Disease Incidence and Symptom Severity. Symptoms of TYLCV on the 10 sampled plants of each of the 10 tomato varieties/ breeding lines were observed and scored. A five-point scale adapted from Friedmann, Lapidot, Cohen and Pilowsky (1998, pp. 1004-1007) was used to score symptom severity at 2, 4, 6 and 8 weeks after transplanting (WAT). The scoring scales were: 0 = No visible symptoms, 1 = Slight yellowing of margins of apical leaflets, 2 = Moderate yellowing and slight curling of leaflet tips, 3 = Extensive leaf yellowing, curling and cupping with some reduction in leaf size, 4 = Very severe stunting of plant and leaf yellowing, pronounced cupping and curling of leaves (Plate 1 and 2). The disease incidence (DI) (number of symptomatic plants as per the number of plants on each plot) was also recorded at 2, 4, 6 and 8 WAT.

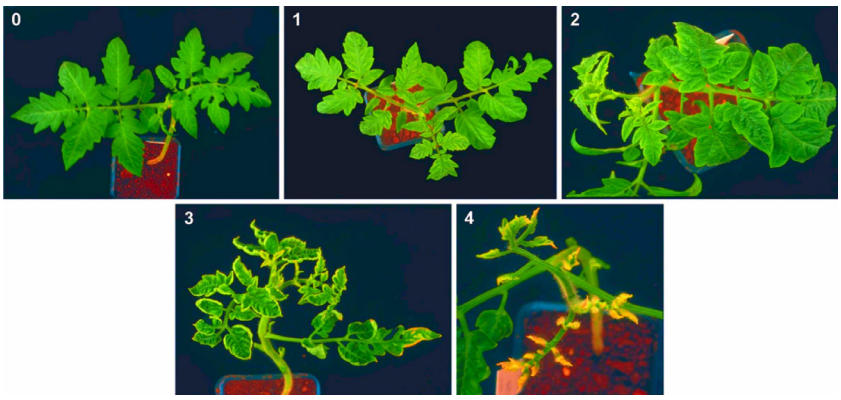

Plate 1. Tomato leaves showing varying degrees of symptom severity from TYLCV infection. Symptom scoring scale: 0-4 according to Friedmann et al. (1998)

Severity of the symptoms was estimated using the formula advanced by Njock and Ndip (2007).

(A) Index of severity of symptoms based on all plants (ISS_{AP}) = $(\sum_{S=0}^{4} (SXs) / \sum_{S=0}^{4} (Xs))$

Where S is severity class (0 – 4)
X is the number of plants giving the score S, and AP is all plants.

(B) Index of severity of symptoms based on diseased plants only.

$(ISS_{DP}) = (\sum_{S=1}^{4} (SXs) / (\sum_{S=1}^{4} (Xs))$

Where DP = diseased plants only

(C) Percent disease incidence was calculated as:

DI % = $100 ((\sum_{n=0}^{4} (SXs) / (\sum_{n=0}^{4} (Xs))$

Agronomic Evaluation and Yield Characteristics of 10 Tomato Varieties/ Breeding Lines. The following data was collected on tagged plants of the 10 varieties/breeding lines during the field trial using Descriptor List for tomato from the International Plant Genetic Resources Institute (IPGRI, 1991). Data was taken on number of days to 50% flowering; number of days to maturity; plant height; number and weight of fruits per plant and yield per hectare (t/ha) was estimated.

Statistical Analyses

Statistical analyses on all studied parameters were performed using GenStat statistical package software (Payne et al., 2007; ver. 12.0), Statgraphics (2010; Plus XV.I) and Microsoft Excel (ver. 2010). Mean of number of whiteflies, fruit number per plant, ISS_{AP} and ISS_{DP} were square root transformed [square root of (x + 0.5)] whereas means of percent disease incidence (DI) were arcsine transformed before

performing ANOVA. Pearson correlation analysis was performed on disease-related parameters of the varieties/breeding lines studied.

RESULTS

Whitefly Populations on 10 Tomato Varieties and Breeding Lines

The mean number of whiteflies counted in Roma, Wosowoso, Cherry Red and Woso x WW was relatively higher compared with Roma x RW and Wild tomato (Table 2). Average whitefly count showed significant differences ($p \leq 0.05$) among the 10 varieties/breeding lines (Figure 1). At 2 WAT, all tomato varieties/breeding lines had relatively high whitefly populations but decreased gradually by 3 WAT except Woso and Cherry Red. Whitefly numbers increased in 4 WAT for all varieties/breeding lines after which it fluctuated till 6 WAT. However, there was a reduction in whitefly counts at 7WAT with the lowest recorded for all the tomato varieties/breeding lines at 8 WAT. In general, whitefly preference for all the varieties/breeding lines was observed during the seven weeks of survey. The highest mean of whiteflies was found on Roma (49.08), whilst Wild tomato had the least (19.58). In the Roma variety, no significant differences were observed between mean whitefly counts from 2 WAT to 5 WAT and between 6 WAT and 7 WAT. However, a significant reduction was observed at 8 WAT. No significant differences were recorded between the average weekly whitefly counts over the whole survey period for Woso (Tab 2). There were relatively higher numbers of whiteflies in the early stages of the survey. Wild tomato recorded the lowest whitefly count throughout the study period.

Generally, fewer whitefly numbers were recorded in both the hybrid and backcross lines in comparison to the parental lines. The highest whitefly number was recorded at 2 WAT where significant differences were observed in the various varieties/breeding lines. At 3 WAT, Woso x Wild, Woso x WW, Cherry Red, Cherry Red x Wild, Cherry Red x CRW did not show any significant differences in whitefly numbers. There were however, significant differences in whitefly numbers at 2,4,5,7 and 8 WAT. Significant difference in weekly whitefly counts were observed in all varieties/breeding lines except Roma and Wosowoso. There were however, no significant difference between the weekly whitefly counts in Roma from 2 WAT-5 WAT and Wosowoso from 3 WAT-8 WAT (% CV: 11.76-15.75).

Table 2
Variation in average whitefly count with time on tomato varieties/breeding lines

Varieties/ Breeding Lines	Weekly Whitefly Counts							
	2 WAT	3 WAT	4 WAT	5 WAT	6 WAT	7 WAT	8 WAT	Mean
Wild	19.58^{e}	19.23^{c}	19.90^{e}	21.00^{d}	21.38^{e}	20.03^{f}	14.65^{e}	19.39^{d}
Woso	43.75^{ab}	44.98^{a}	46.28^{a}	44.70^{a}	45.56^{a}	40.08^{a}	32.15^{a}	42.50^{a}
C-Red	30.33^{cd}	30.63^{b}	34.13^{bcd}	34.78^{b}	34.98^{bc}	33.08^{bc}	20.15^{c}	31.15^{b}
Roma	49.08^{a}	44.03^{a}	45.80^{a}	47.43^{a}	43.80^{ab}	37.75^{ab}	26.80^{b}	42.10^{a}
Hyb-1	35.80^{bcd}	31.03^{b}	30.88^{bcd}	29.68^{bc}	30.63^{cd}	29.60^{cd}	17.30^{cde}	29.27^{bc}
Hyb-2	28.23^{de}	27.35^{b}	29.23^{bcd}	29.98^{bc}	29.95^{cd}	28.50^{cd}	20.18^{c}	27.63^{bc}
Hyb-3	36.30^{bcd}	30.90^{b}	35.28^{bc}	34.25^{b}	28.5^{3cde}	27.55^{cde}	21.05^{c}	30.55^{b}
BC-1	40.88^{abc}	31.25^{b}	37.50^{ab}	31.90^{bc}	32.13^{cd}	28.00^{cde}	16.22^{de}	31.12^{b}
BC-2	28.75^{de}	25.23^{bc}	26.85^{d}	27.78^{c}	26.30^{de}	22.93^{ef}	15.80^{de}	24.80^{cd}
BC-3	31.23^{bcd}	28.55^{b}	27.38^{cd}	27.83^{c}	28.39^{cde}	25.18^{de}	19.55^{cd}	26.87^{bc}
CV%	15.75	14.05	14.48	12.71	13.72	11.76	13.58	15.75

Means in the same column and row followed by the same letter are not significantly different ($p \leq 0.05$)

Disease Incidence of TYLCV on 10 Tomato Varieties/Breeding Lines

Generally, disease incidence was observed on all the tomato varieties/breeding lines from 4-8WAT (Figure 1). Disease incidence for all the varieties/breeding lines increased during the evaluation period till 8WAT when the highest incidence was recorded. The Roma and Woso recorded the highest average percent disease incidences (43.1% and 30.5% respectively) whereas the lowest disease incidence (11%) was recorded by Wild tomato. All varieties/breeding lines recorded no incidence of disease at all or less than 5% at 2WAT and increased gradually to 8WAT except Roma (Figure 1) where 20% disease severity was recorded at 2 WAT.

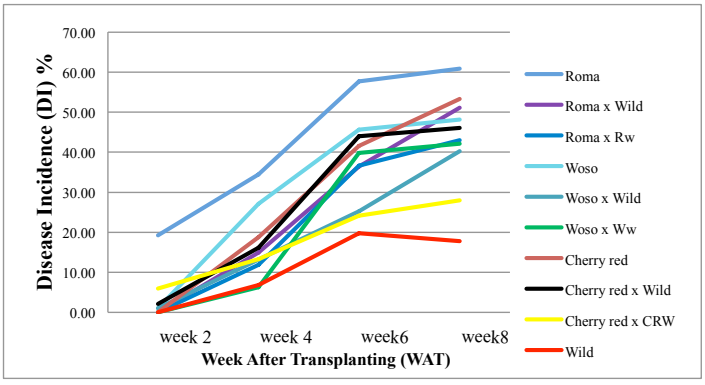

Figure 1. Average Disease Incidence on Tomato Varieties/Breeding Lines Across 8WAT

Symptom Severity of TYLCV on 10 Tomato Varieties/Breeding Lines

In general, symptom severity increased from 2-8WAT for both ISS_{AP} and ISS_{DP} in all the varieties/breeding lines. The Wosowoso gave the highest score in terms of ISS_{AP} and ISS_{DP} (1.9 and 2.27) respectively. Roma recorded the highest symptom severity for ISS_{AP} and ISS_{DP} over the entire study period (Table 3) whilst Wild tomato had the lowest ISS_{AP} and ISS_{DP} values. Wild tomato, Woso x Ww, Roma x Rw, Roma x Wild did not show any symptoms at all. Furthermore, all the backcross lines expressed only mild symptoms throughout the period except Roma x Wild which recorded 1.23 ISS_{AP} at 8WAT. All the F3 hybrid lines expressed mild symptoms (ISS_{DP}<2) during the study period, compared with the parental lines (Table 3). The lowest ISS_{AP} and diseased ISS_{DP} were observed in Wild tomato, and Woso x WW. There were, however, symptom reversion in Wild tomato and Woso x Wild at 6 and 8 WAT. Differences among the 10 varieties and breeding lines with respect to ISS_{AP} and ISS_{DP} were highly significant ($p \leq 0.05$).

Table 3
Variation in TYLCV symptom severity with time on 10 tomato varieties/breeding lines

Varieties/Breeding Lines	Disease Symptom Severity (%)									
	ISS_{AP}					ISS_{DP}				
	2WAT	4WAT	6WAT	8WAT	Mean	2WAT	4WAT	6WAT	8WAT	Mean
Wild	0.00^{b}	0.20^{cd}	0.58^{e}	0.48^{d}	0.31^{b}	0.00^{b}	0.63^{c}	1.26^{de}	1.15^{c}	0.76^{b}
Roma	0.53^{a}	1.33^{a}	2.68^{a}	3.08^{a}	1.42^{a}	1.25a	1.98^{a}	2.77^{a}	3.08^{a}	1.63^{a}
Wosowoso	0.03^{b}	1.05^{ab}	2.13^{ab}	2.48^{a}	1.90^{ab}	0.25^{b}	1.60^{ab}	2.21^{ab}	2.48^{a}	2.27^{a}
Cherry red	0.00^{b}	0.65^{abc}	1.15^{cd}	1.35^{b}	0.79^{ab}	0.00^{b}	1.44^{ab}	1.60^{cd}	1.49^{bc}	1.13^{ab}
Roma x Wild	0.00^{b}	0.45^{bcd}	1.08^{cde}	1.38^{b}	0.56^{ab}	0.00^{b}	1.20^{abc}	1.52^{cde}	1.53^{bc}	1.10^{ab}
Woso x Wild	0.03^{b}	0.35^{bcd}	0.75^{de}	1.10^{bc}	0.73^{ab}	0.25^{b}	1.15^{abc}	1.44^{cde}	1.56^{bc}	1.06^{ab}
Cherry red x Wild	0.08^{b}	0.40^{bcd}	1.50^{bc}	1.45^{b}	0.86^{ab}	0.25^{b}	0.94^{abc}	1.83^{bc}	1.70^{b}	1.18^{ab}
Roma x RW	0.00^{b}	0.20^{cd}	0.65^{e}	1.23^{bc}	0.48^{b}	0.00^{b}	0.75^{bc}	1.12^{e}	1.66^{bc}	0.78^{ab}
Woso x WW	0.00^{b}	0.18^{d}	0.95^{cde}	0.78^{cd}	0.52^{b}	0.00^{b}	0.56^{c}	1.33^{de}	1.22^{bc}	0.88^{b}
Cherry red x CRW	0.13^{b}	0.33^{cd}	0.75^{de}	0.85^{bcd}	0.51^{ab}	0.25^{b}	0.90^{abc}	1.41^{cde}	1.41^{bc}	0.99^{ab}
CV%	217.19	64.87	30.54	31.01	61.95	203.62	49.03	17.05	18.54	48.34

CV = coefficient of variation, ISSAP = index of symptoms severity based on all plants only, ISSDP = index of symptoms severity based on diseased plants only, WAT = weeks of transplanting

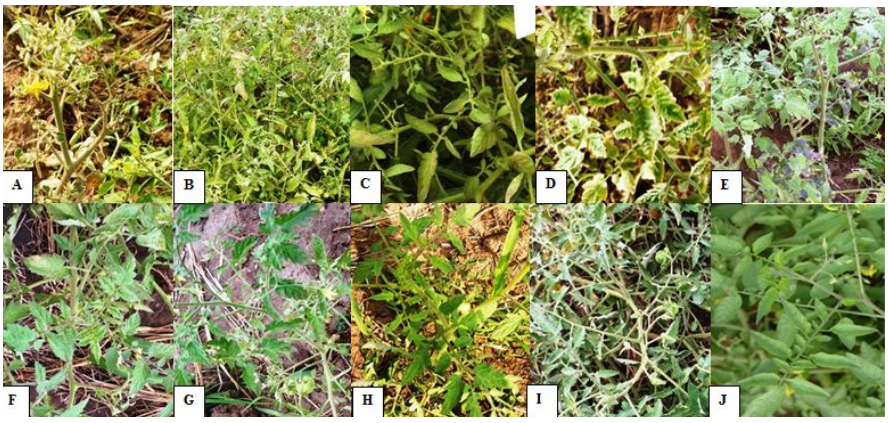

Plate 2. Variation in Leaf Symptoms of TYLCD among Ten Tomato Varieties/Breeding Lines in the Field. (A) Roma, (B) Roma x Wild, (C) Roma x Rw, (D) Wosowoso, (E) Woso x Wild, (F) Woso x Ww, (G) Cherry red, (H) Cherry Red x Wild, (I) Cherry Red x CRW, (J) Wild tomato

Agronomic Characteristics

Days to Flowering, Fruit Maturity, Plant Height (WAT) and Yield. Among the 10 varieties/breeding lines evaluated in the dry season, differences in the mean number of days to first flowering and 50% flowering were highly significant ($p \leq 0.05$) for Roma x RW, Woso, Cherry Red and Cherry Red x CRW. Differences in the mean number of days to first fruit maturity and 50% maturity were also highly significant ($p \leq 0.05$) for all varieties/breeding lines except Roma and Roma x RW (Table 4). In the dry season, Cherry red was the first to flower (41.25 days) but Cherry red x Wild was the first to attain fruit maturity (79.75 days after sowing seed at nursery) while Roma was the last to flower (47 days) and mature (98 days). Variations were observed in plant height recorded on the 10 tomato varieties/breeding lines. Generally, plant height increased with age for all the 10 varieties/breeding lines (Table 4). Average plant height at two, four and eight weeks after transplanting (WAT) represent average days to first, 50% flowering and 50% maturity respectively. Woso recorded the highest plant height at first flowering (30.6 cm), Cherry red for 50 % flowering (47.85 cm) and Cherry red x CRW for 50 % maturity (91.75 cm) (Table 4). The least plant height at first flowering, 50% flowering and 50% maturity were recorded in Wild tomato (20.4, 32.94 and 67.23 cm) respectively. The differences among mean plant heights of the varieties/breeding lines were highly significant ($p \leq 0.05$). Yield was determined by the number of fruits harvested per plant, average fruit weight per plant and total yield extrapolated as tonnes/ha (Table 4). In general, Roma x Rw breeding line attained the highest fruit yield in terms of all yield components. There were highly significant differences ($p \leq 0.05$) among the tomato varieties/breeding lines for average number of fruits per plant and average fruit weight per plant/(g) or average number of fruits per plant and yield (t/ha) (Table 4). The highest number

of fruits per plant, average fruit weight (g) and total fruit yield (t/ha) were achieved in Wild tomato (40.55), Woso (119.26 g) and Roma x Rw (48.49 t/ha) respectively at the end of the growing period. The least number of fruits per plant, average fruit weight (g) and total fruit yield (t/ha) were achieved in Roma (0.7), Wild tomato (11.10 g) and Roma (1.76 t/ha) respectively (Table 4).

Table 4
Days to flowering, fruit maturity, plant height 8 WAT and yield among the 10 varieties/breeding lines

Varieties/ Breeding Lines	Days to Flowering and Fruit Maturity				Plant Height of the Tomato Lines over 8WAT					Yield		
	DFFl	FpFl	DFF	FpFr	2 WAT	4 WAT	6 WAT	8 WAT	Mean	AFP	AFWtP (g)	FY (t/ha)
Wild	44.75^{abc}	50.75^{abc}	81.50^{ab}	90.00^{bc}	20.40^{d}	32.94^{c}	47.18^{b}	67.23^{d}	41.93^{d}	40.55^{a}	2.78^{c}	2.73 x 10^{-6e}
Roma	47.00^{a}	56.50^{a}	98.00^{a}	114.50^{a}	23.43^{cd}	34.38^{bc}	57.35^{ab}	69.58^{cd}	46.18^{cd}	0.70^{d}	13.72^{b}	13.50 x 10^{-6b}
Wosowoso	42.75^{bc}	55.50^{ab}	93.75^{ab}	103.00^{ab}	30.60^{a}	45.35^{a}	67.38^{a}	86.08^{abc}	57.35^{b}	6.15^{cd}	29.82^{a}	29.35 x 10^{-6a}
Cherry red	41.25^{c}	49.50^{bc}	88.00^{ab}	100.00^{ab}	29.50^{ab}	47.85^{a}	69.75^{a}	89.00^{ab}	59.03^{a}	17.65^{abc}	13.03^{b}	12.82 x 10^{-6b}
Roma x Wild	46.00^{ab}	55.50^{ab}	90.00^{ab}	98.25^{ab}	29.50^{ab}	41.40^{ab}	67.20^{a}	88.33^{ab}	56.61^{b}	24.63^{abc}	4.40^{dc}	4.33 x 10^{-6de}
Woso x Wild	44.50^{abc}	55.75^{ab}	93.50^{ab}	102.00^{ab}	30.00^{ab}	39.90^{abc}	67.53^{a}	84.90^{abc}	55.58^{b}	14.95^{bc}	7.85^{c}	7.72 x 10^{-6c}
Cherry red x Wild	43.50^{abc}	51.75^{abc}	79.75^{b}	88.50^{c}	29.10^{ab}	42.70^{a}	65.70^{a}	90.90^{ab}	57.10^{b}	27.64^{ab}	4.59^{de}	4.52 x 10^{-6de}
Roma x Rw	42.75^{bc}	51.25^{abc}	90.00^{ab}	107.50^{ab}	25.18^{bcd}	34.65^{bc}	55.88^{ab}	73.98^{bcd}	47.42^{c}	28.98^{abc}	8.36^{c}	8.23 x 10^{-6c}
Woso x Ww	43.50^{abc}	52.50^{abc}	87.25^{ab}	95.25^{bc}	26.20^{bcd}	41.33^{ab}	60.78^{ab}	79.15^{abcd}	51.86^{d}	15.52^{bc}	9.18^{c}	9.04 x 10^{-6c}
Cherry red x CRW	42.00^{bc}	49.00^{c}	83.00^{ab}	97.75^{ab}	23.08^{cd}	42.15^{ab}	62.55^{ab}	91.75^{a}	54.88^{c}	10.53^{cd}	7.25^{cd}	7.14 x 10^{-6cd}
CV (%)	3.51	4.42	7.06	6.95	17.68	16.74	19.55	16.49	42.18	53.10	76.05	76.05

Means in the same column followed by the same letter are not significantly different ($p \leq 0.05$). DFFl = days to first flowering; FpFl = days to 50% flowering; CV = coefficient of variation; DFFM = days to first fruiting; FpFr = days to 50% fruiting; AFP = average fruits per plant; AFWtP = average fruit weight per plant (g); FY = fruit yield (ton/ha).

Relationship between Disease Incidence, Symptom Severity, Plant Height and Whitefly Count among the 10 Tomato Varieties/Breeding Lines

Generally, all the traits measured showed very low correlation with fruit yield (t/ha) except fruit number per plant which exhibited low correlation with yield (t/ha) (Table 5). Disease severity for all plants (ISS_{AP}), disease severity for diseased plants (ISS_{DP}), average % disease incidence and average whitefly count showed very high negative correlation with fruit yield (t/ha). Percent disease incidence, disease severity for all plants (ISS_{AP}) and disease severity for diseased plants (ISS_{DP}) were moderately negatively correlated while average whitefly count showed negative correlation with number of fruits per plant. Furthermore, average whitefly count, percent disease incidence and disease severity (ISS_{AP}) showed very low positive correlation.

With regards to ISS_{DP}, average whitefly count showed high positive correlation (r = 0.89), percent disease incidence moderately correlating with ISS_{DP} (r = 0.67) while ISS_{AP} showed very high correlation (r = 0.98) with ISS_{DP}. Again, average whitefly count showed very high positive correlation (r = 0.93; r = 0.74) with disease severity (ISS_{AP}). Average whitefly count was highly correlated (r = 0.86) with average disease incidence.

Table 5
Correlation co-efficients of disease-related parameters on 10 tomato varieties/breeding lines

Traits	Average whitefly count	% disease incidence	ISS_{DP}	Fruit/ plant	Fruit yield (t/ha)
Average whitefly count					
Percent disease incidence	0.86**				
ISSAP	0.93***	0.74**			
ISSDP	0.89***	0.67*	0.98***		
Fruit/plant	-0.83**	-0.64*	-0.64*	-0.61*	
Fruit yield (t/ha)	-0.18	-0.18	-0.03	-0.03	0.36

* = significant ($P \leq 0.05$); ** = very significant ($P \leq 0.001$); *** = highly significant ($P \leq 0.0001$) computed using standard linear Pearson correlation

DISCUSSION

The whitefly life-cycle progresses from egg to adult emergence, governed mainly by temperature (Triparthi & Varma, 2002, p. 473-478). In warm climates, the life cycle takes approximately three weeks, but it may take up to two months under cool conditions (Triparthi & Varma, 2002, p. 473-478) with no adult emergence occurring when the temperature drops below 17°C (Czosnek, 2007, pp. 329-342). Generally, whitefly populations on the leaves of the tomato are more in the dry (hot) season than in the rainy (cool) seasons (Canto, Aranda, & Fereres, 2009, pp. 884-894). Thus, it is important to assess disease incidence severity in the dry season where abundance of whitefly populations ar expected as reported on cassava by Appiah et al. (2012, pp.31-37). The study revealed that whiteflies had high preference for Roma, Cherry red, Wosowoso and Woso x WW varieties/breeding lines. This has led to higher disease incidences and invariably higher disease severity for these varieties/breeding lines. Whitefly preference for specific varieties/breeding lines does not necessarily lead to incidence and severity of TYLCV disease as the feeding habits of whiteflies predispose the plant to several other infections other than TYLCV. However, the confirmation of TYLCV in these varieties/breeding lines earlier by TAS-ELISA and PCR (Segbefia, et al., 2015, pp. 17-24) shows that the disease was transmitted by whiteflies. This supports previous observations made by Brown, Costa and Laemmlen (1992, p. 426); Schuster, Mueller, Kring, & Priece (1990, pp. 1618-620) and Asare-Bediako, Wonkyi, van der Puije, Amenorpe and Osei

(2017, pp. 373-378) that direct crop damage occurs when whiteflies feed in plant phloem, remove plant sap, excrete honeydew, which promotes sooty moulds interfering with photosynthesis, and thus, reducing plant vigour. Additionally, higher whitefly numbers were recorded at the early growth stages of the plant and reduced gradually towards the end. This shows preference by whiteflies for younger leaves at two or four weeks after transplanting (WAT) than leaves of matured plants.

Furthermore, the Wild tomato had the lowest whitefly populations compared with other varieties/breeding lines throughout the study period. This may be attributed to resistance-related factors such as small leaf size, smell and /or other physical barriers. Bellotti & Arias (2001, pp. 813-824) reported that in tomato, non-preference of some varieties by whiteflies is due to physical barriers, such as waxy or thick cuticles or the presence of specialised trichomes that inhibit whiteflies from settling and feeding on leaves. The immediate manifestation of a pathogen infecting a plant is the expression of disease symptoms. The TYLCV-induced symptoms usually appear within 2–3 weeks after inoculation (Czosnek, 2007, p. 339). Incidence of TYLCVD on tomato plants is characterised by varied symptoms including upward/downward leaf curling, yellowing of young upper leaves, reduced leaf size, stunting of plants, reduced fruit yield (fruit size and number) and death of plants. Symptom expression, however, varies with viral strain, tomato varieties/ breeding lines, plant age at time of infection and 'enviro-climatic' conditions (Lapidot et al., 2000, pp. 31 7-321; Pico, Diez, & Nuez., 1998, pp. 259-271). In this study, the three backcrossed lines (Roma x RW, Woso x WW and Cherry Red x CRW) exhibited mild disease symptom severity throughout the study period. These were comparable with the Wild tomato (donor parent for TYLCV resistance genes), and showed significant improvements in levels recorded for the three adapted parents (Roma, Wosowoso and Cherry Red) used as recurrent parents in the backcrosses (Segbefia et al., 2015, pp. 17-24).

The resistance obtained using one screening approach may not be equivalent to that obtained using another approach. Thus, a comparison of the use of leaf discs and whole plants in screening for resistance indicated that although leaf disc assays were able to discriminate between immune and susceptible varieties/breeding lines, they were not able to "discriminate between sensitive and tolerant plants which support virus replication and cell-to-cell spread but not its long-distance movement" (Czosnek, et al., 1993, pp. 995-1005). In this study, all the varieties/breeding lines, exhibited a range of TYLCVD leaf symptoms including yellowing, curling and reduced leaf size in the field during the dry season. Based on symptomatology alone, there were no sign of resistance among the cultivated commercial varieties to TYLCVD. This is consistent with reports by Pilowsky and Cohen (2000, pp. 351-353); and Pico, Diez and Nuez (1999, pp. 1006-1012). Among the F3 hybrid lines, Roma x Wild

and Cherry Red x Wild proved to be slightly susceptible. On the other hand, the backcross lines had some level of resistance introgressed into them after the first generation of backcross. The TYLCV disease symptom development generally began two weeks after transplanting to the field. However, most varieties/breeding lines recorded no symptoms except Roma and Wosowoso. Symptom development and severity continued till eight (8) weeks after transplanting, where the highest severity was attained.

Symptom reversal was observed in Wild tomato and Woso x Wild in 6 and 8 WAT culminating in lower ISS_{AP} and ISS_{DP}. This may be a good indication of resistance in these lines, corroborating the report by Czosnek (2007, p.332) that symptoms in resistant plants tend to increase with time and then decrease, unlike those of susceptible plants which normally increase over time and then plateaus. This study has confirmed the successful transfer of resistant genes from the F_3 breeding lines (Roma x Wild, Woso x Wild and Cherry red x Wild) to the backcrossed lines (Roma x RW, Woso x WW and Cherry Red x CRW). All the breeding lines recorded low disease symptoms; conversely, the backcrosses recorded much lower or delayed symptoms, indicating that the transfer of resistant genes from the hybrid lines to the backcross lines was successful.

Plant height of the three F_3 hybrid lines was significantly higher than those of the backcrossed lines. This indicated the gene for tallness in the F_3 hybrid lines has not been transferred to the backcrosses. Differences in plant height among the 10 varieties/breeding lines were not significant ($p \leq 0.05$). All the commercial varieties showed determinate growth pattern whereas the F_3 hybrid lines and backcross lines showed semi-determinate growth pattern similar to the Wild tomato. Flowering and maturity were generally earlier in the backcrossed lines except Roma x RW comparable to the Wild tomato. Though, the F_3 hybrid lines are early maturing which is a desirable trait, they grow tall quickly which requires constant pruning and staking. The parental varieties which are determinate and comparatively shorter require no staking which is an indication that they have been improved upon over the years and are suitable for commercial cultivation. However, their fruits easily touch the ground at maturity making them susceptible to attack by pests. The backcross lines on the other hand, have two main advantages which are earliness to maturity and a longer harvesting period (semi-determinate), a trait desired by local farmers. Further improvement of these backcross lines would make them acceptable to local farmers.

The relevance of TYLCV resistance emanates from its effect on total yield and yield components, relative to uninfected controls (Lapidot et al., 1997, pp. 1425-1428; Lapidot, Weil, Cohen, Segev, & Gaba, 2007. pp. 143-148). In this study, the number of fruits per plant was relatively high in Roma x RW, Roma x Wild and Cherry red x Wild and total fruit yield (t/ha) was relatively high in Cherry red, Wosowoso and Roma x

Wild. These varieties/breeding lines would however perform better in the rainy season as there is a higher incidence of the TYLCV disease in the dry season, which drastically reduces total production with adverse consequences for farmers (Robinson & Kolavalli, 2010, pp.17-19). Similarly, Osei et al. (2010, pp. 315-323) reported that in the rainy season, Ghana is able to produce to meet domestic tomato demand. In terms of weight of fruits, Wosowoso recorded the highest while Cherry red had the lowest among the cultivated commercial varieties. Among the three F_3 breeding lines, Cherry red x Wild was the most prolific, producing the highest number of fruits. Woso x Wild produced the highest total fruit yield among the F_3 breeding lines.

The Wild tomato (*S. pimpinellifolium* L.) produced the highest number of fruits in this study. However, due to smaller fruit size, total fruit yield (t/ha) was low compared with the adapted varieties / breeding lines. In all cases, numbers of fruits recorded by the breeding lines were lower than Wild tomato. However, the total fruit yield (t/ha) of the breeding lines were higher than that of Wild tomato. This indicates that the backcrossing of the F3 breeding lines to the adapted varieties Cherry Red, Wosowoso and Roma resulted in increased fruit size compared to Wild tomato. Therefore, with the combination of desirable attributes such as high level of resistance to TYLCV disease in the field, early maturity, semi-determinate growth habit and large number of fruits, breeders could select Roma x RW, Woso x WW Tomato and Cherry Red x CRW for further improvement in fruit size (weight) towards high fruit yield and tolerance to TYLCVD. The obvious limitation of this study is that diagnosis based on symptom expression alone may be inadequate since other factors (mineral deficiencies) and attack by pests could play a major role in the overall appearance of plants in the field. It is however, relevant as a preliminary step in screening the varieties/breeding lines in the breeding programme against TYLCVD.

In an earlier work (Segbefia et al., 2015; pp. 17-24), TAS-ELISA detected TYLCV in Wosowoso, Woso x Wild, Woso x WW, Roma and Roma x Rw under field conditions. The PCR confirmed the presence of TYLCV in all varieties/breeding lines except Roma x RW. Detection of TYLCV in the symptomless Wild Tomato and the hybrids, Roma x Wild and Woso x Wild and all backcrosses indicate they are symptomless carriers of the virus.

CONCLUSION

Roma recorded the highest population of whiteflies in the dry season and also exhibited the highest symptom severity in all plants (ISS_{AP}) and diseased plants (ISS_{DP}) during the study period. There was symptom reversal in Wild tomato and Woso x Wild at 6-8 WAT for both ISS_{AP} and ISS_{DP} indicating their potential source of resistance. Average whitefly count showed very high positive correlation ($r = 0.93$) with disease severity (ISS_{AP}); while average whitefly count, average percent disease incidence, ISS_{AP} and ISS_{DP} correlated inversely ($r = -0.83$) with yield (t/ha). Roma x Wild, Woso x Wild,

Cherry Red x Wild, Roma x RW, Woso x WW and Cherry Red x CRW could be selected for future breeding work based on their superior resistance to the virus.

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Antioxidant Activity of Natural Pigment from Husk of Coconut

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ABSTRACT

Coconuts grow abundantly in the coastal areas of tropical countries. About 33-35% of the coconut is made of husk which includes mesocarp and exocarp. In Malaysia, the coconut husk is available in large quantities as the residue from coconut production. In previous works, natural pigments from the exocarp and mesocarp were extracted using microwave-assisted extraction. The current study was aimed at investigating the antioxidant activities of these pigments extracts. Quantitative determination of total phenolics and antioxidant capacities of these extracts were assayed for their ability to scavenge DPPH radicals and chelate ferrous ion. The total phenolic content, expressed as mg of gallic acid equivalent (GAE) per gram of extract, was found to be 32.24 mg GAE/g and 8.63 mg GAE/g in the mesocarp and exocarp respectively. The radical scavenging activity measurement, expressed in terms of mmol Trolox equivalent (TE) per gram of extract, was significantly ($p<0.05$) higher in the mesocarp (119.96 mM TE/g) compared with the exocarp (55.27 mM TE/g). Meanwhile, the reducing ability showed significantly ($p<0.05$) higher value in the mesocarp extract (751.89 mM Fe^{2+}/g) compared with the exocarp extract (264.36 mM Fe^{2+}/g). Thus, this study indicated the possible use of pigment extract as a source of natural antioxidant, which has great potential in the food industry and medicinal applications.

Keywords: Antioxidant, exocarp, mesocarp, microwave-assisted extraction and natural pigment

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INTRODUCTION

Cocos nucifera (coconut palm) is a member of the Arecaceae family and is cultivated mainly in the tropical areas which have high humidity, sandy soil, and regular rainfall. Countries such as India, Sri Lanka, Indonesia, and the Philippines are major

producers of coconut (Probir et al., 2013). In Malaysia, the coconut palm is known as *kelapa*, and is the fourth important industrial crop after oil palm, rubber and paddy in terms of total planted area and is one of the oldest agro-based industries (Saif et al., 2015). Coconut fruit has three layers: mesocarp, exocarp, and endocarp. The exocarp (outer layer) and mesocarp (fibrous husk) make up the husk of coconut (Victor, 2013). Coconut is made up of 33-35% of husk, and in Malaysia, it was estimated that 5280 kg of dry husks become available per hectare per year (Tan et al., 2007). Hence, it is possible to make better use of this abundant and cheap agricultural waste to be converted into natural pigment.

Recently, with public awareness and focus on health as well concerns over eco-safety, environmental friendly and nontoxic bio resource products are regaining popularity in different aspects of our lives. This offers a good chance for the reintroduction of natural dyes that could be considered as an alternative to synthetic dyes, which have been known to cause health problems due to their carcinogenic effects (Prusty et al., 2010). Natural pigments are complex organic molecules that give variety of colours to plants and foods. In addition, dyes are also part of the ingredients in cosmetic, pharmaceutical, paper, textile and leather industries (Shahid et al., 2009). the plant dyes are also responsible for significant plant functions. Colour is an essential factor for the choice of the final product among consumers, especially for the pigments used in food industry (Boo et al., 2012). Among all natural dyes, plant-based pigments provide a huge range of medicinal values (Monika et al., 2013). Plants consist of various antioxidants including tannins, flavonoids, and lignin precursors, which act as radical oxidative stress-scavenging compounds (Paramita & Camelia, 2016). For this reason, in vitro antioxidant activities of the mesocarp and exocarp pigment extracts were determined in order to examine any antioxidant component available in the pigment extracts that might have potential to become a natural colorant in food or non-food system. The aim of the current study is to compare the polyphenolic contents and antioxidant activities of sample extracts with standard commercial antioxidants.

MATERIALS & METHODS

Preparation of Samples

C. nucifera was collected from Tanjung Karang, Selangor. Mesocarps and exocarps of the brown-coloured coconuts were utilised in this study. The exocarp was separated from the mesocarp prior to cutting it into smaller pieces and oven dried at a temperature of 60°C for 24 hours. The samples were ground, sieved using a 0.5 mm sieve and kept in a clean plastic container, away from heat and moisture prior to conducting the experiment.

Methods of Extraction

Microwave-assisted Extraction. Extraction of the samples followed Asma Fhadhila et al. (2016). Microwave assisted extraction was performed in an experimental microwave

oven (Samsung, Korea). About 2 g of mesocarp and exocarp samples from the same batch were transferred into a conical flask containing 40 mL of 0.1 M NaOH (ratio of 1:20), each, and heated at 300 W for 2 minutes. The samples were prepared in triplicates. After heating the mixtures in the microwave, they were placed in conical flasks and were allowed to cool down at room temperature and filtered using a filter paper (150 mm [CHM, Germany]). All the filtrates from both treatments were kept at 4°C in the dark prior to analysis.

Determination of Antioxidant Capacity

Total Phenolic Content (TPC). Total phenolic content was determined according to the method described by Santas et al. (2008). A volume of 200 µL extract was mixed with 1.5 mL of Folin-Ciocalteau reagent (1:10 v/v with distilled water) prior to incubation at room temperature for 5 minutes. Later, 1.5 mL of sodium carbonate (Na_2CO_3, 0.566 M) was added to the sample and mixed thoroughly. The absorbance of the mixture was measured at 725 nm using a spectrophotometer (Genesys 20, USA) after 90 minutes of incubation in the dark. Standard gallic acid within the range of 0.000-0.125 mg/mL was treated similarly as the 200 µL of sample extract. The results were expressed as mg of gallic acid equivalents per gram sample.

2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay. The DPPH free radical scavenging activity was determined according to the method proposed by Brand-William et al. (1995). A volume of 60 µM of DPPH solution in methanol was prepared prior to mixing 3.9 mL of the solution with 0.1 mL extracts. The samples were kept in the dark for 30 minutes at room temperature (27°C), and the absorbance was measured using a spectrophotometer (Genesys 20, US) at 517 nm with methanol as the blank. Standard Trolox with the range of 100–500 µM/mL was treated similarly as 0.1 mL of the sample extract. The results were expressed as mg of Trolox equivalents per gram sample. The DPPH radical scavenging activity was also expressed as the inhibition percentage (IP) of free radical by the sample and was calculated based on the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_0 - A_1}{A_1} \times 100$$

A_0 refers to the absorbance of the control reaction containing all reagents except the tested compound, and A_1 refers to the absorbance of the test compound.

Ferric Ion Reducing Antioxidant Power (FRAP). The FRAP assay was performed according to the method described by Benzie and Strain (1999) with slight modifications. Reagents included 300 mM acetate buffer with pH 3.6, 40 mM hydrochloric acid, 10 mM TPTZ solution (dissolved in 40 mM HCL) and 20 mM ferric chloride solution. The working FRAP reagent was freshly prepared on the day of analysis by mixing acetate buffer (100 mL), TPTZ solution (10 mL) and ferric chloride solutions (10 mL) in the ratio of 10:1:1 before incubation at 37°C. As for the blank, 3.0 mL of working

FRAP reagent was mixed with 100 µL of 0.1 M NaOH (solvent used to extract the sample). An amount of 100 µL sample was mixed with 3.0 mL of working FRAP reagent, and the absorbance at time zero (A_0) and after 4 min (A_4) was recorded using spectrophotometer (Genesys 20, USA) at 593 nm. The calculated differences in the absorbance are proportional to the ferric-reducing properties of the antioxidants present in the extracts. For quantification, a calibration curve of ferrous sulphate was prepared with dilutions from 0.1–1 mM. The final results were expressed as mM Fe^{2+} equivalents per gram sample.

Statistical Analysis

All results in this study were expressed as mean ± standard deviation of three replicates. The statistical significance was evaluated using Student's t-test and set at <0.05. Statistical analysis was conducted using SPSS 12 software package for Windows (SPSS Inc. USA.)

RESULTS & DISCUSSIONS

Total Phenolic Content (TPC)

The results of total phenolic content in the mesocarp and exocarp extracts are presented in Figure 1. The content of phenolic compound was significantly ($p<0.05$) greater in the mesocarp (33.24 mg GAE/g) compared with the exocarp (8.63 mg GAE/g). However, studies by Amin and Chew (2006) and Yapo et al. (2013), showed that the cocoa shells and cocoa pod husk were found to contain extremely higher total phenolic content of 112.9 mg GAE/g and 69.0 mg GAE/g respectively compared with mesocarp and exocarp. In addition, walnut husk extracts contained 32–74 mg GAE/g of total phenolic content (Oliveira et al., 2008). However, the phenolic content of corn husk (Dong et al., 2014) and Thai rice husk (Butsat, and Siriamornpun, 2010) was moderately lower than mesocarp and exocarp with a value of 2.98 mg GAE/g and 2.2 mg GAE/g respectively. Total phenolic content of green tea was in a range of 16.02 to 233.68 mg GAE/g (Shrififar et al., 2003).

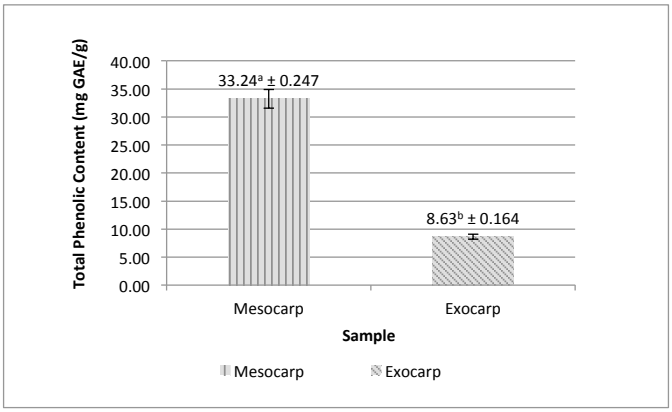

Figure 1. Total phenolic content of mesocarp and exocarp pigment extracts
Note: Values marked with different superscript letters indicate significant differences between mesocarp and exocarp (independent t-test, $p<0.05$)

Chalinee et al. (2016) reported that ethanol and aqueous extracts of coconut (dried fruit) had total phenolic content of 2.21 mg GAE/g and 4.36 mg GAE/g respectively. Pigment extracts of the mesocarp and exocarp have relatively higher values compared with dried fruit coconut. Although the different extraction medium used may contribute to discrepancies between the present result and previous research, the higher total phenolic content of mesocarp and exocarp can be attributed to the extraction method used in this study, which employed microwave-assisted extraction.

Theoretically, microwave radiation loosens the cell wall matrix, causing severed parenchymal cells (Kratchanova et al., 2004). This in turn initiates rapid and extensive opening of the skin tissues, thus leading to improved interaction between the extracting agent and bioactive compound in the extraction procedure. As a result, permeation of the extracting medium solution will be enhanced, leading to effective increase in the yield of bioactive compound being extracted. Improved extraction yields due to microwave heating have also proven for the extraction of flavonoids (Zhang et al., 2013), anthocyanins (Liazid et al., 2011) and phenolic compounds (ballard et al., 2010).

In fact, sodium hydroxide as an extracting medium in this present study may influence the extraction process. As pointed out by Saxena and Raja (2014), many dyes have low water solubility allowing only water-soluble dye components to be extracted, causing low yields of dyes using aqueous extraction. On the other hand, as dyes usually occur in the form of glycosides extractable under an alkaline condition, the alkaline extraction is said to be suitable for dyes having phenolic groups since they are soluble in alkali, thus improving the dye yield. The results are similar to those of Naczk and Shahidi (2006) who reported that the recovery of polyphenols from plant materials is affected by solubility of its phenolic compounds in the solvent used for the extraction procedure. Moreover, solvent polarity contributes to the degree of phenolic solubility.

Phenolic compounds are recognised as antioxidant and scavenging agents against free radicals related to oxidative damage (Fergusion et al., 2006). The oxidation process is one of the most essential routes for producing free radicals in drugs, food and even living systems (Pourmorad et al., 2006). Free radicals cause many human diseases including atherosclerosis, arthritis, Alzheimer's, cardiac reperfusion abnormalities, cancers, neurodegenerative disorders and aging (Sarma et al., 2010). Notably, Yu et al. (2003) pointed out that phenolics have been found to strong antioxidants to hinder the influence of free radicals and reactive oxygen species (ROS), which is the basis of several chronic human infections. Furthermore, there is significant focus in the consumption of certain foods to prevent illness. Thus, diets rich in phenolic compounds can be recommended to improve human health due the effects of phenolic antioxidants (Naczk & Shahidi, 2004).

2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

The antioxidant ability and radical scavenging properties of plants are related to their medicinal properties. In this study, the antioxidant activities of the mesocarp and exocarp pigment extract were measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The DPPH radical is a stable organic nitrogen radical, and the test is quickly and simple which may explain its common use in antioxidant screening (Madhujith & Shahidi, 2006). The mesocarp extract showed significantly ($p<0.05$) higher antioxidant activity (119.96 mM TE/g) compared with the exocarp, which showed 55.27 mM TE/g (Figure 2).

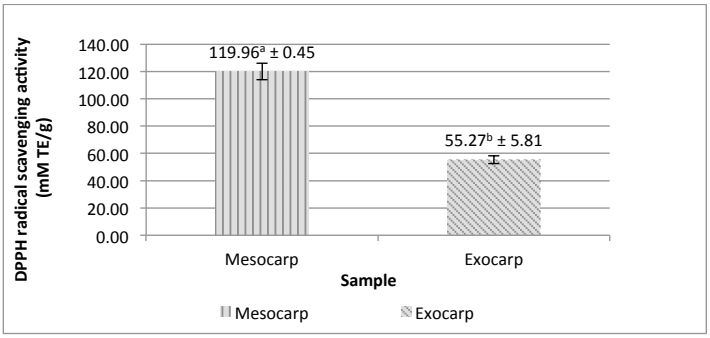

Figure 2. DPPH radical scavenging activity of mesocarp and exocarp pigment extracts
Note: Values marked with different superscript letters indicate significant differences between mesocarp and exocarp (independent t-test, $p<0.05$)

However, the findings from a previous study by Martinez et al. (2012) contradicted with this study. In the earlier study, the DPPH levels of cocoa pod husks and cocoa bean shells were considerably lower than the mesocarp and exocarp with 0.033 mM TE/g for the cocoa pod husk and 0.004 mM TE/g for the cocoa bean shells. High antioxidant activity exhibited in the mesocarp and exocarp may have been due to the existence of colouring pigment in the extract. The presence of colouring pigment in the sample could be due the bioactive compounds such as chlorophyll, carotenoids, and phenolics (Lancaster, 1997).

The antioxidant activities of the mesocarp and exocarp pigment extracts are also expressed and quantified in terms of inhibition percentage (IP). In the present study, the mesocarp extract proved to be a few times less powerful to scavenge DPPH radicals compared with the antioxidant activity of pure standard, Trolox. However, due to the low value of inhibition percentage, results revealed that the exocarp extract was not considered as an effective DPPH radical

scavenger either when compared with Trolox (Figure 3). The higher the amount of antioxidants in the extract, the more the DPPH reduction. High drop of DPPH is connected to the high scavenging activity presented by a particular sample. At a higher concentration, these extracts may display significant free scavenging activities.

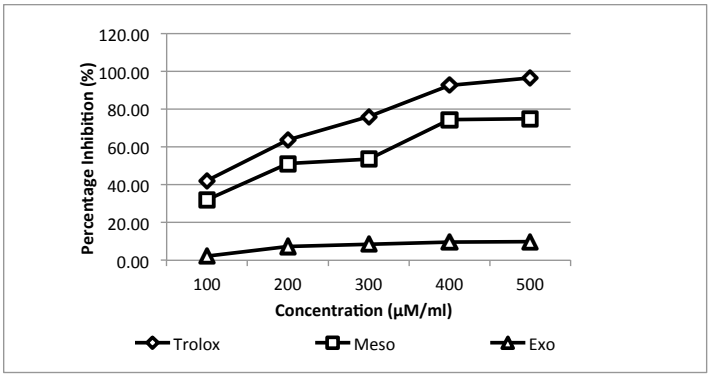

Figure 3. DPPH inhibition of pigment extracts mesocarp and exocarp compared with Trolox

Ferric ion Reducing Antioxidant Power (FRAP)

As shown in Figure 4, the mesocarp pigment extract has significantly ($p<0.05$) higher antioxidant capacity using the FRAP method that exhibited 751.89 mM Fe^{2+}/g compared with the exocarp extract, which showed 264.36 mM Fe^{2+}/g. Dong et al. (2014) reported the FRAP value of cornhusk extracted by 80% ethanol was only 0.002 mM Fe^{2+}/g, whereas the FRAP value of Thai rice husk was in the range of 0.012 to 0.028 mM Fe^{2+}/g(Butsat & Siriamornpun, 2010). On the other hand, Xiang et al. (2016) had isolated compound from Chinese hickory husks and the findings pointed to antioxidant activity in the FRAP assay with moderate values of 10.34–10.91 mM $FeSO^4$/g. Results of the current study indicated that the values of the mesocarp and exocarp pigment extracts were higher than FRAP values of the other types of husk. In addition, these also show that the husk from coconut provides significant antioxidant activity. This means that the husk can no longer be regarded as a worthless part of the coconut. The high antioxidant activities of the mesocarp and exocarp pigment extracts suggest their use in folk medicine.

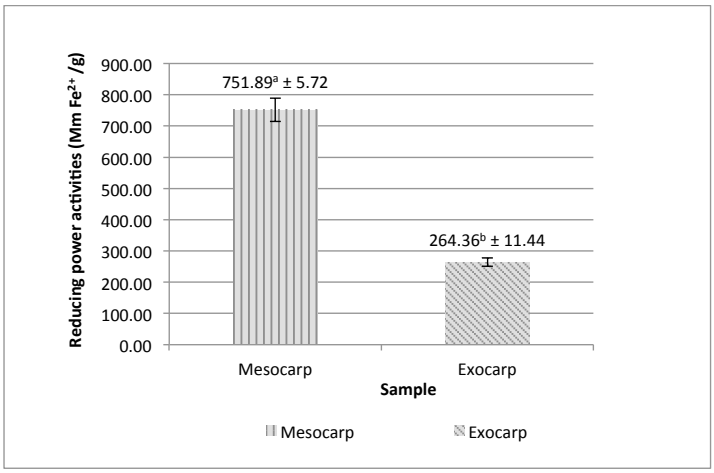

Figure 4. Reducing power activity of mesocarp and exocarp pigment extracts
Note: Values marked with different superscript letters indicate significant differences between mesocarp and exocarp (independent t-test, $p<0.05$)

Generally, the reducing properties are related to the existence of compounds, which exert their action by breaking the free radical chain through donating a hydrogen atom. The FRAP assay determines the change in absorbance at 593 nm due to the formation of a blue-coloured complex of ferrous ion (Fe^{2+}) and 2,4,6-tripyridyl-s-triazine, (TPTZ). In addition, a colourless ferric ion (Fe^{3+}) gets oxidised to ferrous ion (Fe^{2+}) by the action of electron-donating antioxidant (Sumitra et al., 2013). The formation of blue colour evaluated spectrophotometrically at 593 nm is taken as linearly connected to the total reducing capacity of electron-donating antioxidants (Mohd et al., 2013).

The different values among antioxidant assays were attributed to the different chemistry principle as the basis of each method. The DPPH and FRAP methods are based on a single electron transfer (SET) reaction in which antioxidants are oxidised by oxidants, namely metal (Fe III) or a radical (DPPH). As a result, a single electron is transferred from the antioxidant molecule to the oxidant (Mohd et al., 2013). This study pointed to the ability of the mesocarp and exocarp pigment extracts either to quench or to reduce the radicals generated in the assays.

CONCLUSION

This study examined the antioxidant utility of coconut husk, which might be useful in establishing its therapeutic values. The mesocarp and exocarp extract from the husk of coconut showed antioxidant efficacy in all three analyses, namely TPC, DPPH, and FRAP. Thus, coconut husk can be an effective antioxidant despite it being commonly viewed as waste. The study suggests that this extract is a possible source of natural antioxidant that could be of great importance to counter age-associated illness and free radical-related disease.

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Effect of Treatment Methods on the Nutritive Quality of Elephant-Ear Seeds (*Enterolobium Cyclocarpum* Jacq Griseb) as Feed for Ruminant Production

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ABSTRACT

The study was carried out to evaluate the nutritive quality of *Enterolobium cyclocarpum* seeds. Matured pods of *E. cyclocarpum* were handpicked and seeds were manually separated. Seeds were either toasted, boiled or untreated (raw). The experiment was laid out in a completely randomized design consisting of three treatment groups replicated four times with a total seed of 0.5 kg per replicate. The result of the chemical analysis showed that there were significant differences ($P<0.05$) for all the parameters investigated. The crude protein content was significantly highest ($P<0.05$) (24.9%) in boiled *E. cyclocarpum* seeds and least (22.4%) in the raw seeds. Untreated (raw) seeds recorded highest ($P<0.05$) contents for all the secondary metabolites (saponin, tannin, oxalate and trypsin) investigated in this study while boiled seeds recorded lowest. Treatment methods had a significant ($P<0.05$) effect on the *in vitro* gas production of *E. cyclocarpum* seeds with the boiled seeds having highest gas production (72 ml/200 mg DM at 48 hours of incubation). The study showed that boiling of *E. cyclocarpum* seeds improved its chemical composition and gas production, suggesting that moist heat treatment is preferable when making use of seeds in ruminant diets.

Keywords: Anti-nutritional factors, chemical composition, *Enterolobium cyclocarpum* seeds, gas production, processing

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INTRODUCTION

Ruminants face problems on the availability of high quality feeds throughout the year

and negatively affects their performance and productivity. Supplementation of concentrates in the diet of animals can achieve high productivity. However, conventional seed sources such as groundnuts and soybean are scarce and expensive. There is a need to find alternative sources of protein to enhance the nutritional value of poor quality roughage feed provided to ruminants in the dry season, which is both cheap and easily available (Sarnklong et al., 2010).

Forage seeds have been reported to be high in crude protein and hold promise as a feed for ruminants (Babayemi & Bamikole, 2006). Recent trials revealed that some of the indigenous multipurpose tree species especially *E. cyclocarpum* are evergreen and produce seeds that have a very high protein content (Arigbede et al., 2008). The tree is a legume tree which belongs to the family Mimosadeae (Idowu et al., 2013) and has been used in intensive feed garden. As a leguminous multipurpose plant, it has the potential of fixing atmospheric nitrogen into the soil and can also be exploited for feeding ruminants. Studies have shown that the seeds produce higher volatile fatty acids on degradation by rumen microbial organisms (Babayemi et al., 2004). Arigbede et al. (2008) further reported that these seeds are edible and contain substantial nutrients to support high productivity in animals. Although, the seeds are very rich in protein, they are however, constrained by high content of anti-nutritional factors, arising from secondary metabolism in plants.

Anti-nutritional factors can become detrimental to animals if their concentration is high, lowering feed intake and reducing rumen microbial activity and growth (Soetan & Oyewole, 2009). Hence, there is need to reduce or minimise the effects of these compounds in animal feed materials by treating the seeds. Most of the toxic and anti-nutrient effects of phytochemicals compounds in plants could be reduced by several processing methods such as soaking, germination, boiling, autoclaving, fermentation, genetic manipulation and other processing methods (Soetan, 2008). *In vitro* gas production is inexpensive and a not time-consuming method for evaluating nutritive value of feeds for ruminants (Makkar, 2002). Although gas production is a nutritional wasteful product, it provides a useful basis from which metabolizable energy, organic matter digestibility and short chain fatty acids may be predicted (Oloche et al., 2013).

Browse plants seeds are very rich in nutrients, but there is insufficient information on their nutritive value and utilization for ruminants following processing. The objective of this research is to determine the effect of processing *E. cyclocarpum* seeds on their nutritional quality and use in ruminants' diet.

MATERIALS AND METHODS

Experimental Site

The experiment was carried out at the College of Animal Science and Livestock

Production farm, Federal University of Agriculture, Abeokuta, Nigeria. The area lies within the savannah agro-ecological zone of Western Nigeria (Latitude 7° 13 49.46 N, longitude 3° 26 11.98 E, average annual rainfall of 1037 mm). Temperatures are fairly uniform with daytime values of 28– 30°C during the rainy season and 30–34 oC during the dry season with the lowest night temperature of around 24°C during the harmattan period between December and February.

Sourcing, Collection and Processing of Test Ingredients

Matured pods of *E. cyclocarpum* which were planted in the multi-purpose trees garden of the research farm in 2004 were handpicked during the dry season of 2015, after falling off from the tree stands. The collected pods were sun-cured for three days and dehulled to obtain the seeds. The seeds were then divided into three parts and treated by boiling 500 g of seeds in 1 litre of water on hot plate (Stuart, heat stir, CB162, United Kingdom) with four replicates (100°C for 60 minutes), toasting of 500 g seeds per replicates on hot plate (Stuart, heat stir, CB162, United Kingdom) (170°C for 15 minutes) (Ogunsakin, 2014) and untreated (raw seeds) (control). In the boiling method, water was used as the heat medium and toasted seeds were directly heated.

Experimental Design

The experiment was laid out in a completely randomized design consisting of three treatment groups replicated four times with a total seed of 0.5 kg per replicate.

Chemical Composition Analyses

Samples of different treatments of *E. cyclocarpum* seeds were oven dried at 65°C until constant weight was obtained and ground to pass through 1 mm sieve using laboratory hammer mill (Model DFZH-Bühler, Uzwil, Switzerland) and analysed for proximate composition (dry matter (DM), crude protein (CP), ether extract (EE) and ash contents) according to the method of AOAC (2010). Fibre fractions analysis (acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL)) were determined as described by Van Soest et al. (1991). Calcium and Phosphorus contents were determined by atomic absorption spectrophotometry (Fritz & Schenk, 1979). Anti-nutritional factors such as tannins was determined according to the procedures of Jaffe (2003), saponin was determined according to the procedures of Obdoni and Ochuko (2001), oxalate according to Munro (2000) while trypsin was determined as describe by Prokopet and Unlenbruck (2002) cited by Steve and Babatvnde (2013). The *in vitro* gas production was determined following the procedure of Menke and Steingass (1988). Upon recording the final gas volume at the end of incubation (48 h), the lower end of the syringe was connected to the lower end of a pipette containing 4.0 ml of NaOH (10 M). The NaOH (10M) was then introduced from the latter into the incubated contents,

thereby avoiding gas escape. Mixing of the contents with the NaOH solution allowed for the absorption of CO_2, with the gas volume remaining in the syringe considered to be CH_4 (Anele et al., 2011).

Organic matter digestibility (OMD) were estimated as OMD= 14.88 + 0.889 GV+ 0.45 CP + 0.651 ash (Menke and Steingass, 1988). Short-chain fatty acids (SCFA) were estimated as SCFA = 0.0239 GV−0.0601 (Getachew et al., 2000). Metabolizable energy (ME) were calculated as ME= 2.20 + 0.1357 GV+ 0.0057 CP + 0.0002859 EE2 (Menke and Steingass, 1988). Total gas volume (GV) was expressed as ml/200mgDM, CP and ash as percentage DM, ME as MJ/kgDM and SCFA as µmol/g DM.

Statistical Analysis

The variance of the data was analysed and significant treatment means separated using Duncan's Multiple Range Test using SAS (2009) Package.

RESULTS AND DISCUSSION

Chemical composition of different treatments of *E. cyclocarpum* seeds are presented in Table 1. Treatment methods significantly (P<0.05) influenced the chemical composition of *E. cyclocarpum* seeds. Boiled seeds had the highest value of 94.0% for dry matter content while least value of 86.0% was for raw seeds. The crude protein content was highest (P<0.05)

Table 1
Effect of treatment methods on the chemical composition of Enterolobium cyclocarpum seeds

Parameters	Untreated (raw)	Boiled	Toasted	SEM
Dry matter (%)	86.0^{c}	94.0^{a}	92.0^{b}	1.24
Crude protein (%)	22.4^{c}	24.9^{a}	22.8^{b}	0.40
Ash (%)	4.5^{a}	3.9^{c}	4.2^{b}	0.10
Ether extracts (%)	15.0a	10.3^{c}	11.7^{b}	0.77
Neutral detergent fibre (%)	33.0^{c}	41.0^{a}	34.0^{b}	1.36
Acid detergent fibre (%)	12.0^{a}	9.0^{c}	11.0^{b}	0.67
Acid detergent lignin (%)	6.0^{a}	3.0^{c}	4.0^{b}	0.51
Calcium (g kg^{-1})	6.6^{b}	6.6^{b}	8.6^{a}	0.33
Phosphorus (g kg^{-1})	6.0^{b}	7.2^{a}	5.8^{b}	0.28
Saponin (%)	3.1^{a}	2.0^{c}	2.5^{b}	0.17
Tannin (mg kg^{-1})	3.2^{a}	2.0^{c}	3.0^{b}	0.18
Oxalate (mg kg^{-1})	12.8^{a}	10.6^{c}	12.1^{b}	0.33
Trypsin (g kg^{-1})	0.4^{a}	0.1^{c}	0.2^{b}	0.04

$^{a, b, c, d}$: Means within the same row with different superscripts are significantly different (p<0.05) according to Duncan Multiple Range Test
SEM = Standard Error of Mean

(24.9%) in boiled and least (22.4%) in raw seeds. The highest CP content in boiled seeds in this study could have been due to reduction in anti-nutritional factors as reported by Deshpande et al. (2000). The crude protein content of *E. cyclocarpum* seeds in this study was higher than the value reported by Babayemi (2006) for some seeds. The variation could be due to varying times when seeds were collected and their handling. The range of CP of seeds in this study would provide adequate nitrogen requirement by rumen microorganism to maximally digest the main components of dietary fibre leading to the production of volatile fatty acid (Lamidi & Ogunkunle, 2016).

The values obtained for neutral detergent fibre of processed seeds in this study ranged from 33.0 to 41.0% in raw and boiled seeds respectively while acid detergent fibre ranged from 9.0 to 12.0% in boiled and untreated (raw) seeds respectively. Neutral detergent fibre contents of *E. cyclocarpum* seeds in this study followed the same trend as recorded for the crude protein contents. The appreciable NDF value in boiled seeds (41.0%) from this study implies higher dry matter intake for animals (Bamikole et al., 2004). Acid detergent lignin content was significantly ($P<0.05$) highest (6.0%) in untreated (raw) seeds compared with other treatment methods. Lower ADL in the treated seeds in this study could have been due to the effects of heat treatment according to report of Singh and Harvey (2009).

Calcium content of the processed seeds was highest (8.6 g kg^{-1}) in toasted seeds while the contents in the raw and boiled seeds were similar. The contents of phosphorus were highest (7.2 g kg^{-1}) in boiled and lowest (5.8 g kg^{-1}) in toasted seeds. This observation is similar to the work of Sotolu & Faturoti (2008) where higher mineral contents in *Leucaena leucocephala* seeds that were either toasted or put in hot water was reported. The higher mineral contents of treated seeds in this study seems not explainable as one can only speculate on the probable cause. The NRC (2001) recommends that calcium and phosphorus rates should be at least 6.5 g kg^{-1} and 4.0 g kg^{-1} respectively, of the total ration DM for productive cows. Indeed, for the seed processing method in this study, calcium and phosphorus were found to be higher than recommended values.

Values for all secondary metabolites (saponin, tannin, oxalate and trypsin) observed in this study followed a similar trend with the untreated (raw) *E. cyclocarpum* seeds recording the highest contents while the boiled seeds scored the lowest. Treated seeds proved to reduce the secondary metabolites of *E. cyclocarpum*. In this study, boiled seeds gave the least tannin, saponin, oxalate and trypsin contents. This may be attributed to moist heat compared to toasting which is dry heat application. Treatment of seeds, toasting and boiling, have been reported to decrease the anti-nutritional factors of browse seeds (Wiryawan, 1997).

The chemical composition of the treated seeds revealed that they have the potential to fill the gap in ruminant nutrition, especially crude protein content particularly in the

dry season. Supplementing browse plants seeds with grasses could solve the problem of feeding ruminant livestock during this season.

Table 2 presents the effects of treatment on the *in vitro* gas production of *E. cyclocarpum* seeds. Treatment methods had significant (P<0.05) effect on the *in vitro* gas production of *E. cyclocarpum* seeds. In all the treatments, the volume of gas produced consistently increased (P<0.05) from the beginning of 3 hours with increasing hour of the *in vitro* gas incubation. Boiled seeds recorded the highest value while toasted recorded the least for all the incubation periods. Gas production basically results in the fermentation of carbohydrate into volatile fatty acids (Getachew et al., 1999). The amount of gas released when feeds are incubated *in vitro* has been reported to be closely related to digestibility of feed for ruminants (Mebrahtu & Tenaye, 1997). Therefore, the highest gas production observed for boiled seeds suggests a higher digestibility of the seeds than the other treatments. This could be a reflection of a higher proportion of carbohydrate available for fermentation. The high gas production in the present study for all the seeds incubated could be the result of high crude protein contents (Babayemi et al., 2009). The higher CP and other nutrients content in the seeds are essential for growth of rumen micro-organisms that degrade feedstuff prior to gastric and intestinal digestion by the host animal (Reed et al., 1990). Moreover, gas production has been reported to be positively related to microbial protein synthesis (Hillman et al., 1993). This is nutritionally significant to the ruminants because this serve as an indicator to how dry matter is being degraded in the rumen of ruminants.

Table 2
Effect of treatment methods on the in vitro gas production (ml/200 mg DM) of Enterolobium cyclocarpum seeds

Treatments	Incubation period (hours)					
	3	6	12	24	36	48
Untreated (raw)	14.0^{a}	24.0^{b}	38.0^{b}	46.0^{b}	50.0^{b}	50.0^{b}
Boiled	16.0^{a}	30.0^{a}	54.0^{a}	70.0^{a}	70.0^{a}	72.0^{a}
Toasted	6.0^{b}	14.0^{c}	24.0^{c}	34.0^{c}	34.0^{c}	42.0^{c}
SEM	1.63	2.40	4.37	5.32	5.24	4.63

$^{a, b, c....}$ Means within the same column with different superscripts are significantly different (P<0.05) according to Duncan Multiple Range Test
SEM = Standard Error of Mean

Table 3 shows the effects of treatment on the post incubation parameters of *E. cyclocarpum seeds*. The parameters investigated followed similar trend in all the processed seeds. Boiled seeds recorded significantly (P<0.05) highest values for

metabolizable energy (ME), organic matter digestibility (OMD) and short chain fatty acid (SCFA), followed by raw seeds and the least values were in toasted seeds. The SCFA value is an indication that the energy content in feeds will be readily utilized after digestion. The high metabolizable energy, organic matter digestibility and short chain fatty acid that were reported in this study for the differently processed *E. cyclocarpum* seeds, could translate to higher dry matter intake in ruminants for an improved performance.

Table 3
Effect of treatment methods on the post incubation parameters of Enterolobium cyclocarpum seeds

Treatments	Organic matter digestibility (%)	Metabolizable energy (MJ/kgDM)	Short chain fatty acid (µmol/200 mg DM)
Untreated (raw)	68.8[b]	8.6[b]	1.0[b]
Boiled	81.9[a]	10.5[a]	1.4[a]
Toasted	58.1[c]	7.0[c]	0.8[c]
SEM	5.1	1.5	0.1

[a, b, c....] Means within the same column with different superscripts are significantly different (P<0.05) according to Duncan Multiple Range Test
SEM = Standard Error of Mean

The amount of methane (CH_4) in the present study ranged from 24 µmL/200 mg DM in raw to 29 µmL/200 mg DM in boiled *E. cyclocarpum* seeds (Figure 1). Highest anti-nutritional factors in the raw *E. cyclocarpum* seeds could have been responsible for its lowest methane production especially for saponin which had the potential to slightly depress methanogenesis because of its capacity to suppress protozoa, the main butyrate producers in the rumen (Babayemi et al., 2004). Hess et al. (2004) also reported that tannin suppressed methane production from *Calliandra calothyrsu*s, a tropical legume.

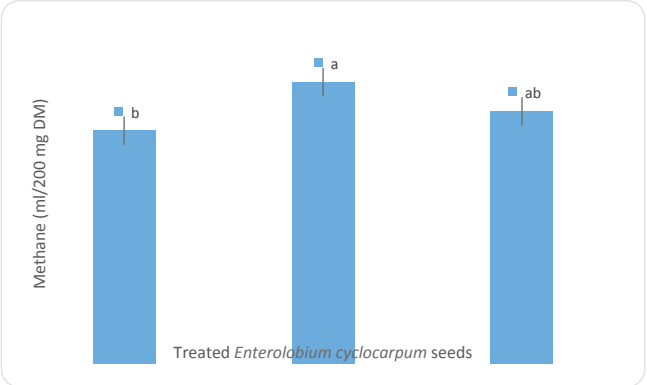

Figure 1. Effects of treatment methods on the methane (ml/200 mg DM) production of *Enterolobium cyclocarpum* seeds following incubation period of 48 h

CONCLUSION

This study found that treating *E. cyclocarpum* seeds by boiling improved its chemical composition and *in vitro* gas production, thus suggesting moist heat treatment of the seeds if they are to be incorporated into animal feeds. Livestock farmers are therefore advised to propagate the tree, since the seeds are rich in nutrients.

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Altitudinal Diversity of Braconid Wasps (Hymenoptera: Braconidae) at Fraser's Hill, Pahang, Malaysia

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ABSTRACT

A diversity study of the subfamilies of Braconidae was conducted at Fraser's Hill, Pahang, Malaysia. Sampling took place at three different altitudes using Malaise traps: lower altitude (<500m), intermediate altitude (501-1000m) and higher altitude (>1000m). A total of 572 individuals of braconids were collected from the three altitudes and comprised 15 subfamilies: Agathidinae, Alysiinae, Blacinae, Braconinae, Cheloninae, Doryctinae, Euphorinae, Helconinae, Lysiterminae, Microgastrinae, Miracinae, Opiinae, Orgilinae, Pambolinae and Rogadinae. There were 435 individuals and 55 species, 84 individuals and 30 species, and 53 individuals and 26 species, with a diversity index of H'= 3.75, 2.91 and 3.01, representing the lower, intermediate and higher altitudes, respectively. The diversity index of the lower altitude (H'=3.75) was significantly different from the intermediate ($p=0.00$, <0.05) and higher altitudes ($p=0.00$, <0.05). There was no significant difference between the intermediate and higher altitudes ($p=0.86$, >0.05). This is probably due to the variability of host and food availability i in lowlands compared with highlands.

Keywords: Altitude, braconid, diversity, abundance

INTRODUCTION

Braconid wasps (Hymenoptera: Braconidae) is a large group of insects with more than 15,000 species distributed throughout the world (Hanson & Gauld, 2006; Quicke, 2014) and occupying different ecological habitats (LaSalle & Gauld, 1993; Shaw & Huddleston, 1991). Braconids are insects that have been proven to be of economic

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and ecological interest (Billah et al., 2008; Whitfield, 2003). Most braconid species have the potential to be used as biological control agents due to their nature of parasitizing other insects (Gillott, 2005; Kimani-Njogu et al., 2001). They tend to have a specific range of hosts specialized by biological and behavioural adaptation (Janzen et al., 2003). The most common hosts for braconids are the larvae of Lepidoptera, Coleoptera and Diptera (Tripplehorn & Johnson, 2005). Braconids are also very sensitive to environmental disturbances which makes them good indicators of diversity and environmental stability (Shaw & Hochberg, 2001). However, in Malaysia, studies on this subfamily is remains undeveloped (Yaakop & Aman, 2013; Yaakop et al., 2009).

Insects usually occupy different niches in a certain habitat and play a functional role in maintaining the dynamics of the ecosystem (Goldstein, 1999). According to Rohner et al. (2015), insect abundance and diversity are affected by both altitude and latitude. For parasitoids, the geographic distribution depends on the plant communities and on their faunal history (Wiens & Donoghue, 2004). This is because of the difference in environmental conditions in lowlands and highlands (Lomolino, 2001).

Abiotic (e.g., temperature, humidity, etc.) and biotic (e.g., competition, migration, etc.) factors can affect the distribution of any species (Eitam et al., 2004). Temperature is a crucial factor that limits distribution especially when altitude becomes a physical factor (Logan, 2001; Parmesan, 2006). Furthermore, wasp diversity is also closely influenced by altitude and latitude (Tan et al., 1990; van der Ent & Shaw, 1998), such that if any change in these factors may affect their diversity. Vegetation covers are strongly associated with insect accumulation, thus attracting various host insects to the wasps (Campan & Benrey, 2004; Sperber et al., 2004). The abilities of parasitoids differ in their surviving period based on the availability of hosts (Eitam et al., 2004). Fraser's Hill is one of the areas known for ecotourism, but there are some activities that can affect the biodiversity of insects. The objective of this study is to compare and measure the differences in braconid diversity and abundance between different altitudes at Fraser's Hill.

METHODS

Study Site

Sampling was conducted at Fraser's Hill in the state of Pahang, Peninsular Malaysia. A few areas have been developed into tourism destinations, but most of the hill forests are still protected and conserved. Three altitudes were selected for this study: lower (<500m), intermediate (501-1000m) and higher (>1000m). The lower altitude is composed of lowland dipterocarp forest, intermediate altitude of hill dipterocarp forest, and higher altitude of montane forest (WWF, 2001).

Braconid Collection

Sampling was carried out by using Malaise traps, which are known for the best ways to capture flying insects passively (Mazon & Bordera, 2008). During the sampling, a total of nine Malaise traps were set up and placed at three different altitudes faced towards the sunlight. For each selected altitude three traps were set up for 30 days before the samples were collected. Sampled insects were then kept in 70% ethanol for further identification in the laboratory.

Species Identification

Identification of braconids was carried out using morphological characters based on taxonomic keys by Achterberg up to subfamily level and to genus and species where possible (Achterberg, 1993; Li et al., 2012; Li et al., 2013). The identification process utilized a stereo microscope, and a photograph of each specimen was taken with a Canon EOS 1000D attached to the stereo microscope (Carl Zeiss Stemi DV4).

Data Analysis

Samples from different altitudes were analysed using PAST software to compare the diversity indices. Diversity Indices viz. Shannon diversity index (H'), Evenness (E) and Richness (R') were analysed to measure the braconids' diversity and species richness at each altitude. The diversity of braconids at the three altitudes was then compared using t-test with p=0.05. Two-way clustering was conducted using computer software PCORD 6 to observe the similarities and differences between the species and altitudes.

RESULTS

A total of 572 individuals were successfully collected throughout the sampling period. They represented 15 subfamilies from all altitudes: Agathidinae, Alysiinae, Blacinae, Braconinae, Cheloninae, Doryctinae, Euphorinae, Helconinae, Lysiterminae, Microgastrinae, Miracinae, Opiinae, Orgilinae, Pambolinae and Rogadinae (Table 1). The lower altitude (<500m) showed the highest number with 435 individuals and 55 species, followed by the intermediate altitude (501-1000m) with 84 individuals and 30 species. The higher altitude (>1000m) showed the lowest number with 53 individuals and 26 species (Table 1). The 15 subfamilies that were found at Fraser's Hill represent 32% of the 46 subfamilies recorded all over the world. Seven of the subfamilies were found in all three altitudes: Alysiinae, Braconinae, Cheloninae, Doryctinae, Microgastrinae, Opiinae and Orgilinae.

Table 1
Subfamilies and genera collected in three different altitudes in Fraser's Hill, Pahang, Malaysia

Subfamily	Genus	Altitude		
		<500m	501-1000m	>1000m
Agathidinae	*Aneurobracon* sp.	0	5	0
	Therophilus sp.	0	0	7
Alysiinae	*Dinotrema* sp.	0	5	1
	Dacnusa sp.	5	0	0
	Heratemis sp.1	4	2	0
	Heratemis malayensis	0	0	1
Blacinae	*Blacus* sp.1	1	1	0
	Blacus sp.2	3	3	0
	Blacus sp.3	6	2	0
Braconinae	*Bracon* sp.1	3	0	1
	Bracon sp.2	5	1	0
	Bracon sp.3	23	0	0
	Braconinae sp.	5	0	1
	Gammabracon sp.	0	1	0
	Habrobracon sp.	10	0	1
	Vipio sp.	6	0	1
Cheloninae	*Ascogaster* sp.	12	1	1
	Chelonus sp.	10	1	0
	Phaneretoma sp.	7	1	0
Doryctinae	*Rhoptrocentrus* sp.	6	0	1
	Spathius sp.1	7	0	1
	Spathius sp.2	10	0	0
	Spathius sp.3	8	0	0
	Spathius sp.4	3	1	0
	Spathius sp.5	9	0	0
Euphorinae	*Centistes* sp.	1	1	0
	*Peristenu*s sp.	8	0	0
Helconinae	*Triaspis* sp.	5	2	0
Lysitermiinae	*Acanthormius* sp.	6	0	0
Microgastrinae	*Alloplitis* sp.	23	4	0
	Apanteles sp.1	2	21	5
	Apanteles sp.2	16	4	2
	Choeras sp.	4	4	4
	Cotesia sp.1	18	1	2
	Cotesia sp.2	4	0	1
	Diolcogaster sp.1	9	1	0
	Diolcogaster sp.2	21	0	0
	Fornicia sp.	7	0	2

Table 1 *(continue)*

Subfamily	Genus	Altitude		
		<500m	501-1000m	>1000m
	Glyptapanteles sp.	7	1	1
	Microgaster sp.	9	0	2
	Parapanteles sp.	39	4	5
	Pholetesor sp.	7	1	1
	Wilkinsonellus sp.	15	7	3
Miracinae	*Centistidae* sp.	5	0	1
Opiinae	*Apodesmia* sp1	8	0	0
	Biosteres sp.	0	1	2
	Bitomoides sp.	7	2	0
	Opiinae sp.1	7	0	0
	Opiinae sp.2	6	0	0
	Opiinae sp.3	9	0	0
	Opius sp.1	5	0	0
	Opius sp.2	9	0	0
	Opius sp.3	4	0	0
	Orientopius sp.	4	2	4
	Psyttalia sp.	7	1	0
Orgilinae	*Orgilus* sp.	5	1	0
	Stantonia sp.	3	0	1
Pambolinae	*Pambolus* sp.	0	0	1
Rogadinae	*Aleiodes* sp.	1	0	0
	Canalirogas sp.	6	2	0
	Rogas sp.1	1	0	0
	Rogas sp.2	4	0	0
	Total	435	84	53

Table 2
Number of individuals, species and diversity indexes sampled from Fraser's Hill, Pahang, Malaysia. Small letter a and b indicates significant difference at p = 0.05

	<500m	501-1000m	>1000m
No. of Individuals	435	84	53
No. of genus	55	30	26
Shannon (H')	3.75[a]	2.91[b]	3.01[b]
Richness (R')	8.89	6.55	6.30
Evenness (E)	0.77	0.61	0.78

This study shows that Microgastrinae has the highest abundance in both species (14) and individuals (257), and can be found in all altitudes. The most dominant genus is also from the same subfamily, *Apantales,* with 50 individuals. In terms of the Shannon diversity index, the lower altitude has the highest H' of 3.75, compared to the intermediate ($p=0.00, < 0.05$) and higher ($p=0.00, <0.05$) altitudes, while there is no significant difference between the intermediate and higher altitudes ($p=0.86, >0.05$) (Table 2).

Based on the two-way cluster analysis, there are three groups that represent more than 75% similarities (Group 1, 2, 3) (Figure 1). Group 1 has the highest number of species (50) which are mostly found in the lower altitude even though some of them can also be accommodated in intermediate and higher altitudes. Group 2 has three species, *Aneurobracon* sp.1, *Gammabracon* sp.1 and *Dinotrema* sp.1 which appear in the intermediate altitude. *Dinotrema* sp.1 also occurs in the higher altitude but at weak probability. Meanwhile, Group 3 has four species, *Biosteres* sp.1, *Heratemis malayensis*, *Pambolus* sp.1 and *Therophilus* sp.1, concentrated in the higher altitude, but there is one, *Biosteres* sp.1, that was also found in the intermediate altitude.

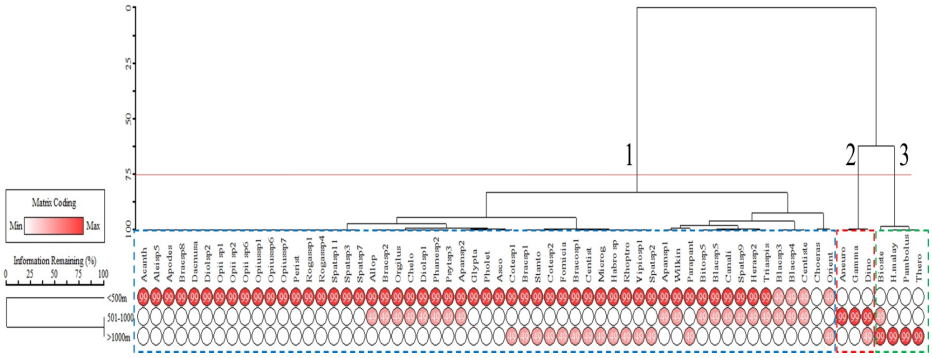

Figure 1. Two-way cluster analysis (dendrogram) of the braconid species collected from different altitudes in Fraser's Hill

DISCUSSION

In this study, 15 braconid subfamilies were found in Fraser's Hill, Malaysia based on Yu et al. (2012). Previous studies from Sivinski et al. (2000) in Mexico, Fernandez-Triana et al. (2013) in New Zealand and Fernandez-Triana et al. (2016) in Costa Rica also discovered some subfamilies in highland areas, such as Alysiinae, Doryctinae, Microgastrinae, and Opiinae, that were similar to this study. A few species from subfamily Opiinae, such as *Fopius arisanus*, are thought to be limited in low temperatures (Snowball & Lukins, 1964), while subfamily *Doryctinae Doryctobracon* crawfordi is sensitive to higher temperatures

than the host, *Anastrepha ludens* (Sivinski et al., 2000). However, there is no solid proof to support that subfamilies Alysiinae, Doryctinae, Microgastrinae, and Opiinae found in this study have been recorded in highlands, because most of the previous studies found them only in lowlands (Abu El-Ghiet et al., 2014; Gadelha et al., 2012; Jimenez-Peydro & Peris Felipo, 2011). This may be a new record for diversity of braconid wasps in highlands in Malaysia, especially for Fraser's Hill.

For overall diversity, the lower altitude (<500m) showed the highest number of braconid individuals compared to both intermediate and higher altitudes (>501m). The lower altitude is said to have denser and more varied vegetation (Adam et al., 2011; Barbieri Junior & Dias, 2012) and generally more abundant insect species groups (Lessard et al., 2011; Nufio et al., 2010; Sanders & Rahbek, 2012). In addition, the altitudinal study of Janzen et al. (1976) shows that species richness of parasitic Hymenoptera was high at 200m is congruent with our study in lower altitudes below <500m is because in most habitats, plant communities determine the physical structure of the environment and hence the distribution and species interactions (Saaksjarvi et al., 2004). The number of small plants such as shrubs and herbs declines as altitude increases, which is another reason lower altitudes has greater diversity of host plants and wasps (Khairiyah et al., 2013).

Higher altitude tends to have more adaptive and specific plants compared to the lower altitude. According to this study, *Apanteles* sp. Were found in all three altitudes. This may be due to its high adaptive character. Besides limited plant variation at higher altitudes, and the construction of tourist conveniences contribute to less braconid abundance and diversity compared to lower ground. Less adaptive braconid species will not survive at higher altitudes (Shaw & Hochberg, 2001), leaving the area less sensitive and adaptive groups. Environmental changes will diminish the host population negatively affecting the braconid population as the habitat area becomes undersized and more disturbed (Bobo et al., 2006; Malmivaara et al., 2002; Roy et al., 2001).

An urbanized forest area that experiences destruction will lead to habitat and species loss (Blair & Launer, 1997; Brown et al., 1996). Poor adaptive species are fragile and cannot survive such disturbances, which will be forced to either leave for other favourable areas, or die (Lien, 2013). The environment at higher altitudes are also influenced by weather conditions and can play an important role in soil characteristics, the composition of plants, population structure and invertebrate composition (Willig et al., 2011). In addition, high altitude environments have low productivity rates (Mani, 2013; Stevens, 1989). Environmental changes can shrink the host population and negatively affect the wasps' population (Idris & Hasmawati, 2002).

Microgastrinae are known for its vast diversity and distribution besides being the most conspicuous group attacking

caterpillars. As exclusive caterpillar parasitoids, the Microgastrinae are one of the most economically important natural enemies of Lepidoptera. This is proved by the utilization of more than 100 species of Microgastrinae in the biological control of lepidopteran pests in industries (Whitfield, 1997). According to Kahuthia-Gathu (2013), the genera *Apanteles* are found to be the common parasitoid for the larvae of *Plutella xylostella*, diamondback moth, the most destructive insect of cruciferous plants throughout the world (Talekar & Shelton, 1993). In Malaysia, these caterpillars are non-native pests of cruciferous crops that were frequently found to be parasitized by a braconid wasp in the genus *Apanteles* (Furlong et al., 2013). Cruciferous plants are economically important being the most common diet in various cultures (Shelton, 2001). The genera *Apanteles* is one of the dominant species with the highest parasitism recorded in reducing the *P. xylostella* population. Besides that, *Heratemis malayensis* has been discovered in highland altitudes corresponding to the taxonomic study by Yaakop et al., (2009) who found the species in the highlands of Bukit Larut, Perak, Malaysia.

Based on the two-way cluster analysis, Group 1 is mostly concentrated at lower altitudes. This is due to the availability of food, temperature and humidity conditions, found in the lowlands (Sanders & Rahbek, 2012). Their numbers could be higher during the rainy season as young new leaves grow, and provide more food for insects (Barbieri Junior & Dias, 2012; Young, 2012). Compared with the lowlands, Groups 2 and 3 are more likely to have a similar habitat which increases in altitude. Species of insects are less rich as the altitude increases because higher altitudes have lower habitat niches, limitation in host plants, and deficiency of food sources (Lomolino, 2001; McCain & Grytnes, 2010). In addition, the good flying ability of braconids makes them capable of finding a suitable habitat for their development (Araujo et al., 2004). From our study, although we can still find a few species of braconid in the higher altitudes, their diversity and abundance tell us that the lowlands are a preferable habitat for the braconid.

CONCLUSION

The braconid wasp's diversity and population were higher in lower altitude mainly because of the availability of food sources. The presence of hosts directly invite the wasps to parasitize and continue their life cycle. Microgastrinae is the most abundant subfamily as the species is widely distributed. As the altitude increases wasp diversity decreases due to less productive vegetation.

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Short Communication

Seroprevalence of *Neospora Caninum* in Sheep and Goats of Gua Musang District in Kelantan, Malaysia

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ABSTRACT

Exposure of *Neospora caninum* parasite in sheep and goats in Kelantan was first reported in March 2016. Out of 10 districts surveyed in Kelantan, Gua Musang was the only district with seropositive animals, suggesting that there might be potential infection of this parasite in sheep and goats in this area. Therefore, a cross-sectional study was conducted from May 2015 to February 2016 to investigate the prevalence and impact of *N. caninum* in sheep and goats in that area. A total of 311 sheep and goat blood samples from 37 farms and 10 animals from each farm were collected. A questionnaire on the risk factors (abortion history, presence of dogs and closeness to the cattle farms) was developed for analysis. Serological test was done using a commercial ELISA kit. Seroprevalence was found to be 0.32% (1/311). Although the results i.e. presence of stray dogs (32.4%; 12/37), abortion history (48.6%; 18/37) and closeness to the cattle farms (27%; 10/37) were rather high, the very low seroprevalence showed that these risk factors were not related to neospora infection. The results suggested that *N. caninum* was not the cause of reproductive failure in sheep and goats in Gua Musang.

Keywords: Gua Musang, Malaysia, *Neospora caninum*, prevalence, sheep and goats

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INTRODUCTION

It is well recognised that *Neospora caninum*, which occurs worldwide, is an important protozoan disease in ruminants. Economic losses due to neosporosis in cattle by reproductive failure, mainly of abortion (Dubey, 1996, p. 28, p. 46; Dubey, 2003, p. 4, pp. 9–10; Dubey & Schares, 2011, p. 94) have been well documented. Similarly, abortion due to neosporosis in sheep and goats naturally (Corbellini, Colodel, & Driemeier, 2001; Kobayashi et al., 2001; Masala et al., 2007; Howe et al., 2008; Ezatpour et al., 2015) and experimentally (McAllister et al., 1996; O'Handley, Liddell, Parker, Jenkins, & Dubey, 2002) have also been reported. Neosporosis can also cause economic losses by reduced weight gain in beef cattle (Barling et al., 2000) and reduced milk yield in dairy cattle (Hernandez, Risco, & Donovan, 2001) but economic losses due to neosporosis have not been reported in sheep and goats. In Southeast Asia, most of the studies on neospora parasite were done on cattle. In this region, seroprevalence of *N. caninum* varied widely from 5.5% in Thailand (Kyaw et al., 2004, p. 255) to 41% in Vietnam (Duong et al., 2008). In Indonesia, seroprevalence in Bali cattle was 5.5% (Damriyasa et al., 2008), while it was 24% in dairy cattle (Sardjana et al., 2015). In a study in the Philippines six out of 17 (35.3%) aborting cows and 10 out of 63 (15.9%) non-aborting cows were seropositive to *N. caninum* (Konnai et al., 2008). Neospora seropositivity was also found to be not related to whether the animal was a heifer or cow (Kyaw et al., 2004, p. 255). IN addition, it has been reported that seropositivity of cattle herd was related to the presence of farm dogs (Arunvipas et al., 2012), while Kyaw et al. (2004) reported no significance difference of neospora seropositivity between herds with dogs or without herds. Although transplacental infection was reported in Thailand (Kyaw et al., 2005) and Malaysia (Chea et al., 2004), no report was found for neospora-related abortion in cattle.

In Malaysia, a few studies on prevalence of *N. caninum* antibodies in cattle have been conducted (Cheah, 2004; Rahman, Manimegalai, Chandrawathani, Premaalathan, & Zaini, 2011), including isolation of the parasite from a newborn calf (Cheah et al., 2004). However, there has been no study of neospora infection in sheep and goats in Malaysia. It was, therefore, considered important to study whether reproductive failure such as abortion in sheep and goats is related to neospora infection.

Neospora antibodies in sheep and goats in Malaysia was first reported in 2016 (Kyaw et al., 2016, p. 43). The study was conducted in 10 districts of Kelantan, and it was found that neospora antibodies in animals had a prevalence of 1.1% (5/472) and a farm prevalence of 6% (3/50). According to the report, all of the seropositive animals and farms were from Gua Musang district only. In Gua Musang, animal prevalence and farm prevalence were found to be 10% (5/50) and 60% (3/5), respectively. These results may indicate possible potential infection of the neospora parasite in sheep and goats

in that particular district and the need for more extensive exploration, including studying the association of important risk factors. Therefore, this study was conducted to observe the seroprevalence of neospora antibodies in a representative sheep and goat population in Gua Musang and the possible association of seropositivity and common risk factors i.e. – the presence of dogs on the farm, abortion history and closeness to the cattle farms.

MATERIALS AND METHODS

Sample and Data Collection

The sheep and goat population in Gua Musang that was studied numbered 7,558 (sheep=3,971; goats=3,587); some of these animals were reared on mixed farms (Department of Veterinary Services, 2012, pp. 41–47). The number of animals in farms varied from three to 30 animals. A cross-sectional study with convenience sampling method was used. This was because some farms were too far in location and it was difficult to arrange for a visit by both the researcher and assistants by the Department of Veterinary Services, Kelantan, to collect samples. A total of 311 blood samples were collected from the jugular veins of the animals (ewes and does) from 37 farms, with approximately 10 animals from each farm being sampled. The calculation was based on the method by Naing et al. (2006). For farms having fewer than 10 ewes or does, blood samples were collected from all the animals. The sera were separated and stored at -20°C until further analysis.

During the blood sample collection, the responses via questionnaire to the three risk factors i.e. the presence of dogs or stray dogs on the farm, abortion histor, and closeness of sheep and goat farms to nearby cattle farms were recorded to investigate whether these potential risk factors were associated with neospora seropositivity. Closeness to nearby cattle farms was arbitrarily fixed at a distance of three km in the same area.

Serological Test

Sera were tested for *N. caninum* antibodies using a commercial ELISA test kit (IDEXX *Neospora* X2 Ab Test) according to the manufacturer's instructions. Briefly, the ELISA kit kept at 4°C was thawed at room temperature for 30 min. The test sera stored at -20°C were thawed at room temperature for 30 min. Ninety µl of sample diluents was dispensed into each well of the microplate. Ten µl of the test sera was dispensed in each well. The positive and negative control was dispensed into the appropriate wells. The final dilution of the mixture was 1:10 dilution. The wells were mixed gently by tapping the plate. The plate was covered with aluminium foil and incubated for 60 min at 37°C. After incubation, each well was washed three times with 200 µl of wash solution. Following the final wash, the plate was firmly tapped against absorbent material to remove liquid content. Then, 100 µl of the conjugate solution was dispensed in each well. The plate was covered with aluminium foil and incubated for 60 min. After incubation, the same washing step was done. After that, 100 µl of TMB substrate

solution from the kit was dispensed in each well. The plate was covered with an aluminium foil and incubated at room temperature for 15 min. Finally, 100 µl of stop solution was dispensed in each well. The result was read at the wavelength of 450 nm using an absorbance reader (Bio-Tek, USA). The sensitivity and specificity of the test kit were 93.7% and 95.3%, respectively. The results were expressed as S/P ratio based on a positive and negative control serum according to the manufacturer's procedure. The optical density readings ≥40% were positive, while ≥30% and <40% were suspect and <30% readings were negative.

$$S/P\ \% = 100 \times [(Sample\ A450 - NCx)/(PCx - NCx)$$

where

PCx = positive control, and NCx = negative control

Statistical Analysis

Descriptive statistics were used. Risk factor analysis was not done as there was only one seropositive animal.

RESULTS

Seropositivity

Only one animal from one farm was seropositive. Seroprevalence, therefore, was 0.32% (1/311) and farm prevalence was 2.7% (1/37). The seropositive farm was a goat farm that had only three animals. There was also only one animal suspected of being seropositive as it gave an optical density reading in the range of 30%-40%.

Risk Factors

The occurrence of all three risk factors was high (Table 1). Thirty-two per cent of the sheep and goat farms had stray dogs. Only one farm had its own dogs. The seropositive farm was a goat farm that had only three female goats of over one year of age. The farm had stray dogs. It had no abortion history.

Table 1
Potential risk factor information of 37 sheep and goat farms in the study

Risk Factors	No. of Farms	%	Remarks
Presence of farm dogs (or) stray dogs	12	32.4	Only one farm had a dog. The rest (11 farms) had stray dogs.
Abortion history	18	48.6	Farms with abortion history were high.
Nearby cattle farms	10	27	Neospora serostatus of nearby cattle farms was not known.

DISCUSSION

The seroprevalence results obtained in the present study conducted in Gua Musang provided a very low percentage (0.32%) compared to that obtained from previous studies (10%). According to the other researchers, seroprevalence of neospora in sheep and goats differed depending on geographic location and range from 0.6% in New Zealand (Reichel, Ross, & McAllister, 2008) to 63% in Jordan (Abo-Shehada & Abu-Halaweh, 2010), while in goats it was from 0.4% in Poland (Czopowicz et al., 2011) to 23.6% in the Philippines (Konnai et al., 2008). The exposure and infection may also depend on the other risk factors.

The dogs are considered a main source of neospora transmission as they are definitive hosts (McAllister et al., 1998). The reason why the prevalence in the present result was lower than that reported in the previous report (Kyaw et al., 2016, p. 43) might been because the spread of the parasite was sporadic. Also, the opportunity of contamination of feed or water by the faeces of the infected definitive host might have been low as most of the farms were intensively reared. On the other hand, the frequency and the number of stray dogs that could get access to the farm might also have been very low. In addition, the dog(s) that had accessed the farms, if at all, might not have been infected with neospora. Although it might be difficult to collect samples from stray dogs, it would be meaningful to do so if serology for neospora antibody were detected in the animals. Since information on the serostatus or prevalence of neospora in cattle in Kelantan is lacking, it is probable that the cattle farms situated close to the sheep and goat farms may not be infected.

The number of farms with high occurrence of abortion indicated that most of the sheep and goat farms in Gua Musang had reproductive problems. This study concluded that the neospora parasite might not have been the cause of any reproductive failure as seroprevalence of *N. caninum* was very low. The abortions might have been due to other abortifacients rather than the neospora parasite. It is recommended to explore the real cause of abortion in sheep and goats in Gua Musang.

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Short Communication

24-Epibrassinolide Mediated Changes on Germination and Early Seedling Parameters of *Vigna Mungo* (L). Hepper Var. Shekhar-2 under Salinity Stress

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ABSTRACT

Salinity mediated inhibition of seed germination and seedling emergence are the main problems in saline areas. An investigation was carried out to evaluate the effect of four different concentrations (10^{-5}, 10^{-7}, 10^{-9} and 10^{-11} M) of pre-soaked 24-epibrassinolide (24-EBL) on the germination traits associated with seedling emergence in *Vigna mungo* (L). Hepper under salt stress. The results revealed that salinity significantly reduced germination traits especially at higher doses of 16 and 20 dsm^{-1}. Radical length, plumule length, radical fresh weight, plumule fresh weight, germination percentage, seedling length, seedling fresh weight and the seed vigour index also decreased with increasing salinity but seeds primed with 24-EBL alleviated the effect of salinity. Under both stressed and non-stressed conditions, 10^{-5}M 24-EBL was found to be most significant, while 10^{-11} M 24- EBL was least significant.

Keywords: *Vigna mungo*, seed priming, salt stress, 24-epibrassinolide, germination traits

INTRODUCTION

Salt stress poses a major challenge to agriculture throughout the world by influencing plant growth and greatly reducing crop yield. Salt stress exerts a serious limiting factor for crop growth and production in arid and semi-arid regions. Sodium chloride (NaCl) is the most soluble and widely distributed salt in world (Munns

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& Tester, 2008). Plants vary greatly in their tolerances to salt. However, the performance of crops under saline conditions depends on seed germination, seedling emergence, establishment and also tolerance at late stages of growth. Salinity creates a lot of hardship for seeds in the germination period either by limiting water absorption by seeds (Dodd & Donovan, 1999) or by affecting the mobilisation of stored reserves (Linn & Kao, 1995). Salinity directly affects the organisation or synthesis of proteins in germinating embryos (Ramagopal, 1990). The effect of salt stress on germination percentage, germination rate and seedling growth varies depending on plant species (Ungar, 1996). Plant growth reduction under salt stress is mainly caused by accumulation of salt, an imbalance in the uptake of mineral nutrients and low soil water potential (Hosseini & Thengane, 2007). Ion nutrition gets disrupted due to excessive Na+ ion accumulation in root surface. Under salt stress, seed germination and early seedling growth are crucial factors limiting crop establishment and yield (Kitajima & Fenner, 2000). Salinity affects the seed germination of pulses like *Glycine max* (Essa, 2002) and *Vigna* spp., (Jabeen et al., 2003). It is well-known that salt stress has a negative impact on seed germination and vigour (Rehman et al., 2000). A low level of salinity inhibits germination by inducing seed dormancy (Khan & Weber, 2008). Salinity causes lower osmotic potential of germination media, and this alters the imbibition of water by seeds (Khan & Weber, 2008), thereby minimising the utilisation of the reserved food of seeds (Promila & Kumar, 2000) and hormonal imbalance (Khan & Rizvi, 1994).

Brassinosteroids (BRs) are universally occurring plant polyhydroxysteroids (Noguchi et al., 1999). BRs play a substantial role in many developmental processes of plants such as seed germination, root growth and flowering (Sasse, 2003). Khripach et al. (1999) reported that in plants, BRs have growth promoting and other regulatory properties. Bajgua and Hayat (2009) reported that BRs also participate in plant response to biotic and abiotic stresses like salinity, cold and drought stress. BR-regulated stress responses occur through induction of protein synthesis, activation or suppression of key enzymatic reactions and the production of various chemical defense compounds (Bajgua & Hayat, 2009). Exogenous application of BRs promoted seed germination and seedling growth of *Brassica napus* and *Arabidopsis thaliana* under salt stress (Kagale et al., 2007). Seed priming is an effective technique for increasing seed germination and seedling growth of many crops under stressful conditions (Farooq et al., 2006; Bajehbaj, 2010).

Black gram (*Vigna mungo* L. Hepper) is grown in the tropical and subtropical regions of the Indian sub-continent for its protein-rich edible dry seeds. It is a source of nutritionally rich protein that complements cereals to provide a balanced diet. Limited genetic variations in black gram germplasm slows the development of breeding varieties

and provides resistance to abiotic and biotic stresses. Therefore, increasing attention is being focussed on enhancing production of black gram by using phytohormones to overcome its limitations. The present research was undertaken to study the effect of 24-EBL on salinity induced changes in seed germination of *Vigna mungo*.

MATERIALS AND METHOD

Certified seeds of black gram (*Vigna mungo* L. Hepper var. Shekhar-2) were procured from CCS Haryana Agriculture University, Hisar, India. The plant growth regulator, 24-EBL, was purchased from Sigma Aldrich Ltd., New Delhi, India. The experiment was performed in the research laboratory of the Botany Department, Kurukshetra University, Kurukshetra. Uniform seeds were surface sterilised with 10% hypochloride for 2 to 3 min to avoid fungal infection and then rinsed with distilled water. The seeds were soaked at 25°C for 4 h in 10^{-5}, 10^{-7}, 10^{-9} and 10^{-11} M 24-EBL. Distilled water was used as the control treatment. The salinity treatments were given up to five levels (0, 8, 12, 16 and 20 dsm^{-1}). A total of 10 seeds were taken in each Petri dish containing a double layer of sterile filter paper and then given the same appropriate amount of distilled water (as control) and different salinity solutions. Seeds were regularly checked for seven days and the germinated seeds were counted. After emergence of radical about 2 mm in length, each seed was considered as germinated. At the end of the test the seed germination percentage (SGP) was determined.

$$\text{Germination percentage (GP)} = \frac{\text{Number of germinated seeds}}{\text{Total no. seeds}} \times 100$$

After the seventh day, plumule length (PL), plumule fresh weight (PFW), radicle length (RL), radicle fresh weight (RFW), seedling length (SL), seedling fresh weight (SFW) and seedling vigour index (SVI) was determined. Plumule length (PL) and radicle length (RL) were measured using a metre scale. The seedlings were blotted using blotting paper to remove adhering water and plumule fresh weight (PFW), radicle fresh weight (RFW) and seedling fresh weight (SFW) of each plant were weighed on an electronic balance to record the respective fresh mass. The seedling vigour index (SVI) was calculated based on the work by Abdul-Baki and Anderson (1970) as given below.

$$\text{Seed vigour index (SVI)} = \frac{\text{Germination \%} \times \text{Seedling length}}{100}$$

Analysis of variance was based on ANOVA procedure using the SAS software. Two-way ANNOVA range tests at the 5% probability level were used to estimate the differences among the means of the different treatments.

RESULTS

Radical Length

In the present study radical length decreased progressively with increase in salinity (Table 1). Salinity decreased radical length by about 18%, 31%, 43% and 54% at S_1,

S_2, S_3 and S_4 levels of salinity, respectively compared to the control. Application of 10^{-5} M EBL increased radical length by 39.4%, 28.7%, 21.5% and 15.5%, respectively at S_1, S_2, S_3 and S_4 levels of salinity corresponding to their controls. Other concentrations of 24-EBL like 10^{-7}, 10^{-9} and 10^{-11} M also increased radical length but the magnitude was less compared to when 10^{-5} M concentration was used.

Table 1
Pre-soaking effect of 24-EBL on radical length, plumule length, radical fresh weight and plumule fresh weight under salt stress

Treatment (M)	Radicle	Plumule	Radicle	Plumule
	Length (cm)		Fresh Weight (gm)	
Control	10.63 ±0.88	7.16 ± 0.19	0.118 ± 0.016	0.086 ± 0.004
EBL 10^{-5}	11.56 ±0.24	7.73 ± 0.14	0.132 ± 0.071	0.102±0.006
EBL 10^{-7}	11.46 ±0.26	7.60 ± 0.21	0.128 ± 0.028	0.098 ± 0.005
EBL 10^{-9}	11.20 ± 0.23	7.53 ± 0.14	0.126 ± 0.026	0.095 ± 0.004
EBL 10^{-11}	11.00 ± 0.25	7.26 ± 0.17	0.121 ± 0.013	0.089 ± 0.002
8 dsm^{-1}	08.70 ± 0.11	5.90 ± 0.11	0.091 ± 0.025	0.068 ± 0.001
8 dsm^{-1} + EBL 10^{-5}	12.13 ± 0.14	8.33 ± 0.88	0.148 ± 0.038	0.107 ± 0.004
8 dsm^{-1} + EBL 10^{-7}	11.83 ± 0.91	8.10 ± 0.17	0.140 ± 0.035	0.106 ± 0.002
8 dsm^{-1} + EBL 10^{-9}	11.50 ± 0.57	7.46 ± 0.14	0.130 ± 0.040	0.096 ± 0.003
8 dsm^{-1} + EBL 10^{-11}	11.30 ± 0.83	6.80 ± 0.19	0.125 ± 0.046	0.081 ± 0.002
12 dsm^{-1}	7.30 ± 0.11	3.93 ± 0.12	0.065 ± 0.005	0.047 ± 0.001
12 dsm^{-1} + EBL 10^{-5}	9.40 ± 0.15	5.26 ± 0.81	0.091 ± 0.008	0.068 ± 0.001
12 dsm^{-1} + EBL 10^{-7}	8.89 ± 0.85	4.83 ± 0.89	0.084 ± 0.004	0.066 ± 0.001
12 dsm^{-1} + EBL 10^{-9}	8.70 ± 0.11	4.53 ± 0.15	0.082 ± 0.006	0.054 ± 0.003
12 dsm^{-1} + EBL 10^{-11}	8.10 ± 0.86	4.33 ± 0.85	0.073± 0.004	0.048 ± 0.002
16 dsm^{-1}	6.03 ± 0.17	2.63 ± 0.81	0.050 ± 0.002	0.024 ± 0.001
16 dsm^{-1} + EBL 10^{-5}	7.33 ± 0.16	3.33 ± 0.14	0.065 ± 0.005	0.031 ± 0.002
16 dsm^{-1} + EBL 10^{-7}	6.83 ± 0.20	3.26 ± 0.12	0.063 ± 0.004	0.030 ± 0.004
16 dsm^{-1} + EBL 10^{-9}	6.46 ± 0.78	3.20 ± 0.18	0.058 ± 0.003	0.028 ± 0.002
16 dsm^{-1} + EBL 10	6.16 ± 0.14	2.90 ± 0.13	0.052 ± 0.002	0.025 ± 0.001
20 dsm^{-1}	4.90 ± 0.11	1.70 ± 0.05	0.038 ± 0.002	0.016 ± 0.002
20 dsm^{-1} + EBL 10^{-5}	5.66 ± 0.88	2.03 ± 0.08	0.045 ± 0.004	0.020 ± 0.003
20 dsm^{-1} + EBL 1^{0-7}	5.53 ± 0.12	1.96 ± 0.12	0.043 ± 0.002	0.018 ± 0.001
20 dsm^{-1} + EBL 10^{-9}	5.36 ± 0.84	1.86 ± 0.04	0.039 ± 0.003	0.017±0.002
20 dsm^{-1} + EBL 10^{-11}	5.03 ± 0.12	1.73 ±0.09	0.038 ± 0.003	0.016 ± 0.001
F value				
Salinity	849.915	1970.2952	845.049	1307.367
Treatment	238.294	57.308	47.045	56.675
Salinity* Treatment	35.118	7.644	6.809	8.528

Mean ± SE was calculated for three replicates. Values with NS are not significantly different at $p<0.05$

Plumule Length

Similar to radical length, plumule length also decreased with increasing salinity. Plumule length experienced the highest decrement at the salinity level of 20 dsm^{-1}. The 24-EBL at 10^{-5} M mitigated salt stress by enhancement of plumule length by 41.1%, 33.8%, 26.6% and 19.4% at S_1, S_2, S_3 and S_4 levels, respectively corresponding to their controls.

Radical Fresh Weight

Salinity decreased radical fresh weight by 22.8%, 44.9%, 57.6% and 67.7% at S_1, S_2, S_3 and S_4 levels, respectively compared to their controls (Table 1). In this study, we found that radical fresh weight of black gram was reduced in the presence of NaCl as a result of salt osmotic effects, which reduced water availability. Seeds of black gram primed with 24-EBL increased the radical fresh weight of the salt-stressed seedlings. The most effective concentration was 10^{-5} M, which increased 62.6%, 40%, 30% and 18.2% radical fresh weight at S_1, S_2, S_3 and S_4 levels, respectively corresponding to their controls.

Plumule Fresh Weight

Reduction of about 20.9%, 45.3%, 72%, 81.3% by salinity at S_1, S_2, S_3 and S_4, respectively in fresh weight of plumule was observed in the present investigation. The effect of salt stress was diminished by seeds primed with different concentrations of 24-EBL. The most effective concentration was 10^{-5} M, which alleviated salt stress as seen in plumule fresh weight of black gram by 57.3%, 44.6%, 29.1% and 25% at S_1, S_2, S_3 and S_4 levels, respectively corresponding to their controls.

Germination Percentage

Germination percentage was decreased by salinity levels and the percentage of decrement was 13.1%, 27%, 34%, and 48.2% at S_1, S_2, S_3 and S_4 levels of salinity, respectively compared to non-stressed conditions (Table 2). In the present study, 24-EBL significantly increased germination percentage under salt-stressed and non-stressed conditions. The most effective concentration of 24-EBL was found to be that of 10^{-5} M, which increased germination percentage by about 15.9%, 32.2%, 36.8% and 53.2% at 8, 12, 16 and 20 dsm^{-1} levels of salinity, respectively corresponding to their controls.

Seedling Length

Seedling length of *Vigna mungo* decreased with an increase in salinity level. Data presented in Table 2 showed the effect of seed pre-treatment by different concentrations of 24-EBL on seedling length during stress. Seeds pre-soaked with 24-EBL alleviated the seedling length in both stress and non-stress conditions, whereby a concentration of 10^{-5} M increased seedling length at S_1, S_2, S_3 and S_4 levels by 40.1%, 30.5%, 23% and 16.6% over controls.

Table 2
Pre-soaking effect of 24 EBL on germination, seedling length, seedling fresh weight and seed vigour index under salt stress

Treatment (M)	Germination (%)	Seedling Length (cm)	Seedling Fresh Weight (gm)	Seed Vigour Index
Control	90.66 ± 0.33	17.80 ± 1.25	0.205 ± 0.032	17.21 ± 1.14
EBL 10^{-5}	100.0 ± 000	19.30 ± 1.18	0.235 ± 0.037	19.3 ± 1.22
EBL 10^{-7}	100.0 ±000	19.06 ± 1.26	0.224 ± 0.075	19.06 ± 1.18
EBL 10^{-9}	100.0 ± 000	18.73 ± 1.19	0.221 ± 0.038	18.73 ± 1.16
EBL 10^{-11}	90.66 ± 0.35	18.20 ± 0.90	0.211 ± 0 037	17.64 ± 1.12
8 dsm^{-1}	80.33 ± 0.38	14.60 ± 0.78	0.159 ± 0.037	12.16 ± 0.92
8 dsm^{-1} + EBL 10^{-5}	90.66 ± 0.42	20.46 ± 0.92	0.256 ± 0.032	19.78 ± 1.12
8 dsm^{-1} + EBL 10^{-7}	90.33 ± 0.47	19.93 ± 0 .83	0.247 ± 0.057	18.6 ± 1.21
8 dsm^{-1} + EBL 10^{-9}	90.00 ± 0.57	18.96 ± 0.97	0.226 ± 0.059	17.08 ± 1.12
8 dsm^{-1} + EBL 10^{-11}	80.66 ± 0.36	17.60 ± 0.10	0.206 ± 0.023	15.47 ± 1.19
12 dsm^{-1}	70.00 ± 0.39	11.23 ± 0.77	0.113 ± 0.035	7.86 ± 0.69
12 dsm^{-1} + EBL 10^{-5}	90.00 ± 0.43	14.66 ± 0.71	0.160 ± 0.020	13.20 ± 0.87
12 dsm^{-1} + EBL 10^{-7}	80.66 ± 0.45	13.66 ± 0.97	0.150 ± 0.033	11.85 ± 0.76
12 dsm^{-1} + EBL 10^{-9}	80.33 ± 0.33	13.20 ± 0.86	0.137 ± 0.043	11.01 ± 0.81
12 dsm^{-1} + EBL 10^{-11}	70.33 ± 0.57	12.46 ± 0.99	0.122 ± 0.055	09.15 ± 0.59
16 dsm^{-1}	60.33 ± 0.55	08.66 ± 0.62	0.075 ± 0.008	05.47 ± 0.45
16 dsm^{-1} + EBL 10^{-5}	80.66 ± 0.57	10.66 ± 0.93	0.096 ± 0.004	09.25 ± 0.58
16 dsm^{-1} + EBL 10^{-7}	80.33 ± 0.38	10.10 ± 0.95	0.094 ± 0.007	08.40 ± 0.49
16 dsm^{-1} + EBL 10^{-9}	70.35 ± 0.31	09.66 ± 0.75	0.086 ± 0.005	07.08 ± 0.46
16 dsm^{-1} + EBL 10^{-11}	70.00 ± 0.33	09.06 ± 0.67	0.077 ± 0.006	06.34 ± 0.52
20 dsm^{-1}	50.00 ± 0.57	6.60 ± 0.350	0.054 ± 0.004	03.29 ± 0.37
20 dsm^{-1} + EBL 10^{-5}	70.66 ± 0.38	7.70 ± 0.590	0.065 ± 0.008	05.90 ± 0.43
20 dsm^{-1} + EBL 10^{-7}	70.35 ± 0.33	7.46 ± 0.480	0.062 ± 0.009	05.47 ± 0.39
20 dsm^{-1} + EBL 10^{-9}	60.00 ± 0.53	7.23 ± 0.390	0.056 ± 0.006	04.33 ± 0.40
20 dsm^{-1} + EBL 10^{-11}	50.33 ± 0.88	6.76 ± 0.280	0.054 ± 0.004	03.62 ± 0.36
F Value				
Salinity	29.951	3643.340	1856.474	573.078
Treatment	12.345	161.837	91.521	47.834
Salinity*Treatment	1.393 N.S.	17.012	12.584	2.535

Mean ± SE was calculated for three replicates. Values with NS are not significantly different at $p<0.05$

Seedling Fresh Weight

Table 2 showed the effect of 24-EBL on seedling fresh weight of black gram during salinity stress. Seedling fresh weight decreased by 22.4%, 44.8%, 63.4% and 76% at four different salinity levels (8 dsm^{-1}, 12 dsm^{-1}, 16 dsm^{-1} and 20 dsm^{-1}), respectively corresponding to their controls. The concentration, 10^{-5} M 24-EBL, was found to be the most effective concentration

in increasing seedling fresh weight by 61%, 41.5%, 28% and 20% at S_1, S_2, S_3 and S_4 salinity levels, respectively relative to their controls in the present study.

Seed Vigour Index (SVI)

Enhanced salinity significantly decreased seed vigour in the present investigation. Reduction in seed vigour was expected because seedling length and germination percentage were decreased by salinity stress. Salinity decreased the SVI by 29.3%, 60.1%, 68.2% and 80.8% as compared to the control. Best results were obtained using a concentration of 24-EBL (10^{-5} M) in alleviation of salt stress (Table-2).

DISCUSSION

The seeds treated with 24-EBL enhanced radical length at all the salinity levels compared to the untreated seeds. High salinity may slow down the uptake of water by the plants, resulting in inhibition of root elongation (Werner et al., 1995). Similarly, HBR alleviated salt-stress mediated inhibition of root elongation in barley (Marakli et al., 2014). This might be attributed to enhancement at the level of nucleic acid and soluble proteins by BRs, ultimately promoting growth (Anuradha & Rao, 2001). Similar results were obtained by Jaleel et al. (2007) in *Catharanthus roseus*, where root length was affected by different salinity levels.

Some growth parameters such as root fresh weight and shoot fresh weight of *Pisum sativum* was decreased by NaCl (Yildirim et al., 2008). Diminution in shoot growth and fresh weight of barley was caused by a salt concentration of 100 mM (Demirkiran et al., 2013). During seed germination water entry occurred through aquaporins. Salinity reduced the radical fresh weight because NaCl is an inhibitor of aquaporin-mediated root water transport (Martínez-Ballesta et al., 2006).

Jamil et al. (2007) also reported reduction in fresh weight of radish plants under salt stress. Our results were also corroborated by Rahim et al. (2012), who reported the negative impact of increasing salinity on the germination of barley.

Our results were in conformity with the germination of *Eucalyptus camaldulensis* seeds under salt stress by 24-EBL (Sasse et al., 1995). The 24-EBL significantly reduced the dormant period of embryos and increased germination percentage in cherry plum and sloe, as reported by Pugachev et al. (2000). Brassinosteroids enhanced seed germination by increasing the growth potential of tobacco seedling embryos (Leubner & Metzger, 2001). Abscisic acid-mediated inhibition of germination in *Arabidopsis* was overcome by brassinosteroids (Steber & Mccourt, 2001).

Reduction in seedling length may have been due to the negative effects of NaCl ion toxicity (Mashadi et al., 1991). Jeannette et al. (2002) reported that increasing salinity reduced the seedling growth of *Phaseolus* spp. Enhanced salinity significantly decreased seed vigour in the present investigation. Reduction in seed vigour due to salinity was expected

because seedling length and germination percentage was decreased by salinity stress (Segatoleslami, 2010).

CONCLUSION

From this study, it can be concluded that higher doses of salt drastically can inhibit germination traits in black gram. Seeds primed with 24-EBL (10^{-5} M) overcame the deleterious effects of salinity stress on the parameters investigated. Application of 24-EBL significantly improved all parameters of seedling length, fresh weight and seedling vigour index, thus improving the tolerance of black gram to salt stress. Our results are expected to contribute to information on alleviation of salt stress in different legumes.

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Short Communication

Light-harvesting Complex and how it Affect Growth of *Arabidopsis thaliana* plants

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ABSTRACT

Light-harvesting complexes (LHCs) control light-dependent energy transfer in photosystem II(PSII). In order to find out if defective LHCs affect plant growth, light-related parameters were compared between a *chlorinal-1* mutant (*ch1-1*; defective LHCs) and wild-type (WT) plants of *Arabidopsis thaliana*. The aim of this study was to assess the effects of LHCs on light-related parameters on the growth of *Arabidopsis* plants. A JUNIORPAM fluorometer was used to measure the parameters such as coefficients of photochemical fluorescence quenching (qp and ql); parameters of non-photochemical quenching (qn and NPQ), the yield of non-regulated energy dissipation of PSII [Y(NO)], the value of the efficient quantum yield of PSII {Y(II)}, and yield of regulated energy dissipation of PSII {Y(NPQ)}. The *ch1-1* mutant showed similar coefficient of photochemical quenching to the WT plants. On the other hand, a non-photochemical quenching, an efficient quantum yield of PSII, and yield of regulated energy dissipation of PSII significantly declined in *ch1-1* mutant compared with the WT plants. The *ch1-1* mutant plants exhibited the value of decreased growth and smaller size of leaf compared with that of WT plants. The percentage of the area, length and width of the leaf of the mutant declined when compared with that of WT plants. These results suggest that defective LHCs regulated growth through affecting light-related parameters of the *ch1-1* mutant of *Arabidopsis thaliana* plants

Keywords: *ch1*-1 mutant, non-photochemical quenching, plant growth, photosystem II, glutathione, light-dependent energy

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INTRODUCTION

Arabidopsis thaliana is widely used to understand molecular biology of various plant traits, involving flower growth and light sensing (Más, 2005). Glutathione (GSH) controls growth and development, stomatal movement, and yield of the *Arabidopsis thaliana* plants (Jahan et al., 2008; Jahan et al., 2014; Jahan et al., 2016) and corn plants (Munirah et al., 2015a). Different stimuli such as atmospheric pollutants, biotic and abiotic stress, hormones, and light-harvesting complexes (LHCs) affect GSH content of the *Arabidopsis thaliana* plants (Sánchez-Fernández et al., 1997; Okuma et al., 2011; Jahan et al., 2016). Antenna complexes in photosystems collect and channel the photons to power the carbon-fixing reactions (Caffarri et al., 2009; Ogawa et al., 2004; Barber, 2006).

External application of GSH increased light-related parameters such as chlorophyll (Chl) content, chlorophyll fluorescence, yield, photosynthesis of corn plants (Munirah et al., 2015a; Syuhada et al., 2014; Inani et al., 2015) and leaf numbers, Chl content and fluorescence of *Arabidopsis* plants (Jahan et al., 2016). On the other hand, it was shown genetically and chemically that deficient GSH levels in guard cells affected stomatal aperture of the *Arabidopsis* plants (Jahan et al., 2016; Okuma et al., 2011). This result might limit photosynthetic activity, growth, water loss and productivity of plants (Syuhada et al., 2016; Jahan et al., 2016; Khairi et al., 2017). Recent results indicate that chlorophyll content is correlated with light and gas exchange parameters of corn plants (Munirah et al., 2015a and b; Syuhada et al., 2014). The LHCs regulate the light reaction in photosystem to modulate the chloroplastic progress (Krol et al., 1995) and LHCs regulate physiological functions of plants (Jahan & Hasan, 2017). The *ch1-1* mutant with defective LHCs in photosystem II (PSII) accepts limited photosynthetic light core complex (Ogawa et al., 2004). Therefore, the mutation of LHCs in the *ch1-1* mutant had resulted in reduced leaf development and lower accumulation of GSH compared with the wild-type *Arabidopsis* plants (Jahan et al., 2016; Jahan et al., 2011). Ogawa et al. (2004) stated that GSH biosynthesis affected leaf development of *Arabidopsis* plants. The effects of GSH on Chl content, photosynthesis rate, and yield of corn plants have been documented (Munirah et al., 2015a; Syuhada and Jahan, 2016). The *ch1-1* mutant showed the presence of defective LHCs in the photosystem core complexes in plants (Takabayashi et al., 2011).

In this short communication, the objective is to evaluate the function of Wild-type (WT) ecotype [Columbia (Col-0)] and *ch1-1* mutant of *Arabidopsis thaliana* plants were collected from Ohio State University, USA and grown in plastic pots. A mixture of 30% peat soil and 70% vermiculite (Vermiculite, Malaysia) by volume were used in preparing a growing media. The light intensity of 80 µmol photon m^{-2} s^{-1}, temperature of 22 ± 2°C, and a day / night cycle of 16/ 8 h were maintained in the growth chamber (Jahan et al., 2016; Jahan et al., 2012). Treatments were laid out as

completely randomised design with five replications (five different plants) unless otherwise stated. Experiments were carried out from March 2016 to Oct 2016.

Measurement of Parameters

A JUNIORPAM fluorometer (Walz, Germany) was used to measure the coefficients of photochemical fluorescence quenching [(qp and ql]; The qp is more consistent with separated light- harvesting antennae of photosystems while ql combined light-harvesting antenna to absorb photon from many reaction centres (Kramer et al., 2004)], parameters of non-photochemical quenching (qn and NPQ), the yield of non-regulated energy dissipation of PSII [Y(NO)], the value of the efficient quantum yield of PSII {Y(II)}, and yield of regulated energy dissipation of PSII {Y(NPQ)} in 5-6-week old leaves of both plants. The qn is non-photochemical quenching coefficient, whereas NPO is an alternative calculation of qn related with the number of quenching centres in the light-harvesting antenna. Data was recorded at mid-day consistently. A CI-202 portable leaf area meter (CID Bio-science, USA) was used to measure the area, length and width of leaves of both plants. The percentages of these leaf parameters of the *ch1-1* mutant plants against WT plants were computed. Plants were grown at different times to determine different parameters throughout the experimental time. Plants were grown under identical conditions where different planting times did not affect the growth of *Arabidopsis* plants. Five plants were randomly selected as replicas.

Statistical Analysis and Accession Number

Student's t-test was used to evaluate the significance difference between mean values at $p < 0.05$ using MS Excel software (Microsoft Corporation). The *Arabidopsis* Genome Initiative numbers for the genes discussed in this article was *CH1-1*, At1g44446.

RESULTS

Figure 1 shows the effect of defective LHCs on light-dependent parameters of the *Arabidopsis thaliana* plants. The coefficients of photochemical fluorescence quenching (qp and ql) were found to be similar in both plants (Figure 1[a]). The parameters of non-photochemical quenching (qn and NPQ) declined in *ch1-1* mutant plants compared with that of WT plants (Figure 1[b]). The qn and NPQ were 0.122 and 0.079 respectively in WT and 0.038 and 0.02 respectively in *ch1-1* mutant plants. The reduction of non-photochemical quenching in the *ch1-1* plants indicated that the mutant plant was more susceptible to the photoinhibition due to the defective LHCs. In addition, the yield of non-regulated energy dissipation of PSII [Y(NO)] was 0.394 in the *ch1-1* mutants which is higher than that of 0.251 in the WT plants (Figure 1[c]). The value of the efficient quantum yield of PSII{Y(II)} and yield of regulated energy dissipation of PSII{Y(NPQ)} decreased in

the *ch1-1* mutants compared with the WT plants (Figure 1[c]). The PSII{Y(II)} and PSII{Y(NPQ)} were 0.631 and 0.117 in WT and 0.561 and 0.045 in *ch1-1* mutant plants respectively. Reduction of Y(II) in the *ch1-1* mutant may be caused by a decrease of maximum quantum yield in PSII. Picture in Figure 1[d] shows the dwarf morphological characters of *ch1-1* mutants relative to that of wild-type plants. Leaves of mutant plants show lighter colour than that of WT *Arabidopsis* plants.

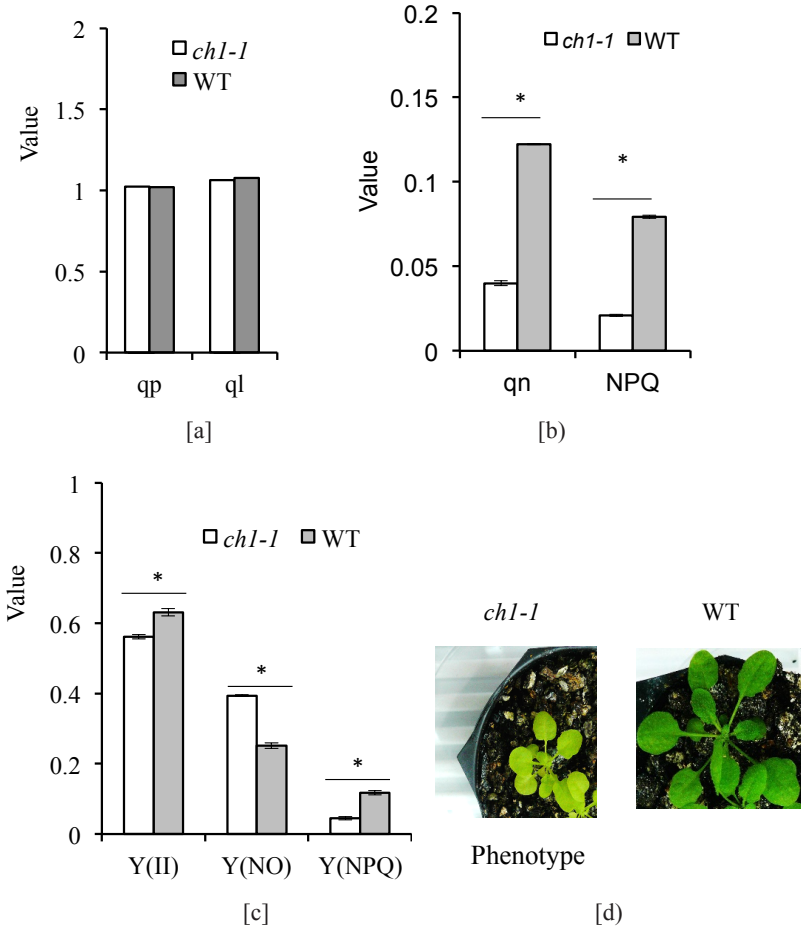

Figure 1. Different light related parameters in wild type and *chlorinal 1 (ch1-1)* mutant plants of *Arabidopsis thaliana.* [a] co-efficient of photochemical fluorescence quenching (qp and ql) in WT (closed bars) and *ch1-1* plants (open bars), [b] parameters of non-photochemical quenching (qn and NPQ) in WT (closed bars) and *ch1-1* plants (open bars), [c] the yield of non-regulated energy dissipation of PSII [Y(NO)], the value of the efficient quantum yield of PSII {Y(II)} and yield of regulated energy dissipation of PSII {Y(NPQ)} in WT (closed bars) and *ch1-1* plants (open bars), and [d] external structure, colour of leaf and leaf development between WT and *ch1-1* mutant plants. Error bars represent the standard error (n=5). The asterisk indicates significant (P values ≤ 0.05) differences between plants by the horizontal line

The size and percentage of the different parameters of the leaves of both plants are shown in Table 1. The length, width, and area of the leaf of *ch1-1* mutants were 1.34 cm, 1.1 cm, and 0.91 cm^2 respectively, significantly lower than the respective figures of 2.23 cm, 1.46 cm and 2.45 cm^2 of WT plants (Table 1). When the percentages of reduction of parameters of the leaf of the mutant was computed, it was found that area of leaf declined to 63% of WT. The trends were also found in length and width, 40 and 25%, respectively (Table 1). Thus, it is suggested that defective LHCs affected parameters of the leaf to suppress the growth of *ch1-1* mutants of *Arabidopsis* plants.

DISCUSSION

This study shows that defective LHCs significantly decreased qn, NPQ, Y(NO), and Y(NPQ) in the *ch1-1* mutants compared with those of the WT plants (Figure 1). This might be related to the antenna function during photoinhibition. Light-dependent reaction boosted photosynthesis rate (Busch et al., 2009) and leaf development in *Arabidopsis* plants (Jahan et al., 2014; Owaga et al., 2004). Plants regulate photosynthesis process by adapting photochemical function in the antenna complexes of photosystems (Jansson et al., 1997) to perform photosynthesis efficiently (Busch et al., 2009), which might increase the growth of *Arabidopsis* plants (Jahan et al., 2014; Table 1). Chlorophyll content and chlorophyll fluorescence are linked to the GSH content that influences the growth and yield of *Arabidopsis* plants (Jahan et al., 2014; Jahan et al., 2016). The *ch1-1* mutants accumulate significantly lower amount of chlorophyll and GSH levels than those of WT *Arabidopsis* plants (Jahan et al., 2016; Jahan et al., 2011). Therefore, guard cells of the *ch1-1* mutants showed higher sensitivity to abscisic acid (ABA) activity compared with the WT plants (Jahan et al., 2008; Jahan et al., 2014; Okuma et al., 2011) that limits photosynthesis rate in *ch1-1* mutants. The above findings indicate that mutation of LHCs affected light-related parameters and photosynthesis rate of the plants. The mutation of LHCs reduced the gaseous movement through the smaller stomatal opening of the guard cells of the *Arabidopsis* plants (Jahan et al., 2016) and affected the growth and phenotype of the *ch1-1* plants (Figure 1[d] and Table 1).

Table 1
The area, length and width of the leaf of ch1-1 and WT plants

Type	Area (cm^2)	Length (cm)	Width (cm
ch1-1	$0.91^b \pm 0.01$	$1.34^b \pm 0.017$	$1.1^b \pm 0.005$
WT	$2.45^a \pm 0.03$	$2.23^a \pm 0.01$	$1.46^a \pm 0.012$
Reduction (%) in *ch1-1* against WT plants	62.8	39.9	24.6

Means + standard errors with different letters within a column were significantly different at $p \leq 0.05$ by t-test

In the photosynthesis process, the light energy is converted into chemical energy (Barber, 2006). The *ch1-1* mutation could cause a lower light-induced efficiency of energy in PSII due to the reduced non-photochemical quenching (qn and NPQ) in the *ch1-1* plants than that of WT plants (Figure 1[b]). Different factors [including biochemical alleviation] affect plant growth through disturbing photosynthetic parameters of rice plants (Khairi et al., 2015; Hisyam et al., 2017). The NPQ is a prominent prophylactic protection strategy for the light reaction in the photosynthetic electron pathway. In the light-harvesting complexes, NPQ scatters additional excitation energy by using xanthophylls and the absorbance of the cross-section of the photosystems (Bailey et al., 2005). The NPQ and photosynthesis showed a positive correlation in plants (Schubert et al., 2006). The yield of non-regulated energy dissipation of PSII in the *ch1-1* mutants was higher than that of the WT plants (Figure 1[c]) indicating that PSII used a smaller amount of light energy due to the mutation of LHCs in the *ch1-1* mutant plants. This mutation confirms the higher energy fraction and photo inactivation of PSII dissipated as heat and fluorescence indicating instability despite the presence of environmental stresses (Busch et al., 2029). The mutation of LHCs might affect the photosynthesis activity (Müller et al., 2004) and movement of guard cells of *Arabidopsis* plants (Jahan et al., 2016; Jahan et al., 2014), in which they are linked to the growth of the *ch1-1* plants (Figure 1[d] and Table 1).

Previous studies have shown that GSH biosynthesis regulated the growth and flowering of the *ch1-1* mutant plants (Jahan et al., 2014; Ogawa et al., 2004)., which confirms the finding of this study that defective LHCs affect the growth of *ch1-1* mutants (Figure 1[d] and Table1). Deficiency of GSH increased stomatal closure of the guard cells (Okuma et al., 2011) and energy reaction in leaves of *Arabidopsis* plants (Owaga et al., 2004) that may affect photosynthesis rate and growth of the mutant plants. Moreover, Jahan et al. (2014) confirmed that the impairment of the growth of the *ch1-1* mutant compared with the WT plants was due to the mutation of LHCs in plants but the authors did not discuss photo parameters in their studies. This study confirms that defective LHCs in *ch1-1* mutant plants regulate light related parameters (Figure 2), which might reduce the photosynthesis rate. In conclusion, defective LHCs impair light-related parameters and GSH biosynthesis to affect the growth of the *ch1-1* mutant plants (Figure 2).

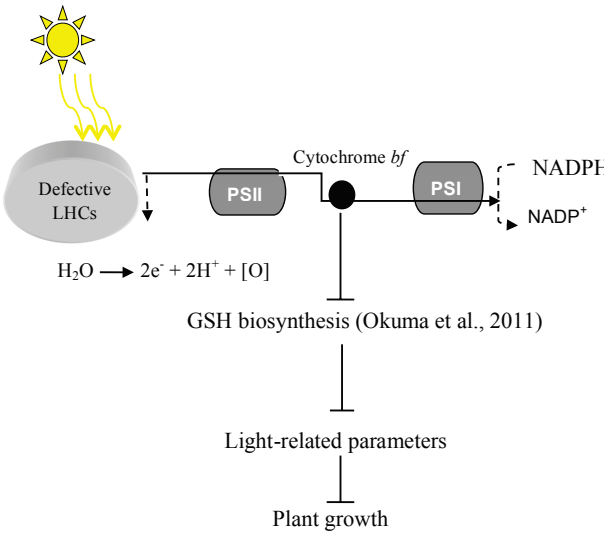

Figure 2. A schematic diagram shows mutation of LHCs affects light-related parameters and GSH biosynthesis in the *ch1-1* mutant plants of *Arabidopsis thaliana*. The arrow indicates energy flow in PSII and broken arrow indicates light reaction in the *ch1-1* mutant. Reverse T-bar indicates suppressive pathways. Defective light-harvesting complexes affect light-related parameters [(Chl content, Chl fluorescence, qn and NPQ, Y(NO), Y(II), Y(NPQ)] regulate GSH biosynthesis in cells of leaves to modify growth of the *Arabidopsis* plants

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Revised: June 2016

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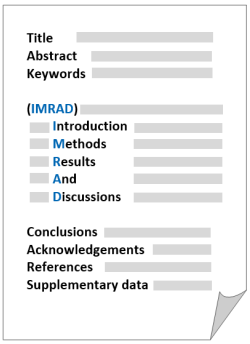

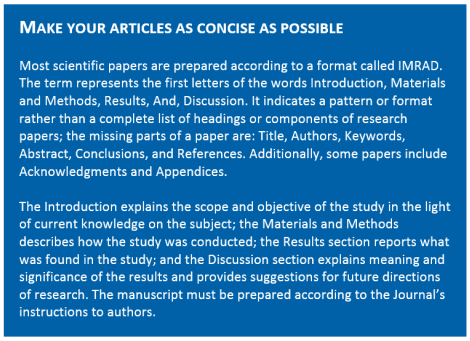

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