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About the Journal

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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.

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

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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.

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ABSTRACTING/INDEXING

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Foreword

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

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

This issue contains **eleven articles**, out of which one is a review paper, eight are regular papers and two are case reports. The authors of these articles come from different countries, namely, **Malaysia, Nigeria** and **Bangladesh**.

The review paper aims to briefly cover four decades of research done on bioactive compounds, nutritional composition, biotechnological applications and antibacterial activities in *Scenedesmus* spp *(Ishaq, A. G., Matias-Peralta, H.M., and Basri, H.)*. The eight articles cover a wide range of topics. In the first research paper, 168 one-week old pullet chicks of Nera® strain were used to evaluate the effects of malted sorgum sprouts in the diet of pullet chickens (*A. O. Fafiolu, A. V. Jegede, O.O. Oduguwa and M. A. Adebule*). The other papers are a study of bioproteins production from palm oil agro-industrial wastes by *Aspergillus terreus* UniMAP AA-1 strain (*Khadijah Hanim Abdul Rahman, Siti Jamilah Hanim Mohd Yusof and Zarina Zakaria*); an assessment of genetic variation in selected germplasms of white jute (Corchorus capsularis L.) (*M. J. Alam, M. E. A. Pramanik, Jannatul Ferdous and M. M. Islam*); an analysis of the characterisation of pathogenesis-related genes and resistance gene candidates in banana (*Musa acminata*) and their expression during host-pathogen interaction (*Chee-Yong Yang, Sathyapriya, H. and Mui-Yun Wong*); a study to determine the phenological stages of torch ginger inflorescence (*Etlingera elatior*) from emergence of rhizome to full bloom using the extended BBCH scale (*Choon, S.Y., Ding, P., Mahmud, T. M. M. and Shaari, K.*); a retrospective study to evaluate the growth and reproductive performance of farmed timorensis deer, *Cervus timorensis* (*M. Alif Zakaria, M. Zamri-Saad, A. H. Hasliza and H. Wahid*); a study to evaluate the copper and zinc levels of various rice soils of Peninsular Malaysia (*Babar, S. K., Khanif, Y. M. and Samsuri, A. W*); and finally, a study to determine the effects of water and nitrogen stress on the vegetative and yield parameters of *Brassica chinensis* var. *parachinensis*, commonly known as choy sum (*Khairun, N. K., Teh, C. B. S. and Hawa, Z. E. J.*).

The first case report describes the use of a standing frontonasal bone flap (SFF) to treat a case of primary sinusitis affecting the right frontal and maxillary sinuses in a horse (*Khairuddin, N. H. and Mimi Armiladiana, M.*) while the second describes the procedure of bladder tube cystotomy to relieve progressing bladder distention in a Saanen buck with obstructive urolithiasis (*Khairuddin, N. H., R. Mansor, R. Radzi and M. Y. Loqman*).

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

Bioactive Compounds from Green Microalga – *Scenedesmus* and its Potential Applications: A Brief Review

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ABSTRACT

Microalgae biomass has been recognised to have great potential as a source of novel bioactive compounds with industrial as well as health promoting applications in human, animal and aquatic lives. Several species of microalgae have undergone various screenings to identify and tap into these valuable resources, among them are the species of the genus *Scenedesmus*. Although it is more commonly known as a source of food for herbivorous zooplankton and in biofuel production because of its high lipid content, *Scenedesmus* has exhibited the potential of being a source of high-value compounds with antibacterial properties. These antibacterial activities have a wide range of applications in various industries that have not been broadly explored and fully exploited. This review aims to briefly cover four decades of research done on bioactive compounds, nutritional composition, biotechnological applications and antibacterial activities in *Scenedesmus* spp.

Keywords: *Scenedesmus*, bioactive compounds, biotechnological applications, antibacterial inhibition

INTRODUCTION

Freshwater microalgae are widely distributed in rivers, lakes and polar waters and they exhibit a diverse range of cellular, morphological, structural and biochemical composition (Chu *et al*., 2004). Many species of freshwater microalgae contain useful chemical compounds and valuable products including high quality proteins, pigments (carotenoids, chlorophyll and phycobiliproteins), lipoprotein, lutein and bioactive compounds that are useful pharmaceutically, as well as for various industrial applications (Chu *et al*., 2004;

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Amaro *et al*., 2011; Wan-Loy, 2012). Among these microalgae is *Scenedesmus*, which belongs to the order Sphaeropleales of the family Scenedesmaceae that is frequently dominant in freshwater lakes and rivers (Borowitzka & Borowitzka, 1988; Guiry, 2014). Many species of this genus are being used worldwide for various purposes due to their ability to adapt to harsh environmental conditions, ability to grow rapidly and ease of cultivation and handling (Lürling, 2003; Pultz & Gross, 2004). Likewise *Scenedesmus* spp. has been used in many biotechnological applications due to its high nutritional content and bioactivities (Chacón-Lee & Gonzalez-Marino, 2010; Guedes *et al*., 2011a). This review aims to collate information from very limited literature available on the great potential of *Scenedesmus* spp. such as antibacterial activities, biotechnological applications and other bioactive compounds it possesses. In doing so, it will reveal the need for further studies to be carried out.

Bioactive Compounds in Scenedesmus *spp.*

Similar to other microalgae, *Scenedesmus* spp. is a rich source of bioactive compounds that are being utilised for the benefit of humanity. They have been exploited for their active metabolites that have been applied in various industries including pharmaceutical, food, cosmetics, energy, aquaculture, medicine and others (Table 1). Some of the bioactive compounds are briefly discussed below.

Astaxanthin

It is considered to be one of the best carotenoid compounds that can successfully protect cells, lipids and membrane lipoproteins from oxidative damage (Ranga Rao *et al*., 2014). It is a sought-after compound as it is used in food, cosmetics and pharmaceutical applications (Kim *et al*., 2011). Astaxanthin products are commonly found in the form of soft gel, capsules, powder, tablet, oil, energy drinks and creams but they are mostly found in combination with other herbal extracts from other sources (Ranga Rao *et al*., 2014). Its other biological functions include; enhances immune response, acts as a strong coloring agent, protects against UV light and is a strong potent antioxidant (Guerin *et al*., 2003). Other carotenoids of importance include β-carotene and lutein, whose most important applications are as natural food colorants and in animal feed (Vilchez *et al*., 1997; Del Campo *et al*., 2000).

Vitamins

Microalgae are known to be a non-conventional source of vitamins because they possess several lipid-soluble and water vitamins in much higher concentrations over known conventional food (Kay, 1991; Zhang & Lee, 1997). According to Abd El Baky and El Baroty (2013), the vitamin content of microalgae such as Vitamin C, B_1 and B_2 is significantly higher than that of higher order plants. The biological functions of Vitamin C includes; strengthens the immune system, traps free radicals, regenerates Vitamin E and activates intestinal absorption of iron (Burtin, 2003). B vitamins (B_1, B_2, B_{12})

TABLE 1
Some valuable metabolites found in *Scenedesmus* spp.

Metabolites	Applications	Study
Vitamin B	Health-Food	Becker (2004); Borowitzka (1988).
Vitamin C	Health-Food additives, Pharmaceutical	Becker (2004); Borowitzka 1988.
Vitamin E	Health-Food, Medicine	Becker (2004); Borowitzka (1988).
Lutein	Animal nutrition, Pharmaceuticals	Tukaj *et al*. (2003); Otto & Wolfgang (2004); Ceron *et al*. (2008); Skjanes *et al*. (2013).
Astaxanthin	Aquaculture, cosmetics, Human nutrition, Medicine	Otto & Wolfgang (2004); Qin *et al*. (2008); Gouveia *et al*. (2008), Jouni and Makhoul, (2012).
Haemagglutinin	Medicine	Chu *et al*. (2004).
β- Carotene	Food colourant, Medicine	Karen *et al*. (2000); Indira & Biswajit (2012); Guedes *et al*. (2013).
Mycosporine-like amino acids, sporopollenin	Cosmetics (UV – screening compounds)	Indira & Biswajit, 2012; Skjanes *et al*. (2013).
Chlorophyll a, b, c	Food colourants, Pharmaceuticals, cosmetics	Karen *et al*. (2000); Gouveia *et al*. (2008); Indira & Biswajit, 2012; Catarina *et al*. (2013).
Polysaccharides	Medicine, Bioethanol, $BioH_2$	Gouveia *et al*. (2008); Skjanes *et al*. (2013).
Extracts with antimicrobial/ antifungal activities	Medicine	Abedin & Taha (2008).
Monounsaturated, polyunsaturated and Saturated fatty acids (Oleic acid, lauric acid, palmitic acid, linoleic acid, α-linoleic acid, stearic acid and others)	Biodiesel, Pharmaceuticals, Animal and Human nutrition, Aquaculture.	Ahlgren *et al*. (1992); Becker (2004); Kim *et al*. (2007); Gouveia and Oliveira (2009); Pandian and David (2012); Mahale and Chaugule (2013).
Amino acids (isoleucine, leucine, valine, lysine, methionine, cysteine, alanine, arginine, aspartic acid, glutamine and others)	Food	Chacón-Lee & Gonzalez-Marino (2010).

are used in the treatment of anemia, and has effect on ageing and chronic fatigue syndrome (Herrero *et al*., 2013).

Polysaccharides

Studies carried out with *Scenedesmus* spp. and other microalgae have shown that certain polysaccharides have medical effects (Skjanes *et al*., 2013). These polysaccharides function as protection against oxidative stress and have efficacy on gastric ulcers, wounds and constipation (Iwamoto, 2004; Spolaore *et al*., 2006).

Mycosporine-like amino acids

These are a group of molecules that consist of an amino acid that is bound to a chromophore and absorbs low wavelength light. These molecules play a vital role in protecting the microalgae against UV radiation (Skjanes *et al*., 2013). For this characteristic, they are being exploited for commercial purposes in cosmetic skin-care products for UV protection (Schmid *et al*., 2006).

Extraction of Biologically Active Compounds from Scenedesmus *spp.*

This is an important aspect to be considered in order to extract and isolate compounds of interest effectively. The general techniques of plant and algae extraction include maceration, hot continuous extraction (soxhlet), microwave-assisted extraction, sonication, supercritical fluid extraction, ultrasound assisted extraction, pressurised liquid extraction and hand grinding with pestle and mortar (Herrero *et al*., 2013). All of these have been also employed in the extraction of bioactive compounds from *Scenedesmus* algae. According to Herrero *et al*., (2013), successful determination of biologically active compounds from plants is also largely dependent on the type of solvent used in the extraction process. The choice of solvent used is influenced by what the extract is intended for and the targeted compounds.

In extracting different bioactive compounds from *Scenedesmus* spp. one of the techniques used is solvent extraction. Among important bioactive compounds extracted by solvent extraction methods from *Scenedesmus* include pigments, fatty acids, antioxidants and others (Table 2).

Antibacterial Activities of *Scenedesmus* spp.

Scenedesmus spp. has been reported to produce antimicrobial substances, which from the pharmaceutical's point of view, are a good source of new bioactive compounds. Although the potential of fatty acids to inhibit the growth and survival of pathogenic bacteria has been recognised for several years, the specific mechanism underlying the bactericidal action of fatty acids in microalgae remains to be fully understood. Nevertheless, they apparently enhance membrane damage that eventually enables cell leakage. Recently, studies of its structure-function relationship make it more evident that these antimicrobial activities rely on both the chain length and the degree of unsaturation (Guedes *et al*., 2011b).

TABLE 2
Different solvent extraction methods used for *Scenedesmus* spp.

Solvent	Extraction method	Compound extracted/ Activity	Study
100% Nanograde Acetone, 2:1 Dichloromethane/Methanol Quartz sand	Ultrasound, Sonication, Mechanical and hand grinding	Pigments and fatty acids	Karen *et al.* (2000).
Acetone, Ethanol Methanol, Diethyl ether	-	Antibacterial and antifungal activity	Abedin & Taha (2008).
1:1 Ethanol/Water	Homogenisation	Antibacterial activity	Catarina Guedes *et al.* (2011).
1:10 Ethanol/Acetone	-	Haemagglutination assay	Chu *et al.* (2004).
1:2 Chloroform/Methanol	Homogenisation	Lipid	Rajiv (2011).
3/1 v/v Ethanol/water Hexane, Ethyl acetate and Hot Water	Grounded with pestle and mortar	Antioxidants (phenols and carotenoids)	Koen *et al.* (2012),
90% Acetone	Hand shaking	Pigments	Beena & Krishnika (2011).
25ml of Diethyl ether, 25ml of petroleum ether.	Hand shaking	Fatty acid	Mahale & Chaugule (2013).
Acetone, Methanol, Dichloro Methane, Diethyl ether, Hexane, Hot Water, Cold water.	-	Antibacterial activity	Beena & Krishnika (2011).
Distilled water 100% ethanol	Ultrasonication	Antimicrobial and anticancer activity	Ördög *et al.* (2004).

Desbois *et al*. (2009) claimed that *Scenedesmus costatum* exhibited antibacterial activity against aquaculture bacteria as a result of their more than 10 carbon atoms in chain length of fatty acids. The Chlorophyta, or green algae, which include *Scenedesmus*, have chlorophyll a, b and several carotenoids (Tomaselli, 2004). Chlorophyll is one of the most valuable bioactive compounds that are being extracted from microalgal biomass and has gained importance as a food additive (Humphrey, 2004). Chlorophylls and β-carotene are major pigments present in microalgae that are known to act effectively as microbial growth inhibitors, and studies have shown that it has antioxidant as well as antimicrobial properties (Humphrey, 2004; Bhagavathy *et al*., 2011). Fan *et al*. (2013) and Jaya *et al*. (2007) reported that pigments from microalgae had antibacterial effect on certain bacteria. Patented application of astaxanthin is also available for preventing bacterial infections (Jouni & Makhoul, 2012). Furthermore, Guedes *et al*. (2011a) reported that *Scenedesmus* spp. is among

the few members of the green algae to produce antimicrobial substances and had active and prominent antibacterial properties that inhibited the growth of several pathogenic strains of bacteria when tested against them. These include *Salmonella* sp., *Pseudomonas aeroginosa, Escherichia coli* and *Staphylococcus aureus.*

Table 3 clearly shows that the antibacterial activity of *Scenedesmus* spp. extracts has positive results but with varying degrees of significant zones of inhibition. It also shows that *Scenedesmus* spp. extracts have higher antibacterial activity against the Gram positive bacteria than the Gram negative bacteria. According to Ördög *et al*. (2004), antibiotics are usually less effective against Gram- negative bacteria because of their more complex multilayered cell wall structure in addition to the presence of lipopolysaccharides on the outer cell wall thereby preventing the penetration of active compounds.

From the table, it is clear that various studies and screenings have been undertaken on this microalga pointing to its potential pharmaceutically. But there is yet to be a conclusive identification and characterisation of specific metabolites responsible for antibacterial activities.

Biotechnological Applications of *Scenedesmus* spp.

Today, industrial and commercial use of microalgal biomass and extracts of biomass has gained a strong foothold in various sectors of human life due to the presence of different useful compounds in them. The rich metabolic content of *Scenedesmus* spp. is being exploited for use in food (Becker, 2004; Toyub *et al*., 2008), aquaculture (Toyub *et al*., 2008), bioremediation (Martinez *et al*., 2000; Omar, 2002), cosmetics (Indira & Biswajit, 2012), pharmaceutical industries (Becker, 2004) and others. Some of these applications are briefly discussed below.

Nutraceutical Applications

Nutritional composition of *Scenedesmus* spp

Similar to higher order plants, the chemical composition of algae is not constant as it is determined by factors like environmental, temperature, pH value, mineral contents, CO_2 supply, population, density, growth phase and algae physiology that can modify its chemical composition (Gouveia *et al*., 2008). According to Yamaguchi (1997), microalgae have the ability to biosynthesise, metabolise, store and also secrete a diverse range of primary and secondary metabolites. Microalgal biomass is made up of different nutritional components of which the main three are proteins, carbohydrates and lipids (oil) (Indira & Biswajit, 2012). The following are some of the reasons why microalgae came to be of such commercial importance due to its nutritional composition: (1) the presence of high protein content in microalgae is the main reason it should be considered as a conventional source of protein, (2) its amino acid pattern compares favourably with other foods, (3) carbohydrates are obtained in various forms such as starch, glucose,

TABLE 3
Antibacterial inhibition of various *Scenedesmus* spp.

Staphylococcus aureus	*Streptococcus Pyogenes*	*Escherichia coli*	*Salmonella typhi*	*Bacillus subtilis*	*Enterococcus faecalis*	Zone of inhibition / Minimum inhibitory concentration	References
8	N/D	N/D	9	2	N/D	mm	Ghasemi *et al.* (2007)
16	16	N/D	N/D	N/D	N/D	mm	Najdenski *et al.* (2013)
18	18	N/D	N/D	N/D	N/D	mm	Najdenski *et al.* (2013)
17	17	N/D	N/D	N/D	N/D	mm	Najdenski *et al.* (2013)
1.0	N/D	R	R	4.0	N/D	cm	Abedin & Taha (2008)
1.5	N/D	R	R	3.0	N/D	cm	Abedin & Taha (2008)
R	N/D	R	R	2.5	N/D	cm	Abedin & Taha (2008)
1.2	N/D	1.5	R	2.0	N/D	cm	Abedin & Taha (2008)
5.0	N/D	N/D	N/D	N/D	N/D	mm	Ördög *et al.* (2004)
0.028	N/D	0.056	N/D	N/D	0.056	mg/ml	Ördög *et al.* (2004)

Note: R- Resistant, N/D- Not done

sugars, other polysaccharides and, (4) its total digestibility is extremely high, which explains why there are no limitations to its use in food and feed (Cornet, 1998; Becker, 2004; Solletto *et al*., 2005).

Scenedesmus spp. are particularly found to contain all essential amino acids and a good amount of protein, lipid and essential minerals (Geldenhuys *et al*., 1988). According to Becker (2004) and Batista *et al*. (2007), *Scenedesmus* contains lipids, proteins and carbohydrates that can compare favourably with other food protein (Table 4).

Human Nutrition

Microalgae for human nutritional requirements are currently being manufactured in different forms such as tablets, capsules, pastilles, liquids and nutritional supplements and are also incorporated into snacks, pastas, candy bars or chewing gum and in beverages (Spolaore *et al*., 2006; Gouveia *et al*., 2008). *Scenedesmus* is among the most used microalgae that has attracted the attention of manufacturers in the food and health-food market (Chacón-Lee & Gonzalez Marino, 2010). Compared to case in, *Scenedesmus* has very high nutritional quality and several toxicological assessments have not revealed any toxic impacts or abnormalities in experiments with test animals (Becker, 1984). Gross *et al*. (1978) carried out a nutritional study by incorporating *Scenedesmus* sp. into the diet of children (5 g/daily) and adults (10 g/daily) and a slight increase in weight was discovered. Subsequently, there was a significant improvement in the weight of four-year-old children who were fed with microalgae compared to those fed with a normal diet. In another study carried out by Natrah *et al*. (2007), a *Scenedesmus* sp. among other microalgae was shown to possess antioxidant properties and biochemical contents that could be applied in the nutraceutical industry.

TABLE 4
Nutrient composition of different *Scenedesmus* spp. (% dry matter)

Scenedesmus spp./Food products	Protein	Carbohydrate	Lipid	Ash	Crude	References
S. obliquus	50-56	10-17	12 -14	N/A	N/A	Becker (2004)
S. dimorphus	8-18	21-52	16 -40	N/A	N/A	Um & Kim (2009); Sydney *et al*. (2010)
S. acutus	50-60	10-17	12 - 14	6 – 10	3 - 10	Soeder & Prabst (1970)
S. quadricauda	47	N/A	1.9	N/A	N/A	Um & Kim (2009); Sydney *et al*. (2010).
S. obliquus	6-12	33-64	11 - 21	N/A	N/A	Batista *et al*. (2007)
S. dimorphus	60-70	13-16	6 - 7	N/A	N/A	Batista *et al*. (2007)
S. obliquus	34.5	N/A	16.13	12.0	6.39	Toyub *et al*. (2008)

N/A- Not applicable

Potential Source of Biodiesel

Due to global warming and exhaustion of fossil fuels, which has become a worldwide problem due to the emission of greenhouse gasses (GHG), attempts have been made to find alternative sources of energy from various biological materials such as plants, animal fat and microalgae (Pandian & David, 2012). They showed in a study that *Scenedesmus* spp. had a high oleic acid content of about 52.8%, making it most suitable for the production of good quality biodiesel.

Bio hydrogen ($BioH_2$) production

Scenedesmus sp. has been used as a feedstock for $BioH_2$ production as a source of biofuel to power both light and heavy-duty vehicles, as well as jet and marine engines (Gouveia *et al*., 2012). Currently, all major car producers offer cars running on hydrogen as fuel; it was discovered that *Scenedesmus* spp. was able to produce hydrogen, and this discovery has led to the search for a way to use this microalgae to convert solar energy into this useful energy carrier (Skjanes *et al*., 2013).

Wastewater treatment

Treatment of waste is an important problem in the world due to the increase in population and industrial activities. Agricultural and municipal wastes contain all the macro and micro-nutrients that are needed for algal growth; therefore, it is economical to grow algae in wastewater (Toyub *et al*., 2008). Because of the special ability of *Scenedesmus* spp. to adapt to different environmental conditions it is being used in domestic and industrial wastewater treatment. It has recently been used in removing heavy metals and in the production of oxygen and in converting waste products into beneficial substances (Abuzer *et al*., 2008).

Cosmetic industry

Several compounds of *Scenedesmus* spp. are used in cosmetic industry as thickening agents, water-binding agents and antioxidants in facial and skin care products (Rajiv, 2011). Pigments such as carotenoids i.e. astaxanthin, phycocyanine and β-carotene (Sánchez *et al*., 2007), mycosporine-like amino acids and sporopollenin are extracted and used as UV screening compounds for skin protection in cosmetics (Indira & Biswajit, 2012: Skjanes *et al*., 2013). Amino acids are known to have a universal function in proteins, but they are also important for skin hydration, elasticity and photoprotection and are included in cosmetics (Lebeau & Robert, 2003).

Pharmaceutical industry

Microalgae have for a long time been used for their therapeutic powers, but scientific investigations for biologically functional compounds started in the 1950s and since then, extensive research has been conducted to find compounds that might result in therapeutically beneficial agents (Mendes *et al*., 2003; Mayer & Hamann, 2005; Cardozo *et al*., 2007; Amaro *et al*., 2011). Furthermore, microalgae has

been found to produce antibiotics as its extracts have proven to be antibacterial, anti-protozoal and antiplasmodial, and it is mostly accredited to compounds belonging to the following chemical classes: indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons (Kellan & Walker, 1989; Ghasemi *et al*., 2004; Ozemir *et al*., 2004; Herrero *et al*., 2006; Cardozo *et al*., 2007).

The three major classes of microalgal photosynthetic pigments (chlorophyll, carotenoids and phycobilins) have demonstrated biological activity in a wide range of biological applications, including prevention of acute and chronic coronary syndromes, muscular dystrophy, atherosclerosis, cataract, rheumatoid arthritis and neurological disorders (Schoefs, 2004; Mimouni *et al*., 2012).

Scenedesmus produces polysaccharides that have been shown to have medical effects that act against oxidative stress (Mohammed, 2008). Other compounds found in *Scenedesmus* that have therapeutic abilities include Vitamin E (Pham-Huy *et al*., 2008), Vitamin C (Skjanes *et al*., 2013), astaxanthin (Olaizola, 2003) and metabolites with antibiotic activities (Chu *et al*., 2004; Ördög *et al*., 2004).

CONCLUSION

Scenedesmus spp. as a rich source of bioactive metabolites presents an advantage and an opportunity for its use in various applications that include aquaculture, cosmetics, pharmaceuticals and human nutrition. Studies had suggested that *Scenedesmus* spp. can produce diverse chemical compounds especially long-chain polyunsaturated fatty acids that can inhibit the growth of pathogenic micro-organisms. However, the active compounds in *Scenedesmus* have not being applied as antibacterial agents to inhibit food-borne pathogens such as *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella* spp. in food preparation and production. Therefore, further studies particularly in the application of the potential of antibacterial compounds in food industry have to be carried out.

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Utilisation of Malted Sorghum Sprouts in the Diet of Pullet Chicks

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ABSTRACT

One hundred and sixty eight (168) one-week-old pullet chicks of Nera® strain were used in a feeding trial to evaluate the effect of malted sorghum sprouts (MSP) in diets of pullet chicks. The trial lasted for 10 weeks. There were three experimental diets containing 0, 150gkg^{-1} or 300gkg^{-1} levels of MSP. Each diet was fed to four replicate groups of 14 pullet chicks. The results showed that feed intake reduced ($P<0.05$) gradually as the level of MSP inclusion in the diet increased. MSP inclusion in diets also led to reduced final weight ($P<0.05$). Crude protein and fibre digestibility decreased significantly ($P<0.05$) with increase in dietary level of inclusion of MSP. The converse was the case for ash digestibility. Birds fed 300 gkg^{-1} MSP had high ($P<0.05$) level of packed cell volume and total serum protein, which was quite unexpected if the final weight and weight gain of the birds are taken into consideration. There was, however, an indication of impairment of protein utilisation when MSP was included as the birds in this group recorded significantly higher serum creatinine. It was concluded that there are some factors inherent in MSP, probably tannin and HCN that impair the utilisation of MSP based diets by pullet chick. It is apparent from the foregoing of inclusion of MSP even at 150g kg^{-1} depressed feed intake and growth. It is, therefore, not advisable to use MSP at a level up to 150g kg^{-1} especially for starting pullets.

Keywords: Starting pullet, malted sorghum sprouts, utilisation

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INTRODUCTION

A major problem facing the poultry industry across the globe is that of escalating cost of conventional feeding stuff. This phenomenon is occasioned by competition between man

and livestock. Profit maximisation cannot be attained unless the birds are fed well formulated diets and at reasonable price. This occurrence had stimulated interest in finding alternative feedstuff that is cheap, readily available and with comparable nutritional qualities with the well known conventional feedstuff. Such alternatives have the potential of reducing feed cost, thereby making poultry enterprise more profitable.

Sorghum spp (Guinea corn) has replaced barley as a raw material in confectionery and brewing industries in many tropical countries (Banjoko, 1990). Malting of sorghum essentially involves soaking and steeping, which is followed by germination of the seed during which amylase enzyme is released. Malt is extracted from the germinated sorghum seeds and the residue consists of sorghum shoots and roots that are referred to as malted sorghum sprouts (MSP) (Aletor *et al.,* 1998). MSP is produced in commercial quantities in Nigeria and an estimated 200,000 metric tonnes of malted and un-malted extracts are produced annually by only one food industry with a sizeable cereal processing plant in Nigeria while the disposal is becoming a problem for the industry (Ikediobi, 1989). MSP has no use at present but efforts for its incorporation into animal feed has been advocated. In fact, feed waste, the disposal of which is becoming a problem for the industry, can be dried, bagged and stored within a short time period.

MSP has potential for use as feedstuff. Ologun *et al.* (1998) showed that up to 40% of sorghum rootlet or malted sorghum sprouts may be fed to rats. Fajemidagba (2000) reported that MSP compared favourably with brewer's dried grain when both were included in practical broiler rations up to 20% level. Oduguwa *et al.* (2001) studied the nutritive value of MSP using rats, and they concluded that the nutritive value was lower compared to soyabean meal reference diet. Morrison (1984) opined that not more than 10 to 15% malt sprouts should be included in concentrate mixture for dairy cows. There is a dearth of information on the utilisation of MSP in the diets of egg type chickens. This study was, therefore, designed to evaluate the effect of feeding MSP in the diet of starter pullets on performance, nutrient utilisation and on some serum metabolites with the intention of assessing the adequacy of MSP as a feed resource for pullet chicks.

MATERIALS AND METHODS

Location

This study was carried out at the poultry unit of the Teaching and Research Farm of the College of Animal Science and Livestock Production, University of Agriculture, Abeokuta, South Western Nigeria.

Diets

A control diet (with NRC, 1994 requirements) containing no MSP was formulated. Two other diets were formulated to contain 150 g kg^{-1}(12.08 MJ kg^{-1} ME, 212.8 kg^{-1} CP) and 300 gkg^{-1}(11.55 MJ kg^{-1}, 219.8 kg^{-1} CP) MSP. The test ingredient was

sourced from a Nigerian brewery located in southwest Nigeria. The gross composition of experimental diet is shown in Table 1.

TABLE 1
Gross composition of experimental diets (g kg^{-1})

Ingredients	Levels of MSP		
	0	150	300
Maize	530.0	380.0	230.0
Soybean meal	180.3	180.3	180.3
Fish meal	40.0	40.0	40.0
Groundnut cake	120.0	120.0	120.0
Brewers dried grain	68.2	68.2	68.2
Lysine	2.5	2.5	2.5
Methionine	3.5	3.5	3.5
Bone meal	30.0	30.0	30.0
Oyster shell	20.0	20.0	20.0
Premix (S)[x]	3.0	3.0	3.0
Salt	2.5	2.5	2.5
MSP	0.0	150.0	300.0
Total	**1000.0**	**1000.0**	**1000.0**
Determined analysis			
Metabolizable Energy ($MJkg^{-1}$)	13.00	12.08	11.55
Crude protein (gkg^{-1})	210.0	212.8	219.8
Crude fibre (gkg^{-1})	36.5	53.5	59.4

[x]The vitamin and mineral premix contained per kg of diet: retinyl acetate,5.12 mg; cholecalciferol, 10 mg; dl-a-tocopherol acetate, 30 mg; menadione, 4 mg; folicacid,1.20 mg; choline, 80,000 mg ; d-pantothenic acid, 19.0 mg; riboflavin, 8.0 mg; niacin, 70 mg ; thiamin, 5 mg; d-biotin, 0.1 mg; pyridoxine, 0.9 mg; ethoxiquin, 28 mg; manganese, 200 mg; zinc, 150 mg; iron, 153 mg; copper, 17.64 mg; iodine, 4 mg; selenium, 0.1 mg.

Experimental Design and Management of Pullet Chicks

A total of 168 one-week-old pullet chicks of Nera black® strain were used for the study. They were divided into three treatment groups of 56 birds each. Each treatment was further divided into four replicates of 14 birds. All the birds were fed a commercial diet for the first seven days before they were distributed into the experimental groups. The pullet chicks were raised in a conventional poultry house with concrete floor containing dry wood shavings as litter material. The birds remained in the deep litter pens throughout the experimental period. Each replicate of 14 birds was housed in a compartment (1.5 x 2M) properly demarcated with wood and chicken wire mesh. Feed and water were supplied *ad libitum*. Litter was changed every two weeks to disallow growth of pathogens. All vaccination and medication procedures were strictly adhered to. The experiment lasted 10 weeks.

Digestibility Trial

Metabolic trial was carried out when the birds were 10 weeks old. Two birds from each replicate of the treatments were selected. The birds were housed individually in specially designed metabolic cages, equipped with separate facility for feeding, watering and excreta collection. Three day total excreta collection followed a three-day acclimatisation period. The excreta collected were weighed and representative samples were taken in well labelled aluminium foil and dried in the oven at 65^0C for 72 hours. The dried excreta samples were milled and analysed for their proximate constituents along with the feed samples using AOAC (1990) methods. Apparent digestibilities of the proximate constituents were then calculated.

Plasma and Serum Biochemistry

Blood samples were collected from three birds selected from each replicate and analysed for some plasma and serum profile. Blood sample was taken by careful puncture of the brachial vein. A pinch (about 5 µg) of Ethylene diamine tetracetate (EDTA) was added to the 2.5 ml blood samples for plasma analysis, which was carried out within three hours of blood collection. Samples for serum analysis were decanted after centrifugation at 3000 rpm for 10 minutes. The packed cell volume (PCV), mean cell volume (MCV), white blood cells (WBC) and red blood cells (RBC) were determined using Wintrobe's microhaematocrit and improved Newbauer haemocytometer as described by Baker and Silverton (1985). Total serum protein was determined according to the methods of Colowich and Koplan (1955) while serum albumin was determined using bromocresol purple method of Varley *et al.* (1980). Serum creatinine was determined using the principles of Jaffe reaction as described by Bousness and Taussky (1945) while serum uric acid was determined using the kit-Quinica clinica spam (Wootton, 1964).

Data Collection

The performance of the birds was monitored on a weekly basis via feed intake, body weight gain and feed-to-gain ratio.

Chemical Analysis

Feed and excreta samples were analysed for their proximate constituents (Dry matter, crude protein, ether extract, crude fibre, ash, nitrogen free extract) according to AOAC (1990) methods. Gross energy in feed and excreta output were determined using adiabatic bomb calorimeter and data used to evaluated the metabolisable energy. The test ingredient (MSP) was analysed for its cyanide content using the method described by Zitnanak (1973). The cyanide content was determined by Ogawaski cyanide ion-electrode CN-1256B.

Statistical Analysis

The design of the experiment was a completely randomised design and all data collected were analysed using Analysis of Variance techniques (Steel & Torrie, 1980). Significant differences between means were separated using Duncan's multiple range test (Gomez & Gomez, 1985). A 5% confidence level was set to test statistical significance.

RESULTS AND DISCUSSION

Proximate Composition of Malted Sorghum Sprouts

The proximate composition of MSP is presented in Table 2. The dry matter value of 842.3 g kg^{-1} was recorded in this study. Crude protein (CP) value of 224.3 g kg^{-1} agrees with earlier reports of Aning *et al.* (1998) and Fafiolu *et al.* (2006) while Akinola (2002), reported CP values of 226 and 243 g kg^{-1} respectively. A lower crude fibre (CF) of 46.7 g kg^{-1} was reported in this study, which is different from 83.0 g kg^{-1} reported by Oduguwa *et al.* (2001). The generally low crude fibre of MSP may be due to the fact that hardening or lignification of the rootlets and shoots has not taken place before growth termination

during malting process at the 5th day of germination. However, Oduguwa *et al.* (2007) reported a relatively high content of neutral detergent fibre in MSP (224 g/kg). This is an indication that non-starch polysaccharides (NSP) may also play a role in limiting the nutritive value of this product.

TABLE 2
Chemical composition of MSP

Component	g kg^{-1}
Dry matter	842.
Crude protein	224.3
Crude fibre	46.7
Ash content	63.0
Ether extract	24.2
Nitrogen free extract	641.8
Ca	1.9
P	3.5
HCN (mg/kg)	1.5

A higher ether extract value of 39.8 g kg^{-1} was obtained by Akinola (2002) for alkaline-treated MSP. Aning *et al.* (1998) also reported 33.0 g kg^{-1} while 22.4 g kg^{-1} was reported in this study. The ether extract values were also understandably generally low because one does not expect tender rootlets and shoots of germinating sorghum seeds to have much oil or any appreciable lipid content for that matter. The ash content of 63.0 g kg^{-1} obtained here differed from the 16.0 g kg^{-1} reported by Aning *et al.* (1998). This difference is, however, high, and may be connected with the type of sorghum used for the MSP. The MSP used in this study was from white sorghum. However, it has earlier been reported that polyphenol levels are high in sorghum with brown pericarp and no testa and very low in unpigmented grains (Nyachoti *et al.*, 1997). The polypehnol in brown pericarp sorghum may have a binding effect on minerals. This, probably, may be the reason for low ash content in Aning *et al*'s (1998) report. Slight contamination with dust and sand particles, which increases the silica content, may also be a possibility for the relatively high ash content observed in this study. The findings, however, agrees with the report of Akinola (2002), who reported ash content of 70.0, 60.0 and 95.0 g kg^{-1} for untreated fermented and alkaline treated MSP.

Oduguwa *et al.* (2007) reported the calcium (Ca) content of MSP was 1.78 g kg^{-1}; the value of 1.9 g kg^{-1} recorded in this present study corroborates this result although an earlier study by Oduguwa *et al.* (2001) reported Ca values in MSP was as low as 0.5 g kg^{-1}. The study revealed that a value of 3.5g kg^{-1} was recorded for phosphorus and 1.5 mg kg^{-1} hydrogen cyanide (HCN) was detected in MSP. This is noteworthy and confirms earlier reports in the literature (RMRDC, 1990) that MSP contains a substance called Dhurin, which is a cyanogenic glucoside that yields HCN on hydrolysis.

Performance and Nutrient Utilisation of Pullet Fed MSP Based Diets (1-10 weeks)

Table 3 shows the performance and nutrient utilisation of pullet chicks (1-10 weeks) fed MSP based diets. Inclusion of MSP depressed ($P<0.05$) feed intake. MSP has a bitter taste, which could be traced to its content of tannin (RMRDC 1990). This may

explain why feed consumption of the birds gradually decreased ($P<0.05$) as the level of MSP in the diet increased. Morrison (1984) had earlier reported that MSP was somewhat bitter and unpalatable. The reduction in feed intake could also have been due to the gritty nature of the resulting diet. The results showed that birds fed the control diet without MSP were heavier ($P<0.05$). The higher final weight observed for the control group over those fed MSP-based diets may be as a result of low intake of MSP diets due to the influence of some anti-nutritional factors (ANFs), which are known to be present in MSP such as tannin and dhurin (HCN) (RMRDC 1990; Aning *et al.*, 1998; Fafiolu *et al.*, 2006; Oduguwa *et al.*, 2007). Abbas and Musharaf (2008) carried out a study in which sorghum seeds were germinated for 3, 5 and 7 days and the effects of germination periods on nutrient contents was determined. The result showed that by the seventh day of germination, tannin increased by 100%. Oduguwa *et al.* (2007) reported that the total tannin content of MSP was 140 g kg^{-1}, out of which only 0.3 g/kg was extractible. Tannins have been described as a group of substances with the ability to bind proteins in aqueous solution. Their multiple phenolic hydroxyl groups lead to the formation of complexes primarily with proteins and to a lesser extent metal ions, amino acids and polysaccharides (Mansoori & Acamovic, 1995, 1996; Makkar, 2001). The implication of this is very important as the availability of a large portion of the protein, and to a lesser extent amino acids and useful metal elements in MSP, would have been compromised because they are in bound form and hence, not easily accessible to enzymatic action. Another effect of tannin in the digestive tract of the birds is that it increases appreciably endogenous losses, a process that is very expensive (Oduguwa *et al.*, 2007). Mucin is a high-molecular weight glycoprotein that covers the entire luminal surface of the gastro intestinal tract (GIT), protecting the underlying epithelium, and it also comprises a significant proportion of the endogenous protein found in the digesta (Lien *et al.*, 2001). Diets high in indigestible materials e.g. NSPs tend to induce structural, morphological and cytokinetic changes in the GIT related to a capacity for high mucin secretion (Jacobs, 1986; Morel *et al.*, 2005). Feeding relatively high content of dietary tannins, a component that is indigestible and highly irritant, may also irritate the gut wall and increase the excretion of gastrointestinal mucin, a physiological waste; HCN is also a well known anti-nutritional factor (ANF) that is toxic and affects protein metabolism in farm animals. (Church & Pond, 1988). The presence of HCN in sprouted sorghum was reported by Ikediobi *et al.*, (1988); they observed that 99% of the cyanide is concentrated in the roots and shoots. The workers explained that some local food and beverages produced from sprouted sorghum grains contained negligible or undetectable levels of cyanide. This apparently is because of prior mechanical elimination of roots and shoots, coupled with heat or water treatment during processing, which they opined, was adequate for detoxifying sorghum-based food and beverage products. Taylor (1983),

TABLE 3
Performance, nutrient utilisation, plasma and serum chemistry of pullets fed MSP based diets

Parameters	Levels of MSP				
	0	150	300	SEM	p-value
Average initial weight(g)	58.8	56.9	56.0	0.41	0.201
Average final weight (g)	898.6[a]	785.2[b]	788.8[b]	22.40	0.00
Average daily feed intake (g)	56.4[a]	52.1[ab]	50.7[b]	2.47	0.033
Average daily weight gain (g)	12.1	10.4`1	10.3	6.55	0.100
Feed to gain ratio	4.7	5.0	4.9	0.39	0.303
Nutrient utilisation					
Dry matter digestibility	0.8213	0.7934	0.8137	1.53	0.110
Crude protein digestibility	0.7157[a]	0.6512[b]	0.6013[c]	0.37	0.028
Crude fibre digestibility	0.7341[a]	0.3704[b]	0.3620[c]	5.90	0.039
Ether extract digestibility	0.9801	0.9725	0.9793	0.23	0.248
Ash digestibility	0.6062[c]	0.6535[b]	0.6994[a]	3.24	0.122
Plasma and Serum metabolites					
Packed cell volume (%)	30.5[b]	32.0[ab]	33.2[a]	0.43	0.021
Mean cell volume (%)	10.2	11.0	11.2	0.27	0.319
White blood cell (mm^3)	4765.0[b]	5225.0[b]	5940.0[a]	1663	0.001
Red blood cell (gm/100ml)	47.0[a]	12.5[c]	14.5[b]	0.72	0.135
Serum total protein (gm/dl)	5.1[b]	5.6[b]	6.8[a]	0.29	0.021
Serum albumin (gm/dl)	2.5	3.0	2.9	0.11	0.228
Serum uric acid (gm/dl)	0.06	0.06	0.97	0.06	0.143
Serum creatinine (gm/dl)	0.48[b]	0.48[b]	0.90[a]	0.06	0.000

working independently, had earlier observed that there was mobilisation of HCN to the root and shoot of sorghum seedling during malting process. Thus, the presence of these ANFs coupled with the effect of NSPs definitely would have affected the growth of birds that received MSP diets (Fafiolu, *et al.*, 2006; Oduguwa *et al.*, 2007). The reduction in feed intake by birds on MSP diets may also be a contributory factor to the slow growth. No difference ($P>0.05$) was obtained in the feed-to-gain ratio and average daily weight gains of the experimental birds.

Nutrient Utilisation

Significant differences ($P<0.05$) were recorded for the digestibility of crude protein, fibre and ash. Crude protein digestibility showed a decrease as the level of MSP increased in diets. This observation confirms the report of Oduguwa *et al.* (2007). Crude protein digestibility was generally low in birds on MSP diets. This observation may not be unconnected with the presence of tannin in diets. Tannins, complex polyphenolic compounds, are known to bind proteins and cations in reactions involving them; hence, the reduction in its digestibility.

Crude fibre (CF) digestibility of the group that received 0 g kg^{-1} (MSP free diet) was significantly higher ($P<0.05$) than that in the other two diets. Holman (1989) reported that fibre present in a feedstuff was opposed to degradation by digestive enzymes secreted by certain organs of the chicks. The diet with 0 g/kg MSP had lower CF levels than the other two diets. The high fibre content of the two diets coupled with the chickens' natural inability to digest fibre may have produced a confounding effect in the birds, leading to very low CF digestibility values compared to those of the control group. The high fibre diets could also have decreased the mean transit time of the digesta (Milton & Demment, 1988) leading to lower digestion of the nutrient components including the fibre itself. Ash digestibility increased significantly ($P<0.05$) as the level of MSP increased in the diets. The elevated values may be as a result of the presence of substantial amounts of minerals in the test ingredient. The dry matter and ether extract digestibility were not significantly ($P>0.05$) affected by dietary treatments.

Plasma and Serum Chemistry of Birds Fed MSP-Based Diets

The haematological parameters of birds fed MSP-based diets are presented in Table 3. The analysis showed that packed cell volume, white blood cell, serum total protein and serum creatinine varied significantly ($P<0.05$) with dietary treatments. The mean cell volume, red blood cell, serum albumin and uric acid were not statistically ($P>0.05$) affected by the level of MSP. Birds fed 300 g kg^{-1} MSP had higher levels of packed cell volume and total serum protein. This is quite unexpected if the final weight and the weight gain observed are put into consideration.

The levels of creatinine in the serum of the group of birds fed 300 g kg^{-1} MSP diet were significantly higher ($P<0.05$) than those of other treatments. High level of creatinine in the blood is an indication of impairment of protein utilisation (Eggum, 1970; Adewusi & Bradbury, 1993). It follows, therefore, that the birds on 300 g kg^{-1} MSP were not able to incorporate the protein in the feed into their tissues despite the high levels in the blood. Perhaps protein was mobilised from other tissues other than from feed to meet the energy requirement of the birds. The same trend was also observed for the serum uric acid although the difference was not significant ($P>0.05$). This indicates physiological protein wastage by the birds.

The above observations point to the fact that some factors in the MSP were the cause of impairment in feed utilisation. Ikediobi (1989) had earlier reported that MSP contained an appreciable level of HCN. Taylor (1983) observed that there was mobilisation of HCN to the root and shoot of sorghum seedlings during the malting process. Tannin has also been indicted as an antinutritional factor that is present in sorghum products and co-products (R.M.R.D.C, 1990, Oduguwa *et al.*, 2001; Oduguwa *et al.*, 2007). The successful removal or alleviation of the effect of these anti-nutritional factors including the fibre will go a long way towards enhancing the utilisation of MSP by monogastric livestock species.

CONCLUSION

The result of this present study showed that the final body weight and body weight gained were best in birds fed 0 g kg^{-1}. It is apparent from the foregoing that inclusion of MSP even at 150 g kg^{-1} depressed feed intake and growth. It is, therefore, not advisable to use MSP at a level of up to 150 g kg^{-1} especially for starting pullets. Lower levels of inclusion could be tested to ascertain the level that these young birds can tolerate in their diet.

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Bioproteins Production from Palm Oil Agro-Industrial Wastes by *Aspergillus terreus* UniMAP AA-1

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ABSTRACT

Presently, the animal feed industry is suffering from inadequate and expensive conventional protein ingredients due to the increasing demand for food and feed products. This has led to the search for unconventional protein sources to fulfil market needs. In this study, the potential of selected palm oil wastes, namely palm pressed fibre (PPF) and palm oil decanter cake for bioprotein production, was investigated. Fermentation process was carried out aerobically in conical flasks with the working mass of 20 g each at 32°C for seven days. The performance of these palm oil wastes as substrates in solid state bioconversion of *Aspergillus terreus* UniMAP AA-1 strain were evaluated. A substrate with higher protein yield was chosen for the subsequent parameter screening using 2-level factorial design. Results showed that the protein content in PPF and palm oil decanter cake was increased up to 401 mg/L and 493 mg/L, respectively post-fermentation. Among the parameters studied, substrate concentration and inoculum size were found to significant affect bioprotein production. The highest protein content of 1683 mg/L was successfully produced from palm oil decanter cake at temperature of 35°C with 50% substrate concentration and 15% of inoculum size, suggesting its potential as an alternative protein source. Thus, this study provides preliminary data for future process optimisation of bioprotein production using the statistical approach.

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INTRODUCTION

Animal feeds with high and balanced nutritional composition determine the productivity of livestock. Presently, good quality of feeds require higher costs due to some limitations in the raw materials of feed and competition with human nutrition (Villa-Boas *et al.*, 2002). Therefore, in order to reduce the cost of animal feeds, the industry uses cheap and locally available feed ingredients such as agro-industrial by-products and waste. These agro-industrial by-products, better known as lignocellulosic wastes, are high in fibre and carbon but lacking in nutrients such as protein and vitamins (Villa-Boas *et al.*, 2002). Hence, the addition of micro-ingredients is essential to improve the nutritional content of the low-cost raw material and other advanced processes, making the final prices of feeds even higher.

One of the most promising approaches to solving this problem is by utilising microorganisms, mainly fungi, to convert agro-industrial waste to produce valuable products (Villa-Boas *et al.*, 2002; Jamal *et al.*, 2007, 2008). These wastes can be regarded as new sources for bioprotein production, which have high nutritional value with higher digestibility, do not compete with food for human consumption and is economically feasible and locally available (Jamal *et al.*, 2009; Gad *et al.*, 2010).

Bioproteins, also known as microbial protein or single cell protein (SCP), are proteins that are obtained by biosynthesis during growth and multiplication of microbial biomass. Bioproteins can be produced using varieties of microorganisms such as fungi, bacteria and algae (Anupama & Ravindra, 2000). In addition, protein produced from microbial sources is cheaper and high in nutritional value (Asad *et al.*, 2000), has a potential use as food additive, is fat binding and can be used as an alternative to costly conventional animal feed (Gad *et al.*, 2010). In addition, bioproteins produced from microbial cells could improve the digestibility of lignocellulosic materials (Villa-Boas *et al.*, 2002).

In this study, agro-industrial wastes from palm oil industries, palm oil decanter cake and palm-pressed fibre (PPF) were chosen as potential substrates for bioproteins production. The utilisation of these substrates is mainly due to their availability and can be obtained at a cheaper cost. As stated before, agricultural wastes are known to be low in protein content and high in fibre. Therefore, in order for it to be high in protein content and to improve digestibility, these wastes are fermented together with a selected fungal strain, *Aspergillus terreus* UniMAP AA-1 (Ahmad Anas & Arbain, 2012). The influence of biotechnological parameters, temperature, substrate concentration and inoculum size upon biosynthesis yield of proteins was also investigated as a basis for process optimisation in the future.

MATERIALS AND METHODS

Sample and Substrates Preparation

Palm oil decanter cake and PPF were obtained from Norstar Palm Oil Mill Sdn. Bhd situated in Kuala Ketil, Kedah,

Malaysia. The substrates were washed and dried in an oven at 60°C for 24 hours. The dried substrate was ground (RT Precision Tech.) and sieved (Retsch) to obtain 500 mm mesh size and was pretreated using 1% NaOH at 90°C for 1 hour as described by Hamisan *et al.* (2009) with some modifications. Then, the pre-treated substrates were washed until the pH was neutral (pH 7) and dried in an oven (Sartorius) at 60°C for 24 hours. The substrates were kept in separate air-tight containers until further use.

Microorganism and Fungal Inoculum Preparation

The *Aspergillus terreus* UniMAP AA-1 strain was obtained from the School of Bioprocess Engineering culture collection (School of Bioprocess Engineering, UniMAP, Perlis, Malaysia) (Ahmad Anas & Arbain, 2012) and was grown on potato dextrose agar (PDA) at 32°C. Inocula were prepared by washing the growing culture with 25 mL of sterile distilled water. The spore suspensions were rubbed and adjusted to final concentration of 10^7 spores per ml. The suspension inocula were kept in a chiller at 4°C for further use.

Fermentative Media Preparation

Evaluation of the potential of palm oil decanter cake and palm-pressed fibre (PPF) as fermentative substrates was carried out in order to determine the best and potential substrate that can be used to produce the highest bioproteins by applying the solid-state fermentation approach. Each substrate was evaluated using a constant amount of substrate concentration, inoculum size and media composition and process condition. The 70% of moisture content was maintained for every 6 g substrate in a conical flask. The 70% was equivalent to 14 mL of solution, which divided into 0.4 mL of inocula suspension and 13.6 mL of growth media solution that contained 0.2% of KH_2PO_4, 0.5% of NH_4NO_3 and 0.1% each NaCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$. The solution was autoclaved prior to usage.

Solid-State Fermentation

Solid-state fermentation was carried out in a 250 mL flask with the working mass of 20 g consisting of 30% substrate, 2% of inocula and 68% of growth media (0.2% of KH_2PO_4, 0.5% of NH_4NO_3 and 0.1% each of NaCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$. Samples were prepared in duplicate and incubated at 32°C for seven days. For the testing of biotechnological process parameters' influence upon yield of protein production, the amount of substrate used was in a range of 30 to 50% (w/w), while 5 to 15% (v/w) of inoculum size was inoculated into the fermentation medium. The temperature of the fermentation process was varied between 30 and 35ºC. These values were tabulated according to data indicated in Table 1 samples were incubated for six days.

Total Protein Determination

The biomass was withdrawn daily for analysis. The samples were dried for 24 hours at 60°C. Dried samples were

TABLE 1
2-Level factorial design for screening of process parameters with actual values and observed results

Run	Temperature (℃)	Substrate (% w/w)	Inoculum size (% v/w)	Protein content (mg/L)
1	30	30	5	582
2	30	30	5	565
3	35	30	5	678
4	35	30	5	715
5	30	50	5	1287
6	30	50	5	939
7	35	50	5	1153
8	35	50	5	1061
9	30	30	15	548
10	30	30	15	611
11	35	30	15	939
12	35	30	15	592
13	30	50	15	1189
14	30	50	15	1481
15	35	50	15	1507
16	35	50	15	1683
17	32.5	40	10	1287
18	32.5	40	10	1238

macerated in a pestle and 50 mL of 1N NaOH was added and incubated at 4^{o}C, overnight. The mixtures were then centrifuged at 8000 rpm for 20 minutes. The supernatant obtained was kept in the refrigerator for further analysis. The protein content in the supernatant was analysed using Lowry method (Lowry *et al.*, 1951).

Screening of Process Parameters

Two-level factorial design was carried out for screening of process parameters using the statistical software package Design Expert Software (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, version 7.1.5) and the statistical analysis of experiment data. The screening process was done in duplicate involving three parameters, temperature, substrate concentration and inoculum size as shown in Table 1. All the parameters values were varied at two levels, which were -1 and +1. Value -1 indicates low level and +1 indicates high level.

RESULTS AND DISCUSSION

The fermentation was performed at constant media composition and process conditions in order to study the potential of palm oil wastes as substrates of the highest bioprotein concentration in fungal biomass. The fermentation was carried out for seven days and protein concentration was analysed daily. The production profiles for each substrate are illustrated in Fig.1.

From Fig.1, it can be observed that the proteins produced by each substrate have the same trend but differ in concentration. Protein production by PPF was gradually increased from 159 mg/L after the first day of fermentation to the maximum concentration of 401 mg/L after five days of fermentation. The protein concentration gradually decreased after six days of fermentation and on day seven, the protein concentration dropped to 199 mg/L.

In comparison, the protein concentration of fermented palm oil decanter cake also gradually increased over the fermentation time. The maximum protein concentration was obtained on the sixth day of fermentation while the lowest protein concentration was observed during the first day of fermentation, which was 146 mg/L. Unlike PPF, the protein content of the decanter cake started to decrease at day 7 and the protein content was 337 mg/L.

Fig.1 depicts that the protein produced by decanter cake was higher than the protein concentration of PPF. It is known that the growth of fungi mainly depends on the carbon, nitrogen and inorganic sources as their nutritional sources, and the main nutrients are carbon sources such as cellulose and hemicelluloses (Amal Nafissa *et al.*, 2008). Oil palm agro-industrial wastes such as PPF and palm oil decanter cake contain high amounts of lignocellulosic materials, which can act as a carbon source for the growth of fungi. According to Mahlia *et al.* (2000), PPF contains 47.2% carbon and 1.4% nitrogen while decanter cake contains 33% carbon and 3.6% nitrogen (Parveen *et al.*, 2010). Since both substrates consist of a sufficient amount of carbon and nitrogen, this factor contributes significantly to their capability in bioprotein production.

Fig.2 describes the comparison of bioprotein production for each substrate.

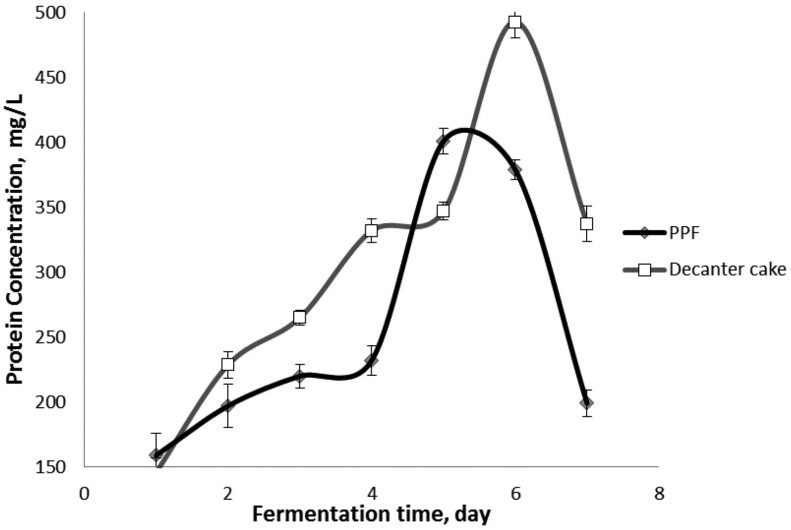

Fig 1: Profile of bioprotein production using different substrates.

The protein content of solid state cultivation on PPF was increased by 2.5 fold while the protein content of palm oil decanter cake was increased over three folds. In addition, the result also showed that the protein increment in palm oil decanter cake was higher than PPF by 85%.

Literature data described incorporations of different type of substrates and microorganisms where protein enrichments were successfully demonstrated. Among these microorganisms, fungi are the most commonly used for bioprotein production as they have the ability to secrete large amount of protein into the growth medium (Jahwarhar *et al.*, 2011). A number of studies were conducted using *Aspergillus niger* with various substrates including coconut dregs, orange waste, empty fruit bunch and palm kernel cake (Marini *et al.*, 2008; Hafiza *et al.*, 2011, 2012, Khadijah Hanim *et al.*, 2012; Alemu, 2013). In addition, *Aspergillus terreus* was employed in combination with rice, *Eichhornia* (water hyacinth) and banana peel (Shahzad & Rajoka, 2011; Jaganmohan *et al.*, 2013), while palm kernel cake was utilised with newly isolated *Rhizopus orizae* ME01 (Mohd Firdaus *et al.*, 2013).

In order to compare bioprotein production, Jaganmohan *et al.* (2013)

TABLE 2
Statistical analysis of 2-level factorial design for each variable

Process parameter	Main effect	F-value	p-value	Confidence level (%)
Temperature	140.75	4.40	0.0599	94.01
Substrate Concentration	633.75	89.17	0.0001	99.99
Inoculum Size	196.25	8.55	0.0138	98.62

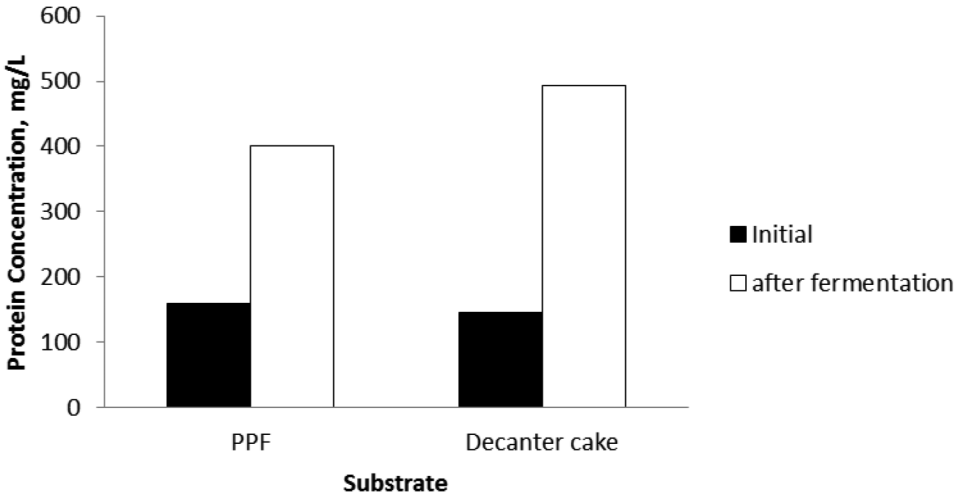

Fig 2: Comparison of bioprotein produced for each substrate.

performed solid-state fermentation using *Aspergillus terreus* with different raw materials namely rice bran, wheat bran, banana peel and combinations of these agricultural wastes for five days at room temperature. *Eichhornia* and banana peel mixture produced the highest mycelial protein, surpassing other substrate combinations with 6 mg/g of yield following process parameters and media optimisation. Conversely, Hafiza *et al.* (2012) studied the application of *Aspergillus niger* for bioprotein production from coconut dregs at 32°C for seven days and achieved a huge protein production of 76.6 mg/g. However, in another study using similar fungi with empty fruit bunches as substrate, the protein produced was significantly low (Khadijah Hanim *et al.*, 2012).

In comparison, protein produced in the present study was slightly lower than that reported earlier (Hafiza *et al.*, 2012, Jaganmohan *et al.*, 2013). The possible reason could have been the difference in nutritional content of the substrates used. As compared to PPF and decanter cake, banana peel contains several important nutrients such as potassium, calcium, sodium, iron, numerous essential amino acids, starch, hemicelluloses and important simple sugars. Moreover, the degradative reactions caused by endogenous enzyme might have increased its sugar content (Saheed *et al.*, 2012). A proximate analysis of coconut dregs revealed that it contained 56.5% (w/v) of carbohydrates, 3.5% (w/v) protein, 24.1% (w/v) of crude fibre and 515 Kcal/100g of energy (Hafiza *et al.*, 2012). These nutritional values obviously are more than those available in the substrate used, thus contributing to a higher fungal growth and subsequently, bioprotein production. However, the low protein yield can be maximised by screening the parameters that influence the yield followed by optimising them using statistical approach.

Since the protein content in palm oil decanter cake increased dramatically after fermentation, the protein content in this substrate has potential to be improved. Thus, decanter cake has shown a better performance as a substrate for bioprotein production and was selected to undergo a screening process prior to optimisation.

Screening of Process Parameters Affecting Bioprotein Production

The screening of process parameters was done according to 2-level factorial design to evaluate the significant process parameters for bioprotein production (Table 1). The highest protein concentration obtained was 1683 mg/L while the lowest protein concentration was 548 mg/L. Analysis of variance (ANOVA) results are presented in Table 2. The R-squared for this experiment was 0.9085. The significant parameters were substrate concentration and inoculum size since the parameters showed a confidence level of above 95% and p-value less than 0.0500 (Table 2). The main effect for each variable was estimated and graphically presented in Fig.3, which revealed that substrate concentration has the most positive effects on the fungal bioproteins production followed by inoculum size. This positive

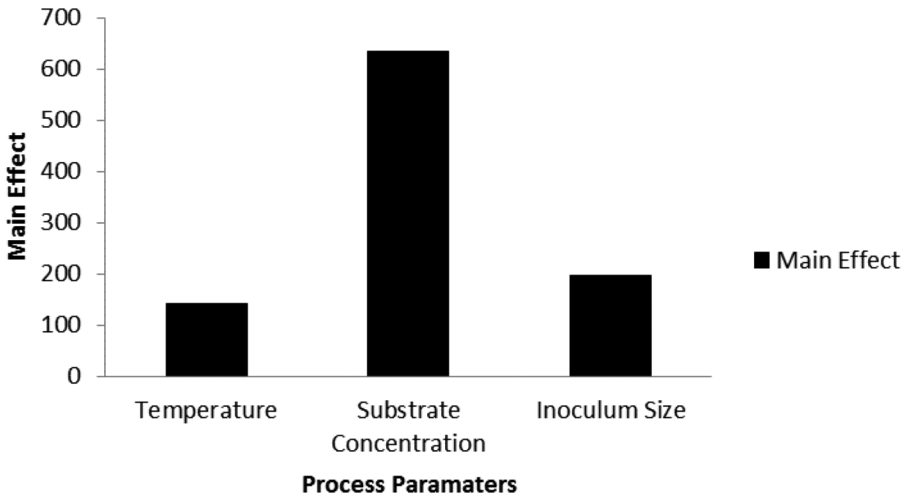

Fig 3: Main effects of process condition on bioprotein production.

effect means that these parameters will increase the fungal bioproteins production by increasing their concentrations and nutritional quality.

The substrate concentration shows the highest positive effect on bioprotein production. Substrate concentration plays a crucial role in solid-state fermentation. Mostly, the substrate concentration used for solid state concentration is around 50-55%, which is equivalent to moisture content of 45-50% (Iluyemi *et al.*, 2006). Variation of moisture content influences microbial growth. Lower moisture content causes reduction in solubility of substrates, low degree of swelling and high water tension (Kheng & Omar, 2005). Increasing moisture content can lead to the reduction of substrate porosity, thus limiting oxygen transfer into the substrat,e which in turn results in decrement of fungi growth and product formation (Abdeshahian *et al.*, 2010). In a previous study by Abdeshahian *et al.* (2010), the cultivation of *A. niger* strain under solid state fermentation produced higher levels of mananase at substrate concentration of 40% to 60% and the production began to reduce when lower levels of substrate concentration was applied (20-30%). This substrate concentration contributed to the highest confidence level of 99.99%, which proved that it is important for optimisation of bioprotein production.

Fig.3 shows that besides the substrate concentration, inoculum size also provided a significant effect on bioprotein production. In a previous study, it was demonstrated that higher inoculum size of *P. chrysosporium* produced more bioproteins (Gad *et al.*, 2010). Higher inoculum size should produce more bioproteins since the term bioproteins itself refers to the total protein extracted from microbial biomass such as fungal, yeasts and bacteria (Handan *et al.*, 2002), provided sufficient substrate concentration is available.

In this study, fermentations were carried out at temperature range of 30 to 35°C. The cultivation temperature was found to be an insignificant factor for bioprotein production due to their confidence level of less than 95%. The temperature for fungal growth varies between species; the most optimum is between 25ºC and 30ºC (Pietikainen *et al.*, 2005). In a study by Jaganmohan *et al.* (2013), it was reported that protein yield and biomass turnover of *A. terreus* increased at 25ºC to 35ºC and decreased gradually to 45°C. Ravinder *et al.* (2003), also studied the effect of temperature on protein yield in *A. oryzae* mutants. They discovered that protein production increased when the temperature was between 20 and 35ºC and decreased rapidly when approaching 45°C. For other species such as *Candida utilis*, *Chetomnium* sp. and *A. niger*, maximum protein production was at 32ºC (Li *et al.*, 2009; Yalemtesfa *et al.*, 2010) Therefore, the selection of temperature range is an important factor when observing fungal growth as to compensate for the diversity between species. In this case, a greater range is expected to provide better profiles, thus significantly justifying the influence of temperature in bioprotein production.

CONCLUSION

This study aimed to explore the potential of palm oil wastes to be used as a substrate for bioprotein production. Based on the experimental results, the solid state bioconversion of palm-pressed fibre (PPF) and palm oil decanter cake with a selected *Aspergillus terreus* UniMAP AA-1 strain successfully increased the protein content of the fermented final by-product from 159 mg/L to 401 mg/L for PPF and from 146 mg/L to 493 mg/L for palm oil decanter cake. Based on this protein production profile, oil palm decanter cake has higher potential thus, should be selected to undergo screening processes. Substrate concentration and inoculum size were identified by 2-level factorial design as important parameters for improving the production of bioproteins by solid-state fermentation on oil palm decanter cake. Protein production dramatically increased to 1683 mg/L following screening at temperature 35°C with substrate concentration of 50% (w/w) and inoculum size of 15% (v/w). This study provides preliminary data for future studies in optimisation of media composition and process condition using the statistical approach.

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Assessment of Genetic Variation in Selected Germplasm of White Jute (*Corchorus capsularis* L.)

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ABSTRACT

Fifty-one genotypes of white jute from different geographic origins were evaluated to study their genetic variability with 11 morphological characters. Significant variation was observed among the genotypes for all the characters. Multivariate techniques were used to classify 51 genotypes. All the genotypes were grouped into six different clusters. Principal component analysis, principal coordinate analysis and canonical vector analysis gave similar results to that of cluster analysis. The highest inter-genotypic distance (1.84) was found between G15, G50 and the lowest distance between G38 and G26. The highest inter-cluster distance (14.37) was observed between cluster I, IV and the lowest distance (2.46) was between cluster III and V. The highest intra-cluster distance was found in cluster I and lowest in cluster V. Considering genetic parameters, high genotypic coefficient of variation (GCV) was observed in branches per plant. High heritability values with moderate genetic advance in percentage of mean were obtained for leaf width, petiole length, nodes per plant. Regarding the cluster distance, inter-genotypic distance and other agronomic performance, the genotypes G47, G33, G48 from cluster I; G27, G17, G23 from cluster III and G13, G40, G45 from cluster II were considered to be better parents for future use in hybridisation programmes.

Keywords: Principal component and coordinate analysis, canonical vector analysis, genotypic coefficient of variation, phenotypic coefficient of variation, white jute

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INTRODUCTION

The crop 'jute' belongs to the genus *Corchorus* and is the most important natural fibre crop next to cotton (Samanta *et al*., 2011). Jute is basically self-pollinated and has 14 diploid chromosomes (2n=14). The genus *Corchorus* contains about 50-60 genotypes, which are distributed throughout the tropical regions of Africa. Jute, the bast fibre, is obtained from the bark of two cultivated species of the genus, namely *Corchorus capsularis* L. and *Corchorus olitorius* L. of the family Tiliaceae. *Corchorus capsularis* L. originates from Bangladesh, India, Myanmar and South China (Singh, 1976).

Currently, the number of recommended jute varieties is limited in terms of meeting the requirements of wide agro-ecological conditions. Most of these varieties are quite old and have a narrow genetic base and are susceptible to various biotic and abiotic stressors such as insects, pests, diseases, drought, water logging and low temperature, among others. All these factors combined with the increasing demand of jute in the world market call for new types of jute to be developed to meet the requirement of various agro-industries. In order to increase the frequency of desired genotypes in breeding progenies, superior parents with high breeding values are needed. However, development of such parents is a long-term and tedious job. Islam and Ahmed (2003) studied variability in jute genotypes and revealed significant differences for all the characters with a wide range of variability. Considerable amount of genotypic variances was obtained for fibre weight per plant, stick weight per plant and plant height. Ahmed *et al*. (1993) reported the phenotypic coefficient of variation was relatively higher than the genotypic one for all characters. Plant height, basal diameter and dry fibre weight had high broad sense heritability estimate coupled with a moderate high genetic advance indicating the success of direct selection (Sardana *et al*., 1990). Node number was found to have low heritability and genetic advance. The quantification of genetic diversity and variability through biometrical procedures has made it possible to make selection based on geographic diversity alone but this is not always justified. Moreover, selection of parents' evaluation of genetic diversity is important to know the source of genes for a particular trait in the available germplasm (Tomooka, 1991). Mostafa *et al*. (2002) reported that high genotypic and phenotypic coefficients of variation were found in dry fibre yield, green weight and stick weight. Under the present context of global environment prospective, jute is getting highest priority as biodegradable agro-industrial crop. Therefore, the investigation was undertaken to assess the variability present in different genotypes and to select the desirable parents for hybridisation programmes.

MATERIALS AND METHODS

Site Description

The study was carried out at the Jute Agricultural Experiment Station of Bangladesh Jute Research Institute (BJRI), Manikganj, Bangladesh from April to

August, 2010. The experimental site was situated in the tropical climate zone, at 23.85 ºN latitude and 90.01 ºE longitude and characterised by heavy rainfall during the months of May to September and scant rainfall during the rest of the year.

Plant Materials

The experimental material comprised 51 genotypes of white jute (*C. capsularis*) including three improved varieties, CVL-1, BJC-7370 and CVE-3. The genetically pure and physically healthy seeds of these genotypes were collected from the gene bank of the Bangladesh Jute Research Institute (BJRI), Dhaka. Accession number and origin of the genotypes are presented in Table 1.

TABLE 1
Accession number and origin of the selected genotypes of white jute (*C. capsularis* L.)

Serial No.	Genotype No.	Accession number	Country of origin/Place of collection
1	G1	890(CVL-1)	Bangladesh
2	G2	860	India
3	G3	4616	Brazil
4	G4	4591	Nepal
5	G5	4872	Thailand
6	G6	4926	China
7	G7	72	Bangladesh
8	G8	4617	Brazil
9	G9	2212	USA
10	G10	1513	India
11	G11	4619	Brazil
12	G12	4700	Brazil
13	G13	4956	China
14	G14	77	Bangladesh
15	G15	4706	Brazil
16	G16	4961	China
17	G17	5125(BJC-7370)	Bangladesh
18	G18	2214	USA
19	G19	4474	Thailand
20	G20	1514	India
21	G21	858	India
22	G22	2215	USA
23	G23	891 (CVE-3)	Bangladesh
24	G24	80	Bangladesh
25	G25	4468	Thailand
26	G26	1832	Bangladesh
27	G27	944	-
28	G28	877	India
29	G29	859	India
30	G30	2020	India
31	G31	2216	USA
32	G32	4472	Thailand
33	G33	78	Bangladesh
34	G34	5060	-
35	G35	4463	Thailand
36	G36	4699	-
37	G37	4710	Nepal
38	G38	2219	USA
39	G39	4879	Nepal
40	G40	2019	India
41	G41	1515	Nepal
42	G42	4951	Nepal
43	G43	70	Bangladesh
44	G44	947	India
45	G45	74	Bangladesh
46	G46	4871	Thailand
47	G47	3693	China
48	G48	865	India
49	G49	75	Bangladesh

cont'd Table 1

50	G50	4615	Brazil
51	G51	861	India

Note: G = Genotype

Seed Sowing, Intercultural Operation and Data Collection

The experiment was laid out in Randomised Complete Block Design (RCBD) with three replications. Each plot had a single row of 3.6 m length. The space between rows was 0.30 m and block-to-block distance was 1.0 m. The genotypes were randomly distributed to each row within each block. Seeds were sown on 2 April, 2010. Thinning and weeding were done twice after 15 and 45 days of sowing to maintain uniform plant population. Insecticide was not applied. Hand-picking was practised to control the hairy caterpillar at larval and pupal stage. The data were recorded on five randomly taken plants from each row of each genotype. Mean values for each characters in each plot were used for statistical analysis.

Statistical Analysis and Genetic Parameters

The analysis of variance was done following the ANOVA test and the mean values were adjusted by DMRT (P=0.05) method (Steel & Torrie, 1981). Phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) were evaluated according to the methods of Johnson *et al*. (1955). Genetic advance (GA) expected and GA as percent of the mean assuming selection of the superior 5% of the genotypes were estimated in accordance with the methods illustrated by Johnson *et al*. (1955) and Robinson *et al*. (1949).

Cluster Analysis

Using Mahalanobis D^2-statistics and its auxiliary analysis assessed genetic divergence among the genotypes studied. Both techniques estimate divergences among a set of genotypes on multivariate scale. The Mahalanobis distance $(D)^2$ values were calculated from transformed uncorrelated means of characters (Bansal *et al*., 1999). The D^2 values of genotypes were arranged in order of relative distance from each other and a method suggested by Bansal *et al*. (1999) was used for cluster formation.

RESULTS AND DISCUSSION

The principal component analysis gave Eigen values for each principal component axes of coordination of genotypes with the first axes accounting totally for the variation among the genotypes, whereas four of these Eigen values above unity accounted for 90.81% (Table 2).

Fifty-one genotypes of white jute were grouped into six different clusters with the application of Mahalanobis's D^2 statistics (Table 3). Ghosh *et al*. (2013) reported five clusters in *C. capsularis* and four clusters in *C. olitorius* while Golakia and Makne (1992) found five and seven clusters, respectively, in groundnuts. These results confirmed the clustering pattern of the genotypes according to the principal component analysis. The results presented

in Table 3 represent the composition of different clusters with their corresponding genotypes and origin included in each cluster. A maximum of 10 genotypes were in cluster V and VI, followed by eight in cluster II, III and IV. There were seven genotypes in cluster I. The genotypes of cluster I produced the highest cluster mean for plant height (2.66), base diameter (19.35), node per plant (54.11), green weight per plant (219.71), stick weight per plant (48.17) and fibre yield per plant (17.35) (Table 4). Cluster II represented eight genotypes of this group that produced the cluster mean for plant height (2.51), base diameter (16.96), green weight per plant (180.37), stick weight per plant (38.04) and fibre yield per plant (11.99). This group contained the second highest cluster mean in branches per plant (2.40) (Table 4).

Inter-genotypic distances obtained from principal component analysis showed that the greatest distance (1.84) was observed between the genotypes G50 and G15 followed by G21 and G2 (1.84), G41 and G2 (1.83), G39 and G2 (1.82), G31 and G2 (1.80), and shortest distance (0.23) was found between the genotypes G36 and G26 followed by G42 and G26 (0.25), G39 and G29 (0.27), G34 and G25 (0.27), G32 and G29 (0.28) (Table 5). Inter cluster distances were calculated from inter-genotypic distances (Table 6). The greatest intra-cluster distance was observed in cluster I (0.83), which was composed of seven genotypes followed by cluster II (0.78) (composed of eight genotypes). Both cluster III (0.74) and IV (0.63) were composed of eight genotypes.

Cluster V showed the shortest intra-cluster distance (0.61), which contained 10 genotypes followed by cluster IV (0.64) (composed of eight genotypes). Cluster VI (0.72) had 10 genotypes. These results revealed that the genotypes in cluster I were distantly related. On the other hand, the genotypes in cluster V were closely related. Cluster III was composed of eight genotypes (Table 3). Genotypes of this group produced the highest cluster mean for leaf length (14.12). This group contained the second highest cluster mean value for plant height (2.60), leaf width (5.27), petiole length (5.04), base diameter (17.68), nodes per plant (52.29), stick weight per plant (40.71) and fibre yield per plant (13.97), respectively. Cluster IV also contained eight genotypes. This cluster had the highest cluster mean for leaf width (5.30) and petiole length (5.10). This group contained a cluster mean value for plant height (2.54), base diameter (17.04) and fibre yield (8.71). Cluster V was composed of the highest 10 genotypes. The highest cluster mean was observed in branches per plant (2.78). This group contained the lowest nodes per plant (47.58). This cluster showed medium mean values for other characters. Cluster VI also contained 10 genotypes. The highest cluster mean was found in leaf angle (78.83). This group contained second lowest cluster mean value for leaf width (4.92), branches per plant (2.03), stick weight (27.40) and fibre yield per plant (9.69) (Table 4).

TABLE 2
Eigen values and percentage of variation in respect of eleven characters in white jute (*C. capsularis* L.) germplasm

Parameters	Eigen values	Percentage of total variation accounted for individual characters	Percentage of cumulative variation
Plant height (m)	7.96	46.10	46.10
Leaf angle (degree)	4.92	28.46	74.56
Leaf length (cm)	1.43	8.30	82.86
Leaf width (cm)	1.37	7.95	90.81
Petiole length (cm)	0.61	3.52	94.33
Base diameter (mm)	0.36	2.12	96.45
Nodes / plant	0.23	1.34	97.79
Branch / plant	0.15	0.87	98.66
Green weight (gm)	0.11	0.66	99.32
Stick weight (gm)	0.09	0.50	99.82
Fibre yield / plant	0.03	0.18	100.00

TABLE 3
Distribution of 51 genotypes of white jute (*C. capsularis* L.) germplasm in six clusters

Cluster	Number of genotypes	Genotype number	Accession number
I	7	8, 12, 14, 15, 33, 47, 48	4617, 4700, 77, 4706, 78, BJC83, 865
II	8	7, 13, 16, 22, 37, 40, 43, 45	72, 4956, 4961, 2215, 4710, 2019, 70, 74
III	8	3, 6, 10, 17, 18, 23, 27, 46	4616, 4926, 1513, BJC7370, 2214, CVE3, 944, 4871
IV	8	11, 24, 28, 32, 35, 36, 49, 50	4619, 80, 877, 4472, 4463, 4699, 75, 4615
V	10	1, 2, 5, 20, 21, 29, 30, 31, 34, 51	CVL-1, 860, 4872, 1514, 858, 859, 2020, 2216, 5060, CVE3
VI	10	4, 9, 19, 25, 26, 38, 39, 41, 42, 44	4591, 2212, 4474, 4468, 1832, 2219, 4879, 1515, 4951, 947

TABLE 4
Cluster means for eleven characters in white jute (*C. capsularis* L.)

Parameters	Cluster					
	I	II	III	IV	V	VI
Plant height (m)	2.66	2.51	2.60	2.54	2.48	2.57
Leaf angle (dg)	77.94	78.77	75.96	76.37	75.10	78.83
Leaf length (cm)	13.34	13.76	14.12	13.53	13.56	13.67
Leaf width (cm)	5.14	5.20	5.27	5.30	4.80	4.92
Petiole length (cm)	4.93	5.04	5.04	5.10	4.55	4.74
Base diameter (mm)	19.35	16.96	17.68	17.04	16.44	17.06
Nodes/plant	54.11	50.02	52.29	49.47	47.58	51.69
Branches/plant	2.21	2.40	2.19	1.88	2.78	2.03
Green weight (gm)	219.71	180.37	137.88	114.32	133.31	160.31
Stick weight (gm)	48.17	38.04	40.71	26.18	29.74	27.40
Fibre yield/plant (gm)	17.35	11.99	13.97	8.71	10.13	9.69

TABLE 5
Ten higher and lower inter-genotypic distance (D^2) between pairs of white jute (*C. capsularis* L.) genotypes of different clusters

10 higher D^2 values	Genotypes Combination (GC)	10 lower D^2 values	Genotypes Combination (GC)
1.8441	G50 & G15	0.2328	G36 & G26
1.8389	G21 & G2	0.2527	G42 & G26
1.8273	G41 & G2	0.2712	G39 & G29
1.8171	G39 & G2	0.2729	G34 & G25
1.7973	G31 & G2	0.2766	G32 & G29
1.7944	G17 & G2	0.2776	G10 & G3
1.7914	G40 & G2	0.2800	G23 & G3
1.7816	G15 & G2	0.2830	G44 & G25
1.7587	G44 & G2	0.2869	G37 & G8
1.7296	G50 & G2	0.2880	G28 & G26

Note: G = Genotype

The two important economic characteristics of jute plant are the fibre and stick yield per plant. In the case of fibre yield, cluster I possessed the highest mean values followed by cluster III, cluster II, cluster V, cluster VI and cluster IV (Table 4). The clustering pattern of genotypes did not follow geographical distribution. The genotypes evolved at one centre even exhibited considerable amount of diversity and was grouped into different clusters, including geographical diversity that may not necessarily be related to genetic diversity. This result is in conformity with the findings of Sinha *et al*. (1991). The probable cause of this situation might be due to frequent movement of plant material through introduction. Varieties developed at the same place have different genetic makeup. Certain entries also possessed similar characters even though they had their origin in different places. One of the reasons could be that the farmers from one place might have used different cultivars from various sources. That is why enormous variability in the materials even in a single location might arise.

To compute the inter-cluster Mahalonobis' (D^2) values, canonical variate analysis was used. The intra and inter-clusters for distance (D^2) values are presented in Table 6. The greatest inter-cluster distance (14.37) was between cluster I and IV, indicating wider genetic diversity between these two clusters followed by cluster I and V, I and III, II and IV, I and VI, II and V. The lowest inter-cluster distance (2.46) was found between the cluster III and V, suggesting a closer relationship among the genotypes followed by IV and V, II and VI, III and IV, V and VI, III and VI and so on included in these clusters. Similar distance was found between cluster III and V, IV and V, II and VI and III and IV, reflecting a close relationship among these clusters (Table 6). However, the maximum inter-cluster distance was recorded between cluster I and IV (14.64). Genotypes from cluster I and IV having the greatest distance if involved in hybridisation might produce a wide spectrum of segregating population. It is the theoretical concept that the maximum amount of heterosis would be obtained in hybrids involving the genotypes belonging to the more divergent origins. However, for a plant breeder the objective is not only to get high heterosis but also to achieve a high level of production by improving and utilising other yield contributing traits so that it could be adjusted in various types of cropping systems rather than getting only high heterosis. The intra-cluster distance varied from 0.61 to 0.84, having the highest in cluster I, which was composed of seven genotypes of diverse origin, while the minimum distance was found in cluster V, which comprised 10 genotypes (Table 6).

The extent of variation among the genotypes in respect of 11 characters was studied and the means value, range and coefficient of variation are presented in Table 7. Variance of the genotypes, genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), environmental coefficient of variation (ECV), heritability and genetic advance are

shown in Table 8. Significant differences were observed among the genotypes for plant height. Plant height ranged from 2.04 to 3.02 m and mean height was 2.56 m (Table 7). The moderate heritability (37.40) together with considerable genetic advance (8.77%) indicated the effectiveness for selection of this character (Table 8). Similar results were also reported by Chaudhury *et al*. (1981) in jute. Significant differences among the genotypes were recorded in leaf angles per plant. The maximum leaf angle was 84.08 dg and the minimum and mean values were 66.30 and 7713 dg, respectively (Table 7). The phenotypic coefficient of variation (8.01) and genotypic

TABLE 6
Average intra (diagonal) and inter-cluster distances (D^2) for 51 white jute (*C.capsularis* L.) genotypes

Cluster	Cluster					
	I	II	III	IV	V	VI
I	**0.835**					
II	5.557	**0.781**				
III	10.920	5.587	**0.737**			
IV	14.367	8.838	3.775	**0.635**		
V	12.032	6.475	2.458	2.504	**0.609**	
VI	8.638	3.285	4.208	6.335	3.831	**0.715**

TABLE 7
Estimation of statistical parameters of 10 different characters of fifty-one different genotypes of white jute (*C. capsularis*)

Characters	Range	Mean	CV%
Plant height (m)	2.04-3.02	2.56	9.03
Leaf angle (dg)	66.30-84.08	77.13	5.50
Leaf length (cm)	11.04-15.26	13.67	8.89
Leaf width (cm)	3.90-6.45	5.09	8.52
Petiole length (cm)	3.82-6.15	4.88	13.17
Base diameter (mm)	14.50-23.58	17.33	11.47
Nodes/plant	37.33-68.87	50.70	11.02
Branches/plant	1.00-10.67	2.26	27.89
Green weight (gm)	97.50-238.1	155.6	12.05
Stick weight (gm)	17.46-63.05	34.27	7.97
Fibre yield/plant (gm).	6.98-28.12	11.70	10.46

(5.82) coefficient of variation were close to each other, indicating less environmental influence in case of leaf angle (Table 8).

The mean value of leaf length showed significant differences among the genotypes. The minimum and maximum leaf length was 11.04 and 15.26 cm, respectively (Table 7). The phenotypic variance (2.08) is higher than the genotypic variance (0.60). Heritability was low (28.96) and genetic advance as percentage of mean was low (6.29) (Table 8). With such low heritability and low genetic advance, selection on leaf length would not be judicious. Leaf area was significantly variable among the genotypes. The mean value for leaf width was 5.09 cm (Table 7). The phenotypic variance (0.51) and genotypic variance (0.33) were close to each other, indicating negligible environment influence on leaf width. Moderate high heritability (63.42) with considerable genetic advance (18.41%) for this trait might be taken into consideration (Table 8) while selecting a suitable line as suggested by Sardana *et al.* (1990). Similar results were found by Ghosdastidar and Bhaduri (1983). The petiole length showed significant differences among the genotypes. It ranged from 3.82 to 6.15 cm with a mean value of 4.88 cm (Table

TABLE 8
Estimation of genetic parameters of 10 different characters of fifty-one different genotypes (*C. capsularis*)

Characters	σ^2g	σ^2p	σ^2e	GCV	PCV	ECV	h^2b	GA (5%)	GA in % of Mean (5%)
PH (m)	0.03	0.08	0.05	6.96	11.38	9.01	37.40	0.22	8.77
LA (dg)	20.15	38.14	17.99	5.82	8.01	5.50	52.83	6.72	8.71
LL (cm)	0.60	2.08	1.48	5.67	10.54	8.89	28.96	0.86	6.29
LW (cm)	0.33	0.51	0.19	11.22	14.09	8.52	63.42	0.94	18.41
PL (cm)	0.20	0.62	0.41	9.27	16.11	13.17	33.10	0.54	10.98
BD (mm)	1.70	5.65	3.95	7.53	13.72	11.47	30.10	1.47	8.50
NP	20.34	51.54	31.20	8.89	14.16	11.02	39.46	5.84	11.51
BP	2.11	2.51	0.40	64.26	70.05	27.90	84.13	2.75	121.41
GW (gm)	1153.67	1507.85	354.18	21.75	24.86	12.05	76.51	61.20	39.19
StW (gm)	15.04	15.91	0.87	33.13	34.08	7.97	94.53	7.77	66.35
Fibre yield (gm)	92.81	105.66	12.85	28.11	29.99	10.46	87.84	18.60	54.27

Note: PH = Plant height (m), LA = leaf angle (dg), LL = leaf length (cm), LW = Leaf width (cm), PL = Petiole length (cm), BD = Base diameter (mm), BP = branches per plant, NP = Nodes/plant, GW = Green weight (gm), StW = Stick weight (gm) and FW = fibre weight per plant (gm), σ^2g = genotypic variance, σ^2p = phenotypic variance, σ^2e = error variance, GCV = genotypic coefficient of variance, PCV = phenotypic coefficient variance, ECV = error coefficient variance, GA = genetic advance

7). The phenotypic variance (0.62) was much higher than the genotypic variance (0.20). The heritability (33.10) was low with low genetic advance (10.98%) (Table 8). With such low heritability and low genetic advance, selection on petiole length was not judicious. The base diameter also showed significant differences among the genotypes. It varied from 14.50 to 23.58 mm and the mean value was 17.33 mm (Table 7). This trait showed higher differences of phenotypic coefficient of variation than the corresponding genotypic coefficient of variation (Table 8). The higher differences of PCV and GCV suggest that the expression of character was mostly under the control of environment. Low heritability (30.10) and low genetic advance (8.50) indicated that the selection for this character would not be effective. The results of this experiment support the findings of Islam *et al*. (2002), who found higher PCV than the corresponding GCV value and heritability coupled with low genetic advance for basal diameter. The node number was significantly varied among the genotypes. The maximum and minimum node numbers were 68.87 and 37.33, respectively (Table 7). The phenotypic coefficient of variation (14.16) and genotypic coefficient of variation (8.89) closely related to each other. It showed moderate high heritability (39.46%) with considerable genetic advance (11.51) (Table 8). Similarly, the greatest genetic advance (35.5%) and highest heritability (52.9%) were found in fibre yield (Ahmed *et al*., 1993). The mean value for number of branches per plant showed significant differences among the genotypes. The highest and lowest branches per plant were 10.67 and 1.00, respectively. The high heritability (84.13%) with high genetic advance (Table 8) indicated that this trait could be taken into consideration while selecting suitable genotypes for a breeding programme. Significant differences were observed among the genotypes in respect of green weight. Green weight ranged from 97.50 to 238.1 gm (Table 7). The estimates of phenotypic variance were very high (1507.85). Heritability (76.51%) and genetic advance (39.19) were also very high (Table 8). Differences between phenotypic and genotypic coefficient of variation were small.

Stick weight ranged from 17.46 to 63.05 gm and mean weight was 34.27 gm (Table 7). The phenotypic (15.91) and genotypic (15.04) variance were close to each other. A minimum difference between phenotypic coefficient of variation (34.08) and genotypic coefficient of variation (33.13) indicate less influence of environmental factors on expression of this character (Table 8). Therefore, selection based on phenotypic expression of this character would be effective for the improvement of this crop. Dry fibre weight showed significant differences among the genotypes and ranged from 6.98 to 28.12 gm (Table 7). The genotypic coefficient of variation (28.11) and phenotypic coefficient of variation (29.99) were close to each other. The heritability (87.84%) and genetic advance (54.27%) were higher (Table 8). The higher heritability with high genetic advance

provided opportunity for selecting high valued genotypes for breeding programmes.

CONCLUSION

Results of the present studies indicated significant variation among the genotypes for all the characters. High heritability coupled with genetic advance was observed in green weight, stick weight, fibre weight, branches per plant and nodes per plant. These characters were under control of additive gene effect and selection for genetic improvement for these might be effective. However, the investigation revealed that no single quantitative trait had major contribution to the fibre yield. An integrated approach of improving quantitative traits would consequently help to increase the yield potential of jute. Considering the cluster, inter-genotypic distance and other agronomic performance, the genotypes G47, G33, G48 from cluster I, G27, G17, G23 from cluster III and G13, G40, G45 from cluster II were considered to be better parents for future use in hybridisation.

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Characterisation of Pathogenesis-Related Genes and Resistance Gene Candidates in Banana (*Musa acuminata*) and Their Expression during Host-Pathogen Interaction

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ABSTRACT

Amplified chitinase gene sequence shared 99% homology with *Musa acuminata* class III acidic chitinase and beta-1,3-glucanase gene sequence was 100% homologous to *Musa* x *paradisiaca* beta-1,3-glucanase. Three nucleotide-binding sites and the leucine-rich repeat (NBS-LRR) type of putative RGCs and one serine/threonine kinase gene were characterised at the amino acid level. Kinase-2 (LVLDDVW) and kinase-3 (GSRIIITTRD) motifs in the nucleotide-binding domain were highly conserved in RGC2 and RGC3 and these genes belong to the non-TIR-NBS class RGCs. RGC1 was also clustered into non-TIR-NBS class RGCs; however, many residue substitutions were present in the kinase-2 and kinase-3 motifs. The sub-domain IX (LTEKSDVYSFGVVL) of serine/threonine protein kinase was highly conserved in RGC5 and it shared highest homology with PTH-2 from muskmelon. RT-PCR analysis revealed the differential expression of PR and RGC genes exhibited by different banana genotypes over sampling time. Chitinase was expressed during banana-FocR4 interaction in all three banana genotypes. However, its expression was high and constant in 'Rastali Mutiara' during banana-FocR4 interaction and resulted in very low disease severity in FocR4 inoculated plants (2%) compared to 'Rastali wild-type (16%) and 'Jari Buaya' (8%) at six weeks after inoculation. This suggests that chitinase may play an important role in disease resistance against FocR4. Besides, our study also shows that 'Rastali Mutiara' can be a potential source of disease-resistant genes for molecular breeding of banana.

Keywords: Banana, *Fusarium* wilt, nucleotide-binding site, PR proteins, resistance-gene candidate, serine/threonine kinase

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INTRODUCTION

Banana is one of the important global food commodities. The commercial and subsistence production is seriously threatened by *Fusarium* wilt caused by soil-borne *Fusarium oxysporum* f. sp. *cubense* (Foc). Foc race 4 (FocR4) is considered economically important as it causes significant plantation losses in banana-producing countries predominantly in the Asia-Pacific region (Aquino *et al.*, 2013). To date, the existing control measures for this disease are not satisfactory.

Banana plants respond to attacks of pathogens by activating defence-related genes from different groups based on biological function and pattern of induction. The production of pathogenesis-related (PR) protein is a vital defence response against biological stress and pathogenic infection. Most PR proteins play a key role in plant defence in response to fungal infection. Previous studies have demonstrated that PR proteins show in vitro antifungal activity either individually or in combination (De Bolle *et al.*, 1993; Melchers *et al.*, 1993; Sela-Buurlage *et al.*, 1993; Koiwa *et al.*, 1997; Saikia *et al.*, 2005; Ye & Ng, 2005; De A Campos *et al.*, 2008; Lu *et al.*, 2012). PR proteins have already been shown to be good candidates for engineering fungal-resistant crops (Punja & Zhang, 1993; Jach *et al.*, 1995; Lin *et al.*, 1995; Tabei *et al.*, 1998; Datta *et al.*, 2001; Kalpana *et al.*, 2006). Productive interactions between chitinase and glucanase transgenes in vivo point to combinatorial expression of antimicrobial genes as an effective approach in engineering enhanced crop protection against fungal diseases (Zhu *et al.*, 1994).

On the other hand, breeding for resistance is the most appropriate approach to control the pathogen in the field. Carlier *et al.* (2000) has reported that sources of resistance to pathogens exist in germplasm across the *Musa* genus. Development of FocR4-resistant cultivars can be possibly done through introgression of Resistance (R) genes into susceptible cultivars (Miller *et al.*, 2008). Joshi *et al.* (2010) reported that more than 50 R genes have been cloned and characterised from mono- and dicotyledonous plants through map-based mapping, transposon tagging and genome homologues analysis. Most R genes identified up to now are members of the cytoplasmic nucleotide-binding and leucine-rich repeat (NBS-LRR) class. It has been reported that NBS-LRR-type R genes confer resistance to a wide variety of pathogens and pests (Dangl & Jones, 2001). According to Meyers *et al.* (1999) and Pan *et al.* (2000), the NBS-LRR-class of R genes is divided into two distinct subclasses based on the presence or absence of an N-terminal with homology to the *Drosophila* Toll and human Interleukin-1 receptors (TIR). The TIR subclass appears to be restricted to dicotyledonous species, whereas the non-TIR subclass is widely distributed in both mono- and dicotyledonous species (Meyers *et al.*, 1999; Pan *et al.*, 2000; Cannon *et al.*, 2002).

Isolation and characterisation of NBS-type sequences, called resistance gene candidates (RGCs), using PCR-based approach based on degenerate primers

have been reported in a great number of plants including banana. For instance, Pei *et al.* (2007) have isolated, characterised and analysed 12 resistance gene analogues (RGAs) in banana (*Musa* spp.). In addition, 20 fragments of RGAs have been isolated from wilt resistant Goldfinger (AAAB) banana (Sun *et al.*, 2009). Besides this, Lu *et al.* (2011) also have reported the isolation and characterisation of four RGAs in commercial banana species.

On top of that, Way (2006) has partially isolated and studied the expression of five putative RGCs from the local banana crop, 'Jari Buaya', in specific interaction of host with pathogen FocR4. However, there was lack of functional study of these RGCs. It would be most useful if we could identify potential defence genes that are involved in the banana-FocR4 interaction. With better understanding of RGCs discovered and its mechanism in disease resistance, we may contribute to disease management based upon genetic improvement in banana. Thus, this study was carried out to screen for the presence of selected defence genes in the genome of various local banana genotypes and relate their expressions during host-pathogen interaction.

MATERIALS AND METHODS

Plant materials and DNA extraction

Four cultivars of local banana (*Musa acuminata*) were used including three cultivated triploid species and one cultivated diploid species. 'Rastali wild type' (AAB) was obtained from Johor Plant Tech Sdn Bhd (Ayer Hitam, Johor). 'Rastali Mutiara' (AAB) and 'Jari Buaya' (AA) were obtained from United Plantations Berhad (Teluk Intan, Perak), while 'Rastali Transgenic' (AAB) was provided by Professor Maziah Mahmood from the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. 'Rastali Mutiara' is known to be tolerant to FocR4, while 'Jari Buaya' is known to be resistant to FocR4 (Chai *et al.*, 2004). For gene expression study, only three banana genotypes were used due to the unavailability of 'Rastali Transgenic'. Genomic DNA was extracted from the lower stem of each healthy banana cultivar (one-month old seedlings) using a Genomic Purification Kit (Fermentas, USA) following the manufacturer's instructions. The concentration of DNA samples was adjusted to 10 ng/µL before use.

Primer Sets

Primers targeted for chitinase and beta-1,3-glucanase were designed based on the conserved regions of similar sequences obtained from GenBank. Primers targeted to Resistance Gene Candidate (RGC) 1, 2 and 3 were designed based on isolated cDNA sequences which were amplified using degenerate primers (Way, 2006). Sequences of RGC1, 2 and 3 belong to NBS-LRR type of Resistance gene. Primers targeted to RGC5 were designed based on isolated cDNA sequences belonging to kinase class. In this study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of banana was used as an internal positive control. Details for the primer sequences of each gene are illustrated in Table 1.

TABLE 1
Primer sequences and PCR annealing conditions used to amplify target genes

Gene	[z]Tm (°C)	Product size (bp)	Forward primer (5'-3') Reverse primer (5'-3')	Genbank accession number/ Reference
GAPDH	58	124	F:GCAGGTCAAGCATCTTTGATGCCA R:ATGTGGCGGATCAGGTCGATTACA	AY821550
chitinase	47	224	F:TGCTGTTATTTGCGTTCCTG R: GTTGTTCCGAGGGTCACAGT	AY525367
beta-1,3-glucanase	48	194	F:CCCTCAGGAACTCCAACATC R:GAGGATGTACTGCGCCAGAT	EU014210
RGC1	55	449	F:ATGGCGCTTCTTCTCATGTCGC R:TCAACAACGAGCTCAAGGAGAA	Way, 2006
RGC2	45	426	F:CCTGTGTCCTTTAGATATTGGGCA R:TGGTAAAATCAAAGCCAGCTTCCG	Way, 2006
RGC3	45	448	F:CCTGTGTCCTTTAGATATTGGGCA R:TCGCTCAGAAGTTGTTCAATGATGG	Way, 2006
RGC5	55	~400	F:CGTACTTCTTCAGCGAGGCGGA R:ACGTCAAGACCACCAACATCC	Way, 2006

[z] Annealing temperature for PCR amplification

PCR Amplification

PCR amplification of each gene was performed in a final volume of 25 µL. PCR-reaction mixture contained 0.2 U *Taq* DNA polymerase, 1X PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM of each primer, 10 ng DNA template and milli-Q of water added up to 25 µL. The PCR reactions were performed using a Biometra T-Professional thermocycler (Goettingen, Germany) and programmed as follows: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 45-55 °C (depending on Tm of primers) for 30 s and 72 °C for 1 min; and an additional elongation period of 10 min at 72 °C.

DNA Sequence Analysis

Amplified products were separated on 2.0% agarose gel and visualised under UV light. PCR products were purified using a Gel Extraction Kit (Qiagen, Germany). DNA sequencing was performed using 3730 x l DNA analyser (Applied Biosystems ABI, USA) by NextGene Company (Selangor, Malaysia). The identity of chitinase, beta-1,3-glucanase and RGCs was analysed by comparison of DNA and amino acid sequences with the GenBank database using BLASTX and BLASTP (Altschul *et al.*, 1990) algorithms. Multiple sequence alignment with Clustal-X (Thompson *et al.*, 1997) was then conducted along with three RGCs (RGC1, 2 and 3), five R genes previously reported in other plant species (tobacco N; *Arabidopsis* RPM1; flax M; rice XA1 and potato RGA2) and one *Musa* AAB resistance gene analogue (banana MRGL2). The same NBS-encoding R genes or RGAs were used for protein phylogenetic analysis. RGC5 was aligned using Clustal-X along with serine/threonine kinase genes of other

plants (muskmelon PTH-2; potato Pto-like; tomato Pto and Fen). The construction of a neighbour-joining tree (Saitou & Nei, 1987) was conducted using the MEGA4.0.2 software, and the reliability of tree branches was evaluated using the Bootstrap method with 1000 bootstrap iterations (Felsenstein, 1985).

Preparation of Inoculum and Inoculation

A pure isolate of FocR4 obtained from the fungal culture collection of the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, was cultured on Potato Dextrose Agar (PDA) and incubated for seven days at 26±2 °C. Spores were harvested with sterile distilled water and adjusted for a stock solution at 4 x 10^5 spores/mL. One-month old seedlings of three different banana genotypes, 'Rastali wild type', 'Rastali Mutiara' and 'Jari Buaya', were inoculated by soil drenching with 100 mL stock solution of FocR4 for each plant and maintained in the glasshouse. The plants were watered daily and fertilised monthly with NPK (15:15:15, W:W:W). Three sampling intervals were carried out at 0, 2 and 4 weeks after FocR4 inoculation (WAI).

Disease development was evaluated based on foliar symptom at weekly intervals until 6 WAI and expressed as percentage disease severity (% DS) using a scale of 0 to 5; 0 = healthy plant; 1 = lowest leaf with yellowish streaks and brown necrosis absent; 2 = less than 25-50% of the total number of leaves with yellowish streaks and brown necrosis present; 3 = more than 50-75% of the total number of leaves with yellowish streaks and brown necrosis present; 4 = 100% of total number of leaves with yellowish streaks and brown necrosis present; 5 = plant collapsed and died due to severe wilting. DS (%) of *Fusarium* wilt was calculated based on the following formula:

$$\frac{\Sigma \text{ (No. of diseased plants in each rating category x Severity rating)}}{\text{Total no. of plants assessed x Highest scale}} \times 100$$

The experiment was conducted in randomised complete block design (RCBD) with four replications, where each replicate comprised 10 seedlings. All data were analysed by ANOVA using SAS 9.0. The mean comparison was performed using least significant difference (LSD) at $p \leq 0.05$).

RNA Extraction and RT-PCR

Total RNA was extracted from the lower stem of various banana genotypes for each sampling interval using RNeasy Plant Mini Kit (Qiagen, Germany). The integrity and concentration of total RNA of each genotype were determined using UV-spectrophotometer (NanoDrop Technologies, USA) and agarose gel electrophoresis. A two-step RT-PCR amplification was performed. First strand cDNA was constructed using Omniscript Reverse Transcription Kit (Qiagen, Germany). Primers targeted to GAPDH, chitinase, beta-1,3-glucanase, RGC1, RGC2 and RGC5 genes were used in this study. Each PCR reaction was performed in a final volume of 25 µL in a PCR-

reaction mixture containing 0.2 unit *Taq* DNA polymerase, 1X PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM of each forward and reverse primers, 50 ng cDNA template and milli-Q water. The RT-PCR amplifications were performed in a Biometra T-Professional thermocycler (Goettingen, Germany) and programmed as follows: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 45-55 °C (depending on Tm of primers) for 30 s and 72 °C for 1 min; and an additional elongation period of 10 min at 72 °C. PCR products were separated on 2.0 % agarose gel and visualised under UV light.

RESULTS AND DISCUSSION

PCR Amplification of Targetted Defence-related Genes and Sequence Analysis

PCR amplification of chitinase, beta-1,3-glucanase and RGCs resulted in a single DNA band of approximately expected size in the gel (Table 1). Identical results were produced in three replicate experiments. Sequences of banana RGCs and chitinase were submitted to the GenBank with accession numbers as follows: RGC1 (KC864792; 449 bp), RGC2 (KC864793; 409 bp), RGC3 (KC864794; 443 bp), RGC5 (KF006850; 365 bp) and chitinase (KC864795; 224 bp). However, the sequence of beta-1,3-glucanase (194 bp) was not submitted to the GenBank due to its short sequence.

DNA sequence analysis of PR genes revealed that the amplified chitinase gene sequence showed 99% homology (E value = 2e-109) to *Musa acuminata* class III acidic chitinase (AY525367). Meanwhile, beta-1, 3-glucanase gene sequence was 100% homologous (E value = 1e-95) to *Musa* x *paradisiaca* beta-1,3-glucanase (EF051254). On the other hand, the nucleotide sequences of the isolated RGC genes were translated into amino acid sequences using the ExPASy Translate Tool (http://web.expasy.org/translate/) and the amino acid sequences of banana Resistance Gene Candidates (RGCs) were compared with the protein sequences deposited in the GenBank using BLASTP algorithm (Table 2). The RGC1 showed 100% identity with NBS-type resistance protein of *Musa* sp. (ACK44409.1 and ABY75803.1), followed by more than 99% identity with NBS-LRR-type disease resistance protein of *Musa acuminata* (ABB96971.1). RGC2 showed 100% identity with NBS-type resistance protein of *Musa* ABB group (ACK44406.1) and *Musa acuminata* AAA Group (ABW96279.1). It also showed 100% identity with putative disease resistance protein of *Musa balbisiana* (CBW30194.1). RGC3 showed 100% identity with NBS resistance protein of *Musa* ABB Group (ACK44406.1), NBS-LRR disease resistance protein of *Musa* AAB Group (CAP66295.1) and NBS-LRR class resistance protein of *Musa acuminata* AAA Group (ABW96279.1). RGC5 showed 100% identity with Pto-like serine/threonine kinase of *Capsicum chinense* (AAQ82660.1) and putative Pto-like serine/threonine kinase of *Solanum sucrense* (AAK82707.1).

TABLE 2
Similarity between banana RGC sequences and GenBank accessions carried out using the BLASTP algorithm

Musa NBS	GenBank protein accession showing the highest similarity	GenBank ID	Identity[z]	E-value
RGC1	NBS resistance protein (*Musa* ABB Group)	ACK44409.1	100	5e-100
	Resistance gene candidate NBS-type protein, partial (*Musa acuminata* subsp. *malaccensis*)	ABY75803.1	100	8e-99
	NBS-LRR type disease resistance protein (*Musa acuminata*)	ABB96971.1	99	8e-100
RGC2	NBS resistance protein (*Musa* ABB Group)	ACK44406.1	100	2e-85
	NBS-LRR class resistance protein (*Musa acuminata* AAA Group)	ABW96279.1	100	1e-24
	Putative disease resistance protein (*Musa balbisiana*)	CBW30194.1	100	1e-81
RGC3	NBS resistance protein (*Musa* ABB Group)	ACK44406.1	100	4e-93
	NBS-LRR disease resistance protein (*Musa* AAB Group)	CAP66295.1	100	4e-43
	NBS-LRR class resistance protein (*Musa acuminata* AAA Group)	ABW96279.1	100	1e-28
RGC5	Pto-like serine/threonine kinase (*Capsicum chinense*)	AAQ82660.1	100	3e-60
	Putative Pto-like serine/threonine kinase (*Solanum sucrense*)	AAK82707.1	100	4e-59

[z] Amino acid identity

Sequence Analysis of Resistance Gene Candidates for Conserved Motif

The amino acid sequences of three RGCs (RGC1, 2 and 3) isolated in this investigation were compared with other known R genes from different plants using the Clustal-X multiple alignment programme. As shown in Fig.1, kinase-2 (LVLDDVW), one of the crucial motifs of the NBS domain, was highly conserved among RGC2, RGC3 and the known NBS-LRR R-proteins. However, a little diversity existed in the kinase-2 motif of RGC1 where it was substituted by VLLDDVW. The same trend has been reported in *Musa* RGA-L (Pei *et al.*, 2007). Besides this, kinase-3 motif (GSRIIITTRD) was present in all RGCs, but more residue substitutions were observed. Furthermore, all RGCs had a conserved tryptophan (W) residue at the end of the kinase-2 domain (Fig.1). Absence or presence of TIR domain is used to classify the NBS-LRR genes into two different subfamilies where subfamily I contains the TIR element while subfamily II lacks it (Meyers *et al.*, 1999; Pan *et al.*, 2000). Several reports have demonstrated that the last residue of the kinase-2 domain can be used to predict with 95 % accuracy whether an RGA belongs to the TIR-NBS or to the non-TIR-NBS family; conservation of tryptophan (W) at this location is tightly linked to non-TIR R genes (RPM1, XA1,

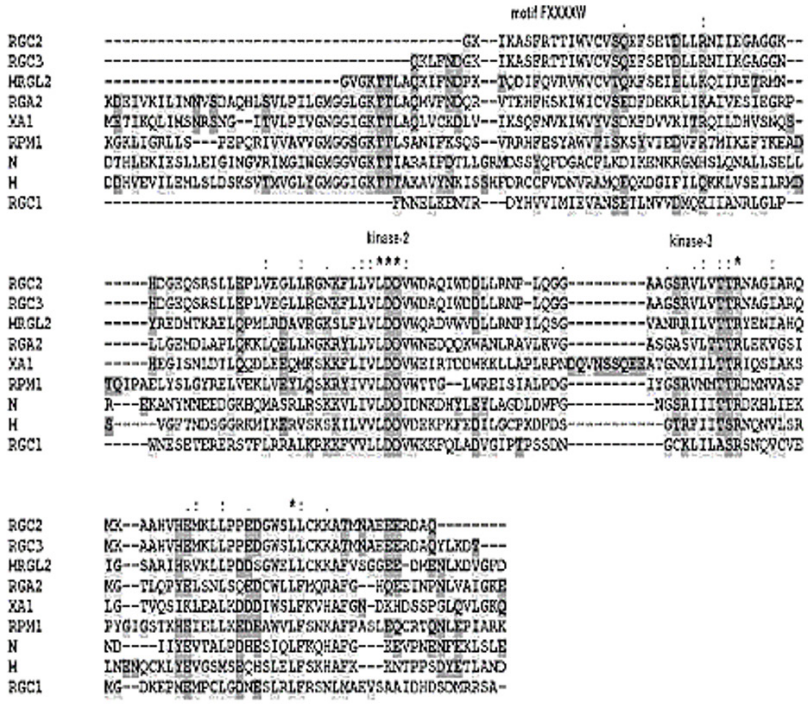

Fig.1: Multiple alignment of amino acid sequences of *Musa* RGCs with known NBS class R genes constructed with Clustal X. The R genes used were tobacco N (GenBank accession No. BAD12594.1); Arabidopsis RPM1 (GenBank accession No. NP187360.1), flax M (GenBank accession No. AAB47618.1); rice XA1 (GenBank accession No. BAA25068.1), potato RGA2 (GenBank accession No. AAP45188.2) and banana MRGL2 (GenBank accession No. HQ 199834.1). The conserved motifs identified are shown in the top panel. The presence of motif FXXXXW before kinase-2 in RGCs (2 and 3), Arabidopsis RPM1, potato RGA2, rice XA1 and banana MRGL2 indicates the non-TIR class as described by Pan *et al.* (2000).

RGA2), whereas conservation of aspartic acid (D) or its uncharged derivative aspartate (N) is characteristic of the TIR class of R genes (N and M) (Meyers *et al.*, 1999; Pan *et al.*, 2000; Jeong *et al.*, 2001; Song *et al.*, 2003). In this study, the conservation of tryptophan (W) residue at the end of the kinase-2 domain in RGC1, 2 and 3 evidently confirmed that R genes in banana were non-TIR-NBS-LRR type. This can be further confirmed with the presence of motif FXXXXW before the motif of kinase-2 in RGCs (2 and 3), RPM1, RGA2, MRGL2 and XA1, which also indicates the non-TIR class as described by Pan *et al.* (2000).

On the other hand, multiple-sequence alignment of RGC5 and known serine/threonine protein kinase genes showed significant homology to the sub-domain IX (LTEKSDVYSFGVVL) of serine/threonine protein kinase gene particularly motif DxxxxG (Fig.2). However, residue substitutes were present in the deduced amino acid of RGC5 along with the sub-domain VII through XI (except sub-domain

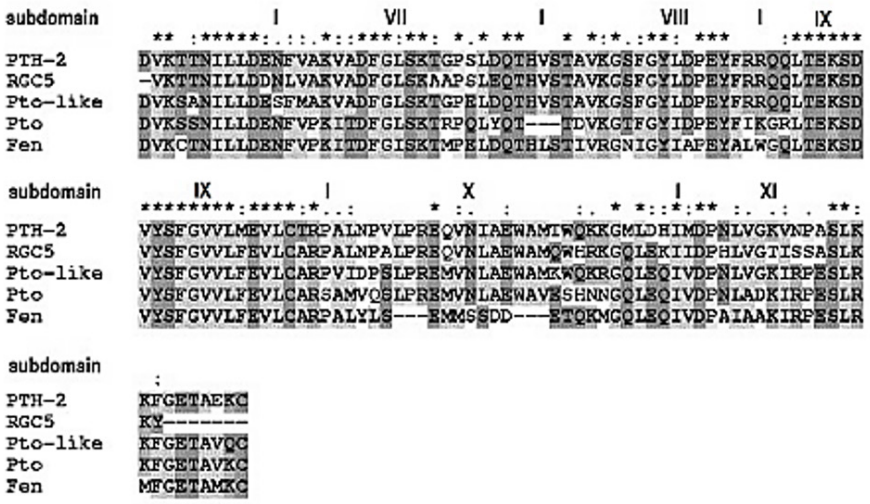

Fig.2: Multiple alignment of amino acid sequences of RGC 5 with serine/threonine kinase genes of other plants constructed with Clustal X. The serine/threonine kinase genes genes used were PTH-2 (GenBank accession No. AAL83882.1) from muskmelon, Pto-like serine/threonine kinase (GenBank accession No. AAK82715.1) from potato, Pto (GenBank accession No. AAB47421.1) and Fen (GenBank accession No. AAF76314.1) from tomato. Roman numerals identify the serine/threonine kinase subdomains as described by Vallad *et al.* (2001).

IX). This phenomenon was also represented in chestnut rose (Xu & Deng, 2010) and bean (Vallad *et al.*, 2001).

Phylogenetic Analysis of RGCs and Other Cloned R Genes

The deduced amino acid sequences of the three RGCs (RGC 1, 2 and 3) and several known NBS-LRR R-proteins from other plant species and banana plants were pooled for phylogenetic analysis. The resulting neighbour-joining phylogenetic tree (Fig.3) indicated that the known R proteins and RGCs could be classified into two groups: TIR- and non-TIR-NBS-LRR R-proteins. All RGCs isolated in this work were grouped into non-TIR-NBS-LRR type. RGC2 and 3 were significantly homologues and clustered within subclass containing non-TIR-NBS-LRR R-proteins. In addition, the amino acids encoded by these RGCs had the largest similarity with MRGL2 from *Musa* (AAB), suggesting that these genes are orthologs. As shown in Fig.3, none of the banana RGCs shared homology with the TIR-NBS-LRR R-proteins, namely N from tobacco and M from flax. The result obtained was in agreement with the hypothesis that the non-TIR subfamily was present in both mono- and dicotyledonous taxa (Pan *et al.*, 2000). On the other hand, RGC1 was divided into a separate subclass of non-TIR-NBS-LRR R-proteins from RGC2 and 3 (Fig.3). This indicated the presence of a diverse gene family coding for proteins with NBS-LRR domains in banana as previously reported by Miller

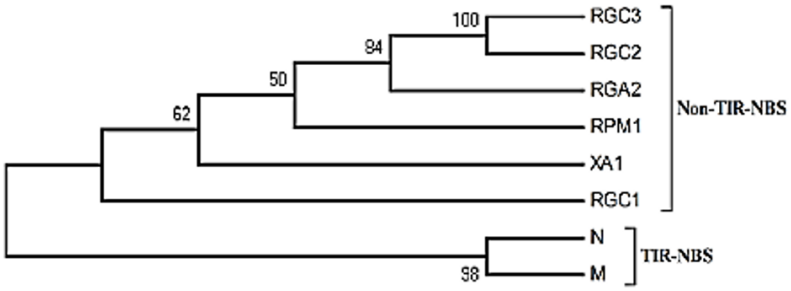

Fig.3: Phylogenetic tree of the deduced amino acid sequences of RGCs based on the neighbour-joining method. The numbers on the branches indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The sources of known R genes were the same as shown in the note of Fig.1.

et al. (2008). The phylogenetic analysis also suggested that RGC1 is probably a new class of non-TIR RGCs in banana. Nevertheless, Pei *et al.* (2007) reported that this phenomenon was dependent on the known NBS-LRR R-proteins included in the analysis as references and requires a more comprehensive study.

Meanwhile, RGC5 was clustered together with PTH-2, a resistance gene homolog of muskmelon and Pto-like serine/threonine kinase from potato (Fig.4). However, phylogenetic analysis revealed that RGC5 had close relationship with PTH-2 from muskmelon, but not with Pto resistance gene from tomato.

Expression of Defence-associated Genes during Host-Pathogen Interaction

The GAPDH gene, two PR genes and four RGCs were detected in the genome of all four banana genotypes with the exception of beta-1,3-glucanase gene, which was not detected in 'Jari Buaya' (Fig.5).

RT-PCR analysis revealed the differential expression of PR and putative RGC genes exhibited by different banana genotypes over time (Fig.6). RGC3 was not used because the gene sequence showed an exact match with RGC2 through sequence analysis. The expression pattern of defence-associated genes in 'Rastali wild-type' (A) and 'Jari Buaya' (C) was similar, except for RGC2. As shown in Fig.6, the band intensity of chitinase was high in 'Rastali Mutiara' (B) compared to 'Rastali wild type' (A) and 'Jari Buaya' (C) before (0 WAI) and after FocR4 inoculation (2 and 4 WAI). This could be correlated with the absence of disease symptoms throughout the period of study (Fig.8). The induction and rapid accumulation of *SolChi*, a gene encoding an acidic isoform of class III chitinase upon infection with *Fusarium oxysporum* f.sp. *lycopersici* in a genotype-resistant tomato suggested its putative role in defence against fungal pathogens (Amaral *et al.*, 2012).

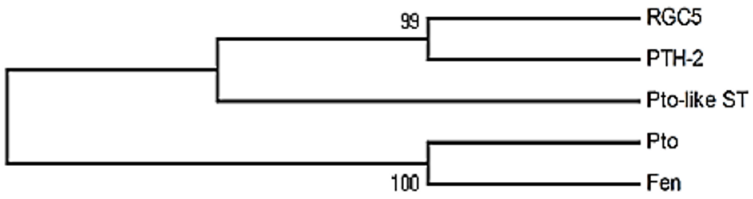

Fig.4: Phylogenetic tree of the deduced amino acid sequences of RGC5 with serine/threonine kinase genes of other plants based on the neighbour-joining method. The numbers on the branches indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The sources of serine/threonine kinase genes of other plants were the same as shown in the note of Fig.2.

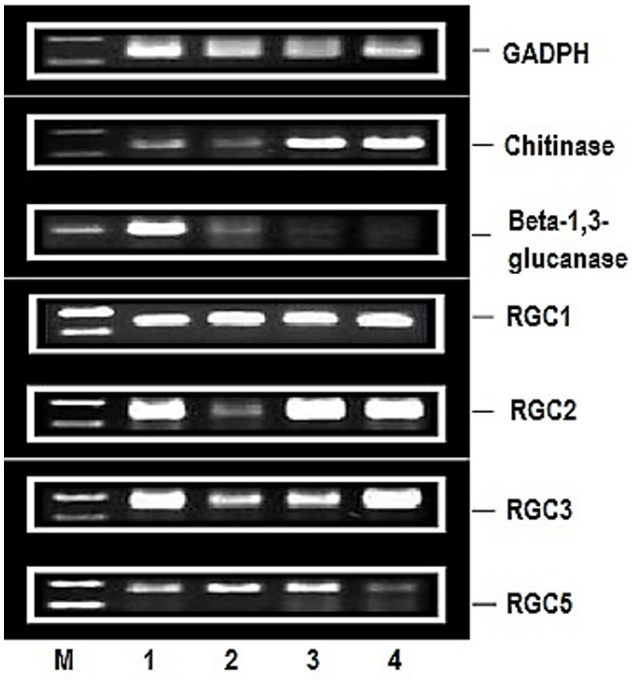

Fig.5: Detection of defence-associated genes and internal control (GAPDH) in various banana genotypes using 10 ng of genomic DNA. Lane M = 100 bp DNA ladder, 1 = 'Rastali wild type', 2 = 'Rastali Mutiara', 3 = 'Rastali Transformed', 4 = 'Jari Buaya'. The sizes of amplified products after sequencing were as follows, GADPH = 390 bp, chitinase = 224 bp, beta-1,3-glucanase = 194 bp, RGC1 = 449 bp, RGC2 = 409 bp, RGC3 = 443 bp, RGC5 = 365 bp.

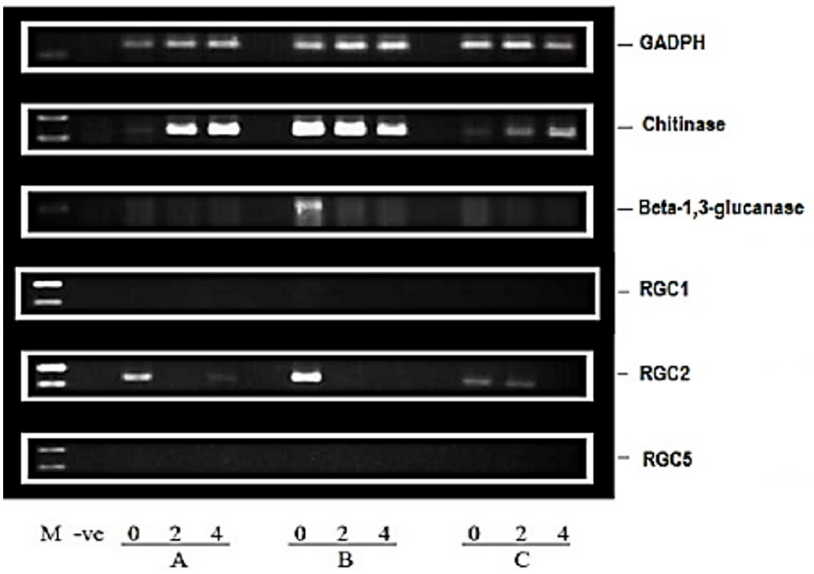

Fig.6: Expression of defence-associated genes and internal control (GAPDH) in various banana genotypes after challenge-inoculation with FocR4 at different intervals. Lane M = 100 bp DNA ladder, -ve = without templates, 0-4 = sampling weeks, A = 'Rastali wild type', B = 'Rastali Mutiara', C = 'Jari Buaya'. The sizes of amplified products after sequencing were as follows: GADPH = 124 bp, chitinase = 224 bp, beta-1,3-glucanase = 194 bp, RGC1 = 449 bp, RGC2 = 409 bp, RGC3 = 443 bp, RGC5 = 365 bp.

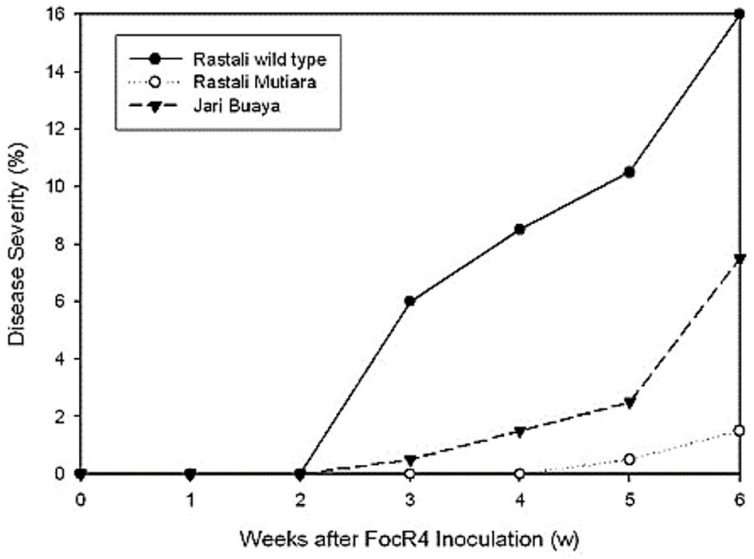

Fig.7: Disease severity of various banana genotypes according to weeks after challenge-inoculation with FocR4.

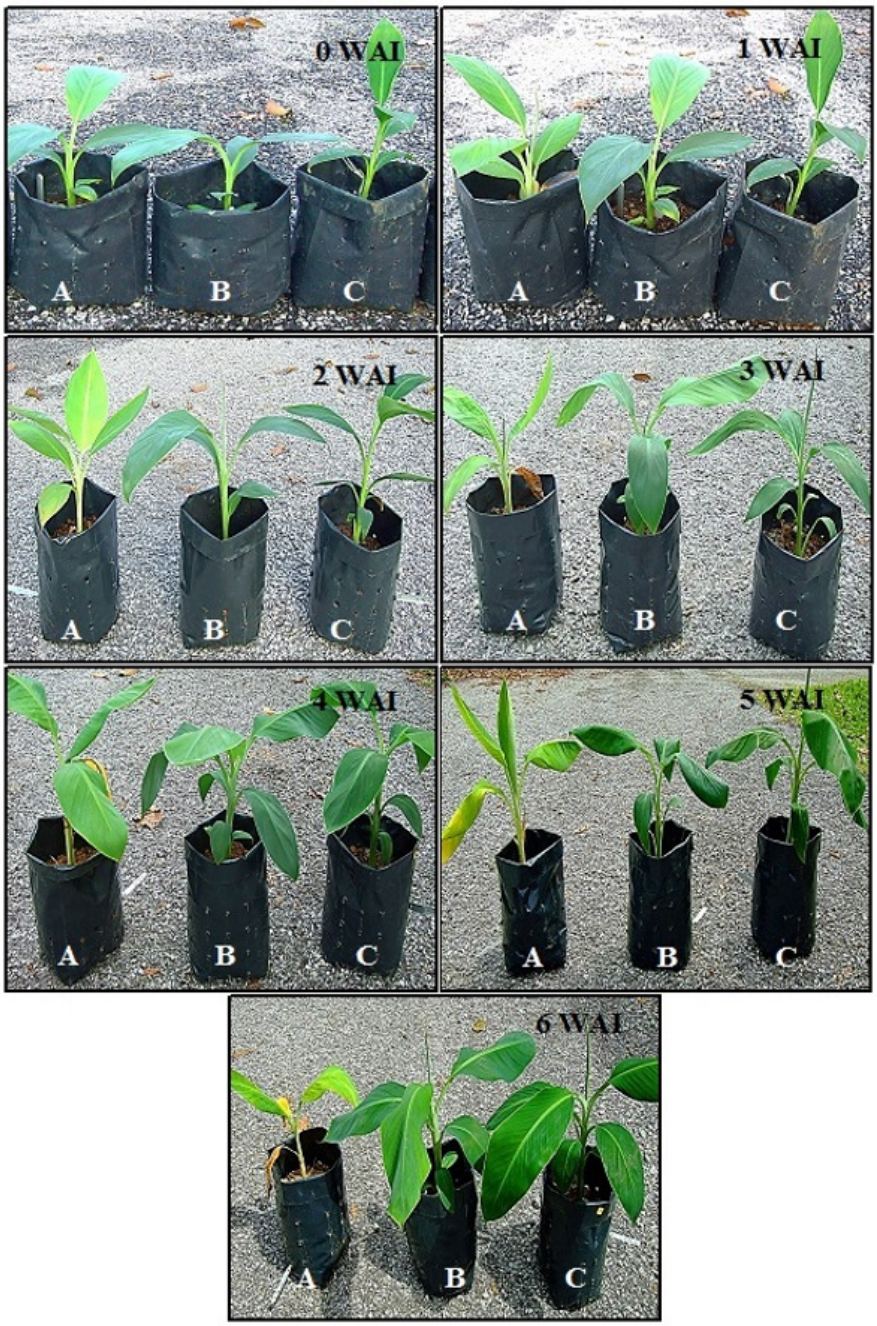

Fig.8: *Fusarium oxysporum* f. sp. *cubense* R4-inoculated banana (*Musa acuminata*) plants from three different cultivars under glasshouse conditions at 6 weeks after inoculation (WAI), A: 'Rastali' Wild-type, B: 'Rastali Mutiara', C: 'Jari Buaya'.

Likewise, Malafaia *et al.* (2013) also have associated the expression of chitinase with resistance of resistant tomato cultivar against *Fusarium* wilt. Moreover, the upregulation of chitinase in both 'Yueyoukang 1', the resistant-banana cultivar and 'Brazilian', the susceptible-banana cultivar after FocTR4 infection, has been related to plant defence in banana roots (Bai *et al.*, 2013). Hence, the higher and constant expression of chitinase against FocR4 in 'Rastali Mutiara' may possibly contribute to the lower percentage of disease severity and also explain the tolerant nature of this cultivar as claimed by the Food and Agriculture Organization (FAO) of the United Nations (Chai *et al.*, 2004). On the other hand, temporal change of chitinase expression in both 'Rastali wild type' and 'Jari Buaya' as shown in Fig.6 suggested that a defence mechanism has likely been activated in these cultivars upon FocR4 infection; however, it had negative correlation with disease resistance to *Fusarium* wilt (Fig.7).

Beta-1,3-glucanase only expressed in 'Rastali Mutiara' at 0 WAI and its expression was not detected in 'Rastali wild type' (A) and 'Jari Buaya' (C) during any period of sampling. This was inconsistent with the previous finding reported by Bai *et al.* (2013). According to Ebrahim *et al.* (2011), different clones of the same plant species can exhibit different production of beta-1,3-glucanase after pathogen inoculation. For instance, the activity of beta-1,3-glucanase enzyme increased in the tolerant clone of *Hevea brasiliensis* upon infection with *Corynespora cassiicola*, while in the susceptible clone it decreased (Philip *et al.*, 2001). Nonetheless, a more comprehensive study should be done on the expression of beta-1,3-glucanase in 'Rastali Mutiara', a tolerant variety against FocR4, to determine the factors affecting its down-regulation during host-pathogen interaction.

The expression level of RGC2 was high before inoculation in 'Rastali wild-type' (A) and 'Rastali Mutiara' (B). Conversely, its expression was down-regulated after inoculation at 2 WAI followed by slight up-regulation at 4 WAI in 'Rastali wild-type' (A). On the other hand, the expression of RGC2 was not detected after inoculation in 'Rastali Mutiara' (B) at 2 and 4 WAI. In 'Jari Buaya' (C), the expression of RGC2 was similar before inoculation and 2 WAI, but was not detected at 4 WAI. The expression of RGC2 in Rastali Mutiara (B) was negatively correlated with disease assessment and recorded a lower disease incidence in 'Rastali Mutiara' compared to 'Rastali wild-type' and 'Jari Buaya' (Fig.7). Resistance gene candidates from different plant species have been previously associated with resistance to phytopathogens. For example, expression of RGA1, RGA2, RGA5 and RGA23 was associated with resistance to *Plasmopara viticola* in grapevine (Wang *et al.*, 2013). Moreover, Peraza-Echeverria *et al.* (2007) have reported the association of RGC2 isolated from *Musa acuminata* subsp. *malaccensis* with resistance against *Fusarium oxysporum*. Although RGC2 has not been correlated with resistance to *Fusarium* wilt disease in this study, its

potential role in disease resistance could be tested in future using new technologies of the post-genomic era, such as RNA interference (RNAi) as proposed by Waterhouse and Helliwell (2003).

Furthermore, RGC1 and RGC5 were not expressed during pathogen-host interaction even though it was detected in the genome of all banana genotypes studied (Fig.5). Previous studies have shown that most of the R genes encode nucleotide binding site (NBS), leucine rich repeat (LRR) motif and serine-threonine protein kinase. However, Meyers *et al.* (1999) have reported that this type of NBS motif and serine-threonine kinase not only encoded in R genes but also encoded as important genes involved in development and signal transduction. From this result, we propose that RGC1 and RGC5 were not involved in activation of defence response against pathogen attack in *Musa acuminata*-FocR4 interaction. In future research, we will focus on cloning and sequence analysis of full-length *Musa* RGC1 and RGC5 in order to acquire a better understanding of its function in banana.

CONCLUSION

In this study, two PR proteins and four RGCs were isolated from different banana genotypes. All RGCs that were characterised at the amino acid level may provide a basis for cloning the full length of disease-resistant gene where only a couple of cases of R genes in banana have been reported so far. In addition, the expression pattern exhibited by these PR proteins and RGCs during host-pathogen interaction was also demonstrated in this study. Chitinase, with constant expression over time in all 'Rastali' genotypes studied, may play an important role in disease resistance against FocR4. Intense expression of chitinase in 'Rastali Mutiara' and occurrence of very low disease severity (2%) in FocR4 inoculated plants demonstrated that this cultivar can be a good source of resistance.

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Phenological Growth Stages of Torch Ginger (*Etlingera elatior*) Inflorescence

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ABSTRACT

Torch ginger (*Etlingera elatior*) inflorescence with its showy appearance can be used as a cut flower. However, the development of inflorescence has not been studied. The aim of this study is to determine the phenological stages of torch ginger inflorescence from the emergence of rhizome until full bloom stage using the extended BBCH scale. The growth stages of the inflorescence are categorised into peduncle elongation, inflorescence emergence, flowering and senescence. It takes about 60 days for the inflorescence to emerge from rhizome and develop to reach full-bloom stage with true flower opening. This study provides important information in understanding this crop.

Keywords: BBCH scale, cut flower, developmental stages, phenology, Zingiberaceae

INTRODUCTION

Torch ginger (*Etlingera elatior*), also known as torch lily, wild ginger or Philippine wax flower, belongs to the family of Zingiberaceae. It is indigenous to Malaysia and Indonesia. In Malaysia, it is called '*kantan*'. Torch ginger is usually propagated by rhizome and seed. It takes about 12 months after planting to start flowering but a longer time is needed when seeds are used. The torch ginger inflorescence is borne on a long, slender and leafless peduncle arising from the rhizome of the ground (Ibrahim & Mood, 2001). The leaves are grown from separate stalks along the rhizome. The torch ginger inflorescence mainly consists of three colours namely red, pink and white. The pink torch ginger is commonly planted in villages while the white and red are rare. The red/pink/white portion of the torch ginger inflorescence is actually the involucral

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bracts. The true flower is cone-shaped and appears between the large waxy bracts.

In Malaysia, the torch ginger inflorescence at tight bud stage is commonly used in culinary dishes. The young inflorescence bud is a compulsory ingredient as flavouring for '*asam laksa*', a popular local dish. It is also an important element in many Malay, Nyonya and Thai dishes. Other traditional usage of the torch ginger plant includes the use of the fruit to treat earache and the leaves for healing of wounds and for bathing by post-partum women to remove body odour (Chan *et al*., 2009). Studies have shown that the leaves contain antioxidant and antibacterial properties (Chan *et al*., 2007). The leaves, peduncle, inflorescence and rhizome have been used for essential oil production (Sohail & Akhtar, 2011).

Recently, besides commercial production as cooking ingredients, this crop is gaining recognition as an ornamental and landscaping plant in urban areas. In fact, when left to bloom, it is one of the most beautiful and striking inflorescences of tropical flowering plants, and brightens up any garden. The extravagant and showy inflorescence of the torch ginger at each stage can also be used as a cut flower. It has been used in floral arrangements in countries such as Australia, Hong Kong, Thailand and the United States of America. The use of the torch ginger inflorescence as a cut flower is relatively young in Malaysia. There is only a small number of florists using the cut inflorescence as bouquet fillers. However, at farm price, the margin of the cut flower is five times higher than that of the tight bud. Therefore, diversifying the usage of the torch ginger inflorescence as cut flowers rather than only for culinary purposes would help to increase the marginal revenue of growers.

In Malaysia, the torch ginger inflorescence is frequently harvested at tight bud stage. Therefore, the developmental stages of the inflorescence are rather vague. No study has been done to identify the phenological stages during the development of the torch ginger inflorescence. By understanding the phenological growth stages of the torch ginger inflorescence, it would be easier to access a suitable timing for applying fertiliser and for watering. Improper of maintenance activities will cause the failure of inflorescence development.

Therefore, the aim of this study was to determine the phenological stages of the torch ginger inflorescence from the emergence of the rhizome until full-bloom stage. In this study, the extended Biologische Bundesanstalt, Bundessortenamt and Chemical industry (BBCH) scale (Meier, 2001), with 10 principal growth stages, numbered from 0 to 9, was used to describe the changes of the morphological characteristics of the torch ginger inflorescence. Developing a uniform phenological scale would be a good reference for researchers to identify the growth stages for further study of this crop. Besides, it is also beneficial to growers for more efficient agricultural practices to enhance the inflorescence production. To our knowledge, this is the first report describing the phenological growth stages of the torch ginger inflorescence using BBCH

scale and perhaps, also the first report about the Zingiberaceae family.

MATERIALS AND METHODS

Pink torch ginger plants planted at Field 2, Faculty of Agriculture, Universiti Putra Malaysia, Malaysia since November 2011 were used in this study. The mean daily temperature during the observation period was 23-35°C with monthly precipitation of 250-300 mm. The inflorescence growth performance of 20 torch ginger inflorescences was observed daily from January to May 2013. The principal growth stage of the torch ginger inflorescence as described in BBCH scale (Meier, 2001) was determined by observing the morphological changes and days taken for the inflorescence to emerge from rhizome until full-bloom stage when true flowers were seen. Fertilisation was done monthly with NPK Blue (12:12:17:2) at 500 g/clump while watering and weeding were carried out when necessary.

RESULTS

From observation, a torch ginger plant flowers only once. The mean of the inflorescence peduncle length is about 0.7 m. The leafless torch ginger inflorescence is unable to carry out photosynthesis. It is presumed that the growth and development of the inflorescence depends on the stored carbohydrates that are translocated from the mother plant. The phenological stages of the torch ginger inflorescence were summarised and are described in BBCH scale.

Principal Growth Stage 3: Peduncle Elongation

30 Beginning of peduncle growth (Fig.1).
32 Peduncle reaching final length of 20% (Fig.1).
34 Elongation continues reaching final length of 40% (Fig.1).
39 Peduncle reaching final length, elongation process stops and inflorescence bud forms (Fig.1).

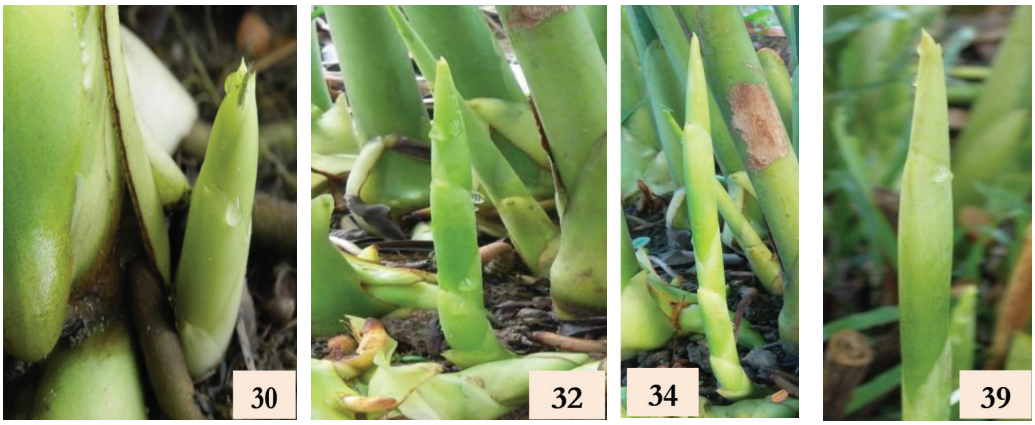

Fig.1: Principal growth stage 3: Peduncle elongation.

Principal Growth Stage 5: Inflorescence Emergence

50 Inflorescence bud starts swelling (Fig.2).
52 Inflorescence bud swelling, pink pigment starts to deposit in the bracts (Fig.2).
56 Inflorescence bud continues swelling until tips form (Fig.2).
59 Bracts visible with intense pink colour (Fig.2).

Principal Growth Stage 6: Flowering

60 Beginning of bract separation and increase in size (Fig.3).
62 Opening of bracts at 2-tip (Fig.3).
64 Opening of bracts at 6-tip (Fig.3).
66 Opening of bracts at 8-tip, causing increase of bract size and layers (Fig.3).
68 Opening of bracts, outer layers of bracts unfold revealing a cone-shaped inflorescence head (Fig.3).
69 Full bloom, also the end of flowering stage, all bracts unfold and true flowers show. The longevity of a true flower is just 24 h (Fig.3).

Principal Growth Stage 9: Senescence

90 Opening of first ring of true flowers complete (Fig.4).
95 Full bloom: 50% of true flowers from inflorescence head opened (Fig.4).
99 End of blooming: All true flowers opened; inflorescence head turned brown and dry. True flowers failed to develop into fruit and seed (Fig.4).

DISCUSSION

In general, it took about 30 days for an inflorescence to emerge from rhizome and develop until inflorescence bud swelling. The development of inflorescence began from elongation of the peduncle and the swelling of the inflorescence bud. During the flowering stage, it took another 30 days from tight-bud stage to reach full

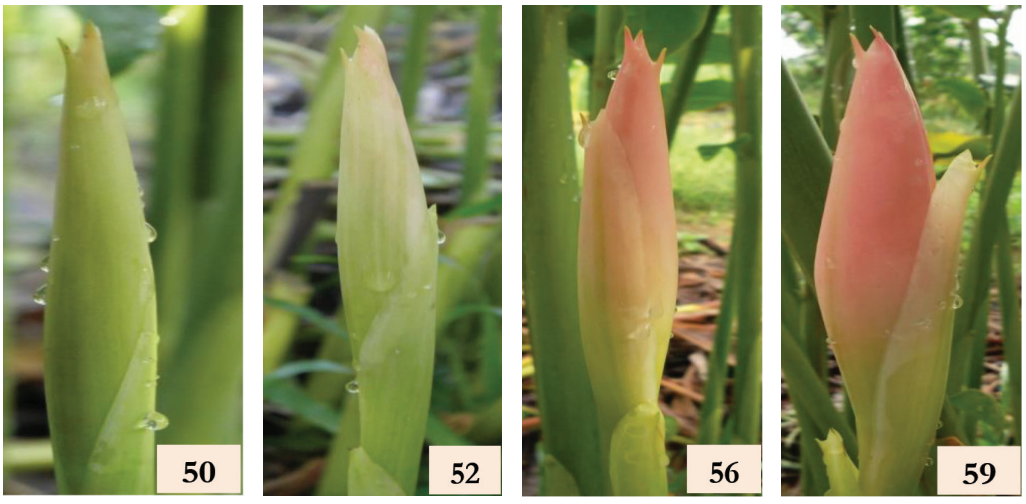

Fig. 2. Principal growth stage 5: Inflorescence emergence.

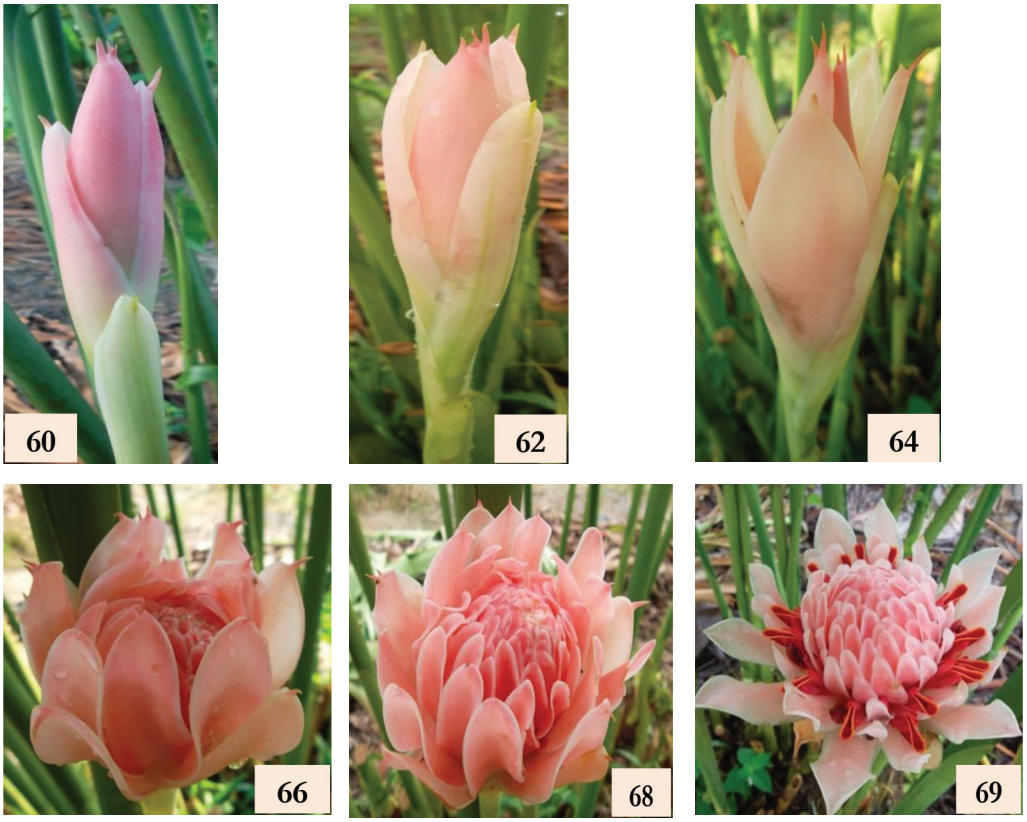

Fig.3: Principal growth stage 6: Flowering.

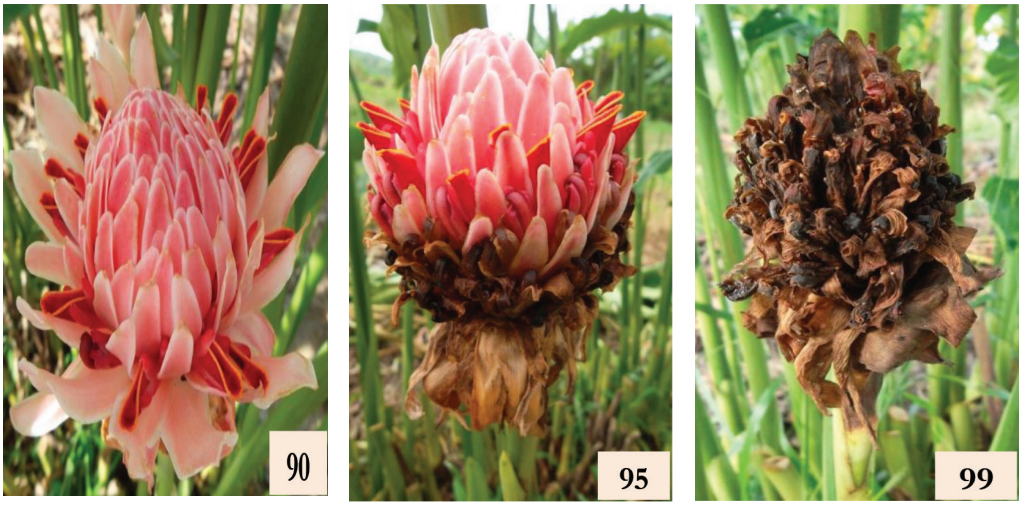

Fig.4: Principal growth stage 9: Senescence.

bloom where true flowers were shown. True flowers were growing in a ring and the opening of true flowers was from outer to inner of inflorescence head. From the opening of the first ring of true flowers, it took about 24 days for all true flowers to open ring by ring. At this stage, the inflorescence head turned brown and dried. The flower failed to develop into fruit and seed due to the absence of the spider-hunter bird, the pollinator, in the study site.

CONCLUSION

Torch ginger inflorescence showed four of the 10 principal growth stages according to the BBCH scale. The growth stages began with the peduncle elongation, continued with inflorescence emergence and flowering and ended with senescence. The results of this study showed a clear picture of the inflorescence development that provided important information in understanding the crop. This finding could further be used to establish the torch ginger as a cut flower.

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Growth and Reproductive Performances of Farmed Timorensis Deer, *Cervus timorensis*

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ABSTRACT

A retrospective study was conducted to evaluate the growth and reproductive performances of timorensis deer, *Cervus timorensis*, that were kept in farm. All 140 animals were allowed to graze at all times while supplemented feed of approximately 1 kg/animal/day was provided except during the rainy season between October and December when supplementation was 2 kg/animal/day. Available farm records between 2011 and 2014 were collected and analysed for growth and reproductive performances. Most fawning occurred between October and December (average rainfall 413 mm) with an apparent peak in December. Average annual fawning percentage for the past four years was 57% while the average rate of mortality for fawns and adults was 5.4% and 13.5%, respectively. The average daily weight gain for up to one year of age was 0.042±0.02 kg but after one year it was 0.052±0.01 kg. The average body weight of adult timorensis at 24 months old was 48.5±1.4 and 44.5±0.9 kg for male and female, respectively. Monthly rainfall showed significant ($p<0.05$) negative correlation with the ADG but showed positive correlation ($p<0.05$) with fawning. Body weight gain was high during the dry months of May and August (average rainfall 150 mm). There seemed to be a clear breeding season for timorensis deer in this study, which was within the moderate months of March to April (average rainfall 236 mm) when body weight started to increase.

Keywords: On-farm evaluation, timorensis deer, growth, reproductive status

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INTRODUCTION

In Malaysia, deer farming was first started in 1977 by farming Sambar deer in Perak and Sabah (Idris *et al*., 1996). This was followed by Selangor in 1987 and Negeri Sembilan in 1988 through integration with oil palm (Idris *et al*., 2000). The deer farm at Universiti Putra Malaysia was established in 1998 as a showcase farm for the public, a model for farmers and a teaching farm for students. Following attempts at farming, deer population has grown rapidly: in 1986 with 300 deer, 1991 with 9,360 deer, 1998 with 10,000 deer and 2003 with 11,000 deer.

Timorensis deer (*Cervus timorensis*) is indigenous to the Indonesian archipelago. It was later introduced to Southeast Kalimantan, New Guinea, the Bismarck Archipelago, New Caledonia, Australia and New Zealand (de Vos, 1982). There are two main subspecies of timorensis deer: the Javan and Moluccan. Timorensis hinds are aseasonal polyestrous breeders (de Vos, 1982). Both hinds (females deer) and stags (males deer) attain sexual maturity at 18 months old and the body weight of hinds at first mating is approximately 46 kg (Van Mourik, 1986).

Growth is an important factor for meat production as it determines the overall productivity of the herd and the economic return from livestock enterprise. Furthermore, birth weight is an important aspect that influences the pre-weaning growth of the young and has a positive correlation with subsequent live body weight development (Le Bel *et al*., 1997). Similarly, weaning weight has strong influence on growth rate, survival and reproductive performance (Sriyanto *et al*., 2010).

Reproductive performance is a necessity for any successful livestock production programme (Woodford & Dunning, 1992). Reproductive rate can be influenced by conception rate, litter size, young mortality and interval between parturitions. Currently, there is a lack of documentation on the growth and reproductive performance of farmed deer in Malaysia. Therefore, efforts should be made to highlight current deer performance in Malaysia and recommend possible suggestions to improve performance. This paper describes the growth, mortality and reproductive performance of timorensis deer, *Cervus timorensis*, kept in farm.

MATERIALS AND METHODS

Study Farm

A deer farm was selected for the study. At the time of study, there were 140 timorensis deer that ranged between 2 months and 17 years old. Total land area was approximately 20 hectares but was divided into several grazing plots in which the shrubs were left intact. The animals were left grazing in the paddocks at all times but were provided supplemented feed during the rainy season between October and December.

Feeding and Breeding Protocol

The deer were reared using an extensive system where they were allowed to graze on *Bracharia decumbens* and *Setaria*

splendida. Cut and carry Napier and guinea grass was provided in the paddock at approximately 1 kg/deer/day. Goat pellet was also provided in the paddock every two days at the rate of 1 kg/animal/day, and the amount was increased to 2 kg/animal/day during rainy season. Body weight was measured three times a year: in April, August and December.

Disease prevention activities included quarantine, mass immunisation and herd health. All newly arrived animals were quarantined before being introduced into the farm proper. All deer were vaccinated against foot and mouth disease (FMD).

Breeding protocol involved natural breeding at stag to hind ratio of 1:4-5. The stag was allowed to run with the breeder hind at all times. Similarly, fawns were allowed to run with their mothers until weaning at three months old. In this farm, breeding seasons were between February and April and between June and August. Animals were only culled following disease problems.

Study Parameters

The reproductive and fawn growth parameters were calculated using the following formulas (Dryden, 2000):

Reproductive parameters

1. Fawning percentage =

   $$\frac{\text{Number of fawning}}{\text{Number of breeder females}} \times 100$$

2. Monthly fawning percentage =

   $$\frac{\text{Number of fawn born in the month}}{\text{Total number of fawn born in the year}} \times 100$$

3. Mortality percentage =

   $$\frac{\text{Number of deaths}}{\text{Total number of animals}} \times 100$$

Fawn growth performance parameters:

1. Weaning weight (kg)

2. Average daily gain from birth to weaning (kg/day) =

   $$\frac{\text{Weaning weight – Birth weight (kg)}}{\text{Weaning days (days)}}$$

Data Collection and Processing

Analyses were made on 130 births between 2011 and 2014. Data were collected from personal observation, previous survey results and secondary data from the Taman Pertanian Universiti. Case histories and records were analysed to obtain information on the study parameters such as age at first parturition, parturition interval, abortions and udder problems and breeding performance.

Collected data were organised, summarised and analysed using SPSS statistical package method and presented in tables and graphic forms. Data on growth parameters (body weight and ADG) were analysed using an independent t-test to detect differences between the genders. Reproductive performance records were analysed to calculate mean and range values

of fawning percentage and fawn sex ratio. Correlation between risk factor and farm performance was analysed using Pearson's correlation.

RESULTS

Growth Performance

The average weaning weight of male fawns was 20.67±1.0 kg (ranged between 9 and 31.5 kg) and the female was 20.29±0.8 kg (ranged between 12.5 and 36.5 kg). There was no significant ($p>0.05$) differences between the weaning weight of both sexes.

At six months of age, the average body weight of the fawn was 23.52±1.0 kg while at 1 year old the weight was 36.23±0.9 kg. Therefore, the average daily weight gain for up to one year of age was 0.042±0.02 kg while the daily weight gain after one year of age was 0.052±0.01 kg.

The average body weight of the adult timorensis at 24 months old was 48.5±1.4 and 44.5±0.9 kg for males and females, respectively. The overall mean body weight was 45.2±1.2 kg. The heaviest stag was 96.5 kg and the heaviest hind was 91.0 kg at 120 months old.

Significantly ($p<0.05$) higher daily gain was recorded between May and September, when the average rainfall was low (142 mm) while rainy months between October and April revealed low daily gain. There was significant ($p<0.05$) negative correlation ($r= -0.74$) between the monthly rainfall and daily weight gain (Fig.1).

Mortality

Within the study period, 7 (5.38%) fawns below 1 year old died. There was no fawn mortality in 2011 and 2014 but the mortality rate was 2% in 2012 and 26.8% in 2014. Mortality was markedly higher during the monsoon months of October, November and December. The main causes of mortality among deer in this farm were dog attack (57.14%) and traumatic injury (28.57%).

Mortality among adults of more than 1 year old revealed an increasing pattern from 2010 to 2013, mainly due to dog attack and traumatic injury. A total of 74 deer (average 19 animals/year; 13.5% anually) were reported dead over the four-year study period. Other causes of death included post-capture myopathy, suspected urea poisoning, tuberculosis and old age.

Reproductive Performance

The gestation period was estimated to be between 8 and 9 months and the age at first fawning was between 2 and 3 years old. The average annual fawning percentage was 57% (ranged between 50% and 70%). Fawning was significantly ($p<0.05$) high during the rainy season between October and December when average rainfall was 390 mm. Back calculation revealed that the breeding season started between March and April when the average rainfall was 237 mm when the animals started to gain body weight (Fig. 1). In fact, between 2011 and 2014 all fawning was within those months. There was moderate positive correlation ($r=0.54$) between rainfall and fawning percentage (Fig. 2).

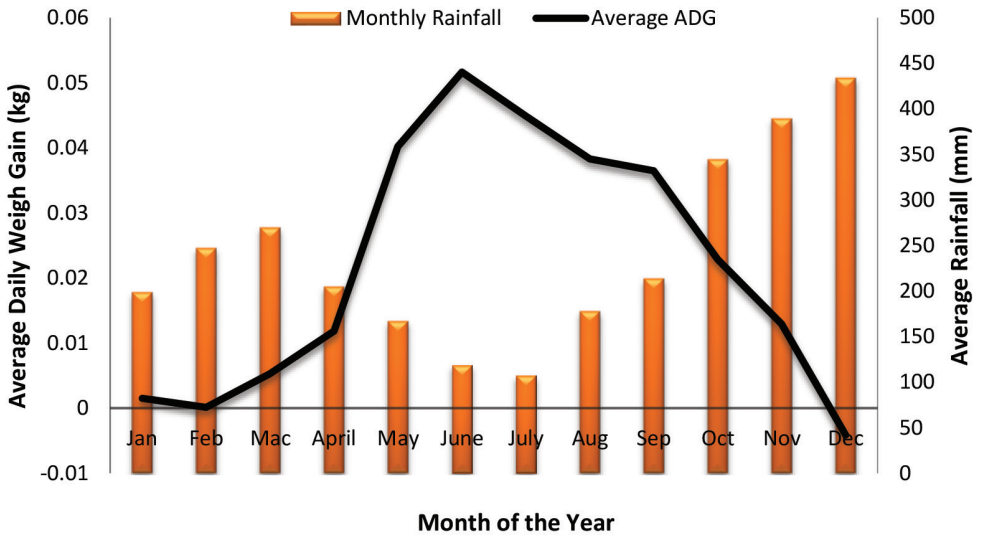

Fig. 1: Correlation between monthly rainfall and average daily gain showing moderate negative correlation (r=-0.74)

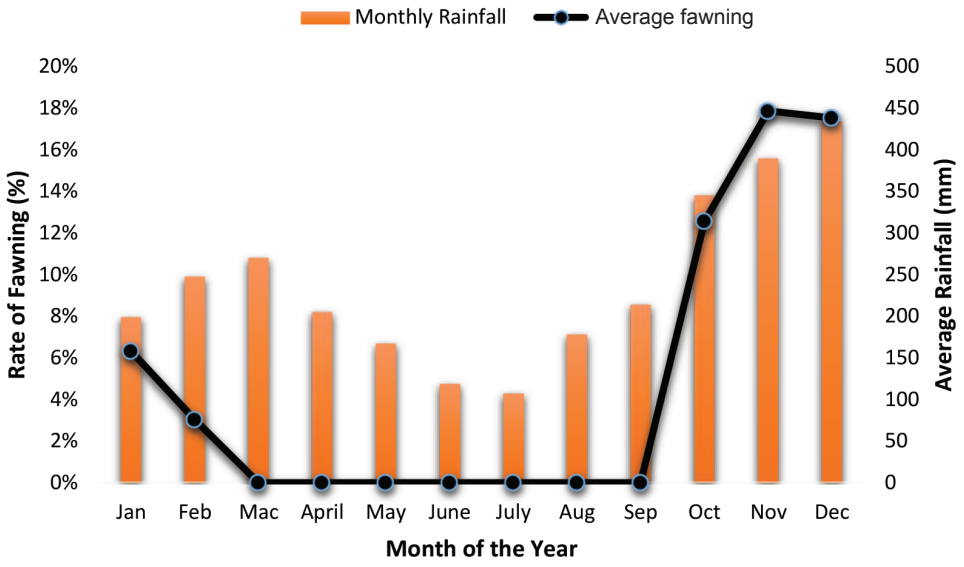

Fig. 2: The pattern of fawning according to monthly rainfall showing moderate positive correlation (r=0.54)

DISCUSSION

Weaning weight is an important economic trait in meat production since it has a strong influence on growth rate and survival (Moore *et al*., 1988) while growth traits are strongly associated with reproductive performance (Mohammadi *et al*., 2013). Since seasonal variation in growth rate has been reported in the tropics, as observed in this study because feed supply varies remarkably (Barry *et al*., 1991), the reproductive performance might be affected according to the season (Mohammadi *et al*., 2013).

Previous studies showed that growth rate was predominant during the early stages of growth and is strongly influenced by breed, milk yield of the hind and the environment under which the animals are maintained including the availability of adequate feed supply in terms of both quantity and quality (Decruyenaere *et al*., 2009). However, sex and birth type showed no significant effect on the post-weaning weight as observed in this study (Moore *et al*., 1988). Nevertheless, in temperate countries, an adult stag can achieve an average weight of 100-140 kg while the females weigh 50-90 kg (Reyes, 2015); these are much higher than those observed in this study. Modification in the feeding management of the farm might improve the body weight (Dahlan & Iskandar, 2013).

Fulbright and Ortega (2006) reported mortality rate between 10 and 15% among white-tailed deer, which is similar to the results of this study. Predatory attacks by dog was the most common (57.14%) cause of deaths in this study. Similarly, Ballard (2011) reported that 58% of the mortality of white-tail deer was due to predatory attacks by wolves. Therefore, the death rate and cause of mortality among deer in this study were within the parameters observed elsewhere.

First fawning in this study ranged between 2 and 3 years old, thus maturity was calculated to be between 22 and 28 months old. This is slightly later than reported by Van Mourik (1986), who found that both hinds and stags attain sexual maturity at 18 months old. However, this study revealed that the fawning percentage was 57%, while the white-tailed deer recorded an average of 41% fawning rate (Fulbright & Ortega, 2006). Similarly, estrous synchronisation efforts in this farm resulted in 50% pregnancy (Mahree *et al*., 2015).

The fawning season in this study was in December while Schmidly (2002) reported the fawning season for Texas deer as being in May. Seasons for breeding and subsequent fawning are related to the feed intake that changed partly due to seasonal differences in the types of plant that can be grazed (Masuko & Souma, 2009). It is believed that the period of moderate rain between March and April in this study enhanced the growth of grass (Thorvaldsson *et al*., 2005; February *et al.,* 2013) and provided opportunity for the breeder deer to consume enough dry matter. With supplementation, provided at the rate of 1 kg/animal/day in this study, the body condition of breeders was enhanced to made them ready for breeding and they eventually fawned 8 to 9 months later

in October to December. Therefore, it is suggested that March to April be recognised as the breeding season for deer in this farm even though de Vos (1982) concluded that timorensis hinds are aseasonal polyestrous breeders. The breeding behaviour of captive timorensis deer was reported to be similar to that of deer in the natural environment (Samsudewa & Capitan, 2011) while estrus behaviour remained similar to other deer species (Mahre *et al*., 2013). Furthermore, there are no differences in size, shape and surface structure between spermatozoa of different stages and different months of the fertile seasons to influence the stag reproductive performance (Mahre *et al*., 2014). This suggested breeding season resulted in a fawning season between October and December, during which time control of predators, particularly stray dogs, and records can be updated.

CONCLUSION

This study revealed that both male and female timorensis showed equal fawn growth performance, as sex difference was not significant. Furthermore, the reproductive and growth performances of timorensis deer obtained in this commercial farm were comparable while a few other parameters were lower than those of other studies.

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Evaluating the Cu and Zn Status of Various Rice Soils of Peninsular Malaysia

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ABSTRACT

Malaysia is taking efforts to be self-sufficient in rice production. However, the country is facing low availability of Cu and Zn in its soils. Even though these elements are required in small quantities for normal plant growth, their role in maximising yield is very impressive. The present study was conducted to assess the micronutrient Cu and Zn contents in selected paddy soils of Malaysia. Investigations carried out showed that marine alluvium [Guar, Sedaka, Keranji, Kuala Kedah, Kangkong, Sedu, Rotan and Kundur (Kedah)] and reverine alluvium; [Chempaka, Lubok Itek, Lating, Batu Hitam and Machang soil series (Kelantan)] were low in Cu and Zn contents. All soils were acidic in nature, with their pH values ranging from 4.3 to 6.5. Based on the results, extractable Cu and Zn concentration of soil varied within the range of 0.09-1.70 and 0.51-2.10 mg kg^{-1} in the soil series of Kuala Kedah and LubukItek, respectively. It was manifested that the micronutrient contents were below the critical level (Cu 0.1-1.0 mg kg^{-1} and Zn 0.5-3.0 mg kg^{-1})according to the method of extracting analysis; Mehlich-I. The sampling depth has shown a variation and the subsurface soil sampling exhibited lesser Cu and Zn contents as compared to the surface soil sampling. Findings of the research indicate that micronutrient deficiency occurs in the main rice regions of Malaysia, and this can be corrected by applying micronutrient fertiliser.

Keywords: Micronutrients, acidic soils, pH, copper, zinc, paddy

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INTRODUCTION

Malaysian rice production is facing many constraints. Among them, the availability of Cu and Zn in the soils is limited (Liew *et al*., 2010). Even after realising the deficiency of micronutrients in some part of Malaysian soils, the micronutrient contents still have

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to be investigated across the different soil series belonging to paddy fields. Though these elements are required in small quantities for normal plant growth, their role in maximising yield is very impressive. The incidence of Zn deficiency has been widely reported worldwide (Behera *et al.*, 2011), including in Malaysia (Hafeezullah, 2010). It is mostly predominant in calcareous soils with high pH (Katyal & Vlek, 1985). It has also been reported to occur in heavily weathered and leached acid soils (Alloway, 2008). The micronutrients Cu and Zn are involved in several essential processes in plants, especially in cereals. Zinc is required in various biochemical processes in rice plant, such as synthesis of nucleotide and cytochrome, metabolism of auxin and production of chlorophyll; as well as enzyme activation and maintenance of membrane integrity. In particular, copper serves as effector, stabiliser and inhibitor, and also as catalyst of oxidation reaction. It is particularly important in N, proteins and hormone metabolism. At the same time, it is involved in photosynthesis and respiration mechanisms, which ultimately affect pollen formation and fertilisation (Dobermann & Fairhurst, 2000). Rice is sensitive to micronutrient deficiency; therefore, a lack of such nutrients in rice can cause a drastic decline in production.

Meanwhile, positive effects of Cu and Zn have been proven, particularly in cereals (Brennan, 1991; Penney *et al.*, 1991). The deficiency of micronutrients has become a major constraint to productivity, durability and unremitting life of the soils (Bell & Dell, 2008). Micronutrient availability in soil is subjective to numerous factors (Shuman, 1986); the vital one is soil pH (Corey & Schulte, 1973; Anderson & Christensen, 1988). Total quantity of micronutrients and their availability is entirely different. The analysis of total quantity of micronutrients cannot serve as an indicator of their availability to plants (McLaren *et al.*, 1984). Besides that, the lack or excess of micronutrients in plant parts serves as a predictor in establishing the deficiency or toxicity level of an element. Therefore, it is of the utmost importance to determine the distribution of micronutrients in plant parts (Gupta *et al.*, 2008). There are several extracting methods for determination of micronutrients. Critical values of each micronutrient may vary according to the extraction method. Particularly for rice soils, the critical values along with the method of analysis mentioned by Dobermann and Fairhurst (2000) are: 0.6 mg Zn kg^{-1} (1N NH_4 acetate, pH 4.8), 0.8 mg Zn kg^{-1} (DTPA method), 1.0 mg Zn kg^{-1} (0.05N HCl), 1.5 mg Zn kg^{-1} (EDTA method) and 2.0 mg Zn kg^{-1} (0.1N HCl) whereas the critical values of micronutrients using the method of analysis, Mehlich-I are: Zn 0.5-3.0 mg kg^{-1}, 1.0-2.0 mg kg^{-1} extracted with Mehlich-III, 0.2-2.0 mg kg^{-1} extracted with DTPA and Cu 0.1-1.0 mg kg^{-1} extracted with Mehlich-I and 0.1-2.5 mg kg^{-1}extracted with DTPA (Jones Jr, 2001).

The interacting factors involved in the availability of micronutrients are crop selection, organic matter, pH and parent material, which may affect typical ranges

in the critical levels of micronutrients. Furthermore, the application of NPK fertilisers, along with intensive cultivation of high yielding varieties, has ultimately resulted in micronutrient deficiency in soils and plants (Cakmak, 2002). Micronutrient fertiliser practice is usually negligible in agriculture systems, particularly in Malaysian soils, due to their acidic nature. This is in spite of the chemical changes of rice fields that are somehow divergent against normal acidic soils. Flooding influences mainly the availability of essential nutrients due to depletion of O_2 and chemical changes, particularly in redox potential and pH, which eventually suppress the availability of Cu and Zn (Fageria *et al.*, 2003). Malaysian soils are often acidic in nature, apart from the low availability of Mo, Cu and Zn (Ratnaprabha *et al.*, 2011). Studies have proven the effects of micronutrient deficiency and the positive impacts of micronutrient fertiliser application in rice production in Malaysia (Hafeezullah, 2010; Saleem *et al.*, 2010). Malaysian farmers emphasise on subsidised fertiliser without micronutrient fertiliser application, which eventually results in low rice production; however, the application of micronutrients has revealed its positive response on growth and yield of rice (Liew et al, 2010). Earlier investigations also reported the deficiency of some essential micronutrients that are required for normal rice growth on the riverine alluvium of Kelantan (Soo, 1975). However, attention was not given to these particular rice areas of Malaysia; it is known that continuous cropping results in micronutrient deficiencies. Incidences of micronutrient deficiencies are due to intensive cultivation, leaching of nutrients and depletion of soil fertility because of the loss of top soil (Somani, 2008). Double cropping with high yielding varieties due to a well-developed irrigation systems is commonly observed in Malaysia (Ho *et al.*, 2008).The long-term continuous cropping of high yielding varieties from the same piece of land causes obstacles in availability of soil micronutrients; hence, balanced fertiliser practice is a dire need for better crop production (Wei *et al.*, 2012). The current study was designed to evaluate the status of the micronutrients Cu and Zn of various rice soils of Peninsular Malaysia, which can be used as a guideline for fertiliser application.

MATERIALS AND METHODS

Sampling Location

Soil samples were collected from 13 different soil series situated in the Northwestern (Kedah) and Northeastern (Kelantan) states of Peninsular Malaysia by applying the simple random soil sampling method. The soil samples were collected at two different depths (0-15cm and 15-30cm) of paddy fields. Further details in relation to the name of the soil series, location, taxonomy, texture of soil (estimated by the pipette method) and cropping practices in these soils are given in Table 1. The sampling sites are situated in the tropical climate with varied average annual precipitation. The temperature is relatively consistent throughout the course of the year, with a maximum of 32 ^{0}C and a minimum of 23 ^{0}C.

TABLE 1
Soil series, taxonomy, texture, GPS location and land use in particular areas

Soil series	Latitude and Longitude	Taxonomy	Texture	Land use
Chempaka	N-05-97370, E-102-29944	Ultisol	Clay loam	Paddy-Paddy
Lubok Itek	N-05-94769, E-102-29465	Entisol	Clay loam	Paddy-Paddy
Lating	N-05-93200, E-102-27113	Ultisol	Sandy loam	Paddy-Paddy
Batu Hitam	N-06-22462, E-102-09477	Ultisol	Silty clay loam	Paddy-Paddy
Machang	N-05-76018, E-102-20285	Oxisol	Sandy clay loam	Paddy-Paddy
Guar	N-05-52098, E-100-27678	Inceptisol	Silty clay	Paddy-Paddy
Sedaka	N-05-53407, E-100-26313	Inceptisol	Silty clay	Paddy-Paddy
Keranji	N-06-08836, E-100-31481	Entisol	Silty clay	Paddy-Paddy
Kuala Kedah	N-06-13422, E-100.29527	Inceptisol	Silty clay	Paddy-Paddy
Kangkong	N-06-22744, E-100-26120	Entisol	Silty clay	Paddy-Paddy
Sedu	N-06-20451, E-100.33191	Entisol	Clay	Paddy-Paddy
Rotan	N-06-14233, E-100-31320	Inceptisol	Silty clay	Paddy-Paddy
Kundur	N-05-98005, E-100-43354	Inceptisol	Silty clay	Paddy-Paddy

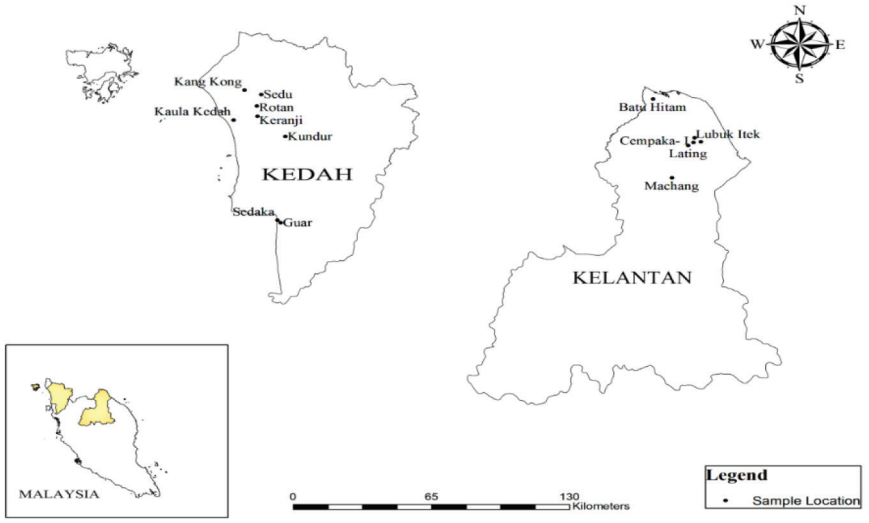

Fig. 1: Map of the Kedah and Kelantan showing the sampling locations

Soil Sampling and Preparation

A total of 26 representative surface and subsurface soil samples (six from each soil series) were collected from the paddy fields to represent the 13 soil series, namely, Cempaka, Lubuk Itek, Lating, Batu Hitam, Machang, Guar, Sedaka, Kerenji, Kuala Kedah, Kangkong, Sedu, Rotan and Kundur of Kelantan (riverine alluvium) and Kedah (marine alluvium). The soil samples were air-dried, then ground using mortar and pestle and passed through a 2-mm sieve, before being placing in polyethylene bags for further analysis.

Soil Analysis

Soil pH was determined 1:1 with soil water ratio (v/w) using a pH meter (Jones Jr, 2001). Soil texture was determined by employing the pipette method (Gee & Bauder, 1986). Available Cu and Zn in soils were extracted by using Mehlich-I. (soil to solution ratio 1:5, soil 5g and 25 mL of double acids; 0.05 N HCl and 0.025 N H2SO4), with a shaking time of 15 minutes at 180 rpm using a mechanical shaker. The contents were then determined on Atomic Absorption Spectrophotometer (Jones Jr, 2001).This method is selected on the basis of its characteristics; this method is particularly designed for acidic soils (like Malaysian soils), specifically, soils with CECs less than 10 meq/100 g of soil and pH less than 7.0 and are relatively low in O.M (< 5%) contents (Davis *et al.*, 1995).

Statistical Analysis

Descriptive statistics of the extractable Cu and Zn contents and pH were obtained. A correlation study was conducted to assess the relationships between pH, Cu and Zn. The collected data were analysed by using Analysis of Variance ANOVA in Completely Randomised Design (CRD), followed by Tukey's test for comparing means of the parameters using Statistic version 8.1.

RESULTS AND DISCUSSION

Soil pH

Findings on pH, Cu and Zn contents from 0-15 and 15-30 cm depths of various rice soils of Malaysia are presented in Tables 2 and 3, respectively. It is manifest from the data that all the soils collected from Kedah and Kelantan were found to be acidic in nature. The soil of Lubuk Itek (Kelantan) is slightly acidic in nature, with pH values ranging from 4.1 to 6.5. pH values are influenced by soil depths; as the sampling depth increases, so do the pH values. Standard error of mean for soil pH in the selected soil series indicated the acidity trend in all the soils from both regions, except for Lubuk Itek, with a pH value of 6.5 (Fig.2). A major reason behind the measurement of soil pH was to modulate the behaviour and micronutrient availability to plants in soil. This means pH value can affect the availability of trace elements (Öborn *et al.*, 1995). Soil pH and redox potential are two most considerable factors in the flooded condition of paddy fields which effects on nutrient cycling.

TABLE 2
Soil pH, extractable Cu and Zn contents in soils at depth of 0-15 cm

	Soil series	pH			Cu mg kg^{-1}			Zn mg kg^{-1}		
		Range	Mean	SD	Range	Mean	SD	Range	Mean	SD
1	Guar	4.25-4.52	4.37	0.13	0.75-0.92	0.81	0.09	1.00-1.67	1.39	0.34
2	Sedaka	4.24-4.42	4.31	0.09	0.54-0.65	0.58	0.05	1.10-1.51	1.33	0.21
3	Keranji	4.50-5.01	4.80	0.26	0.89-0.91	0.91	0.02	1.40-1.51	1.47	0.06
4	Kuala Kedah	5.00-5.65	5.29	0.32	0.10-0.12	0.11	0.01	0.88-0.92	0.90	0.02
5	Kangkong	4.10-4.69	4.61	0.11	0.69-0.76	0.73	0.03	1.00-1.60	1.23	0.32
6	Sedu	4.12-4.76	4.50	0.33	0.81-0.93	0.88	0.06	1.20-1.70	1.46	0.25
7	Rotan	4.32-4.98	4.75	0.37	0.88-0.91	0.89	0.01	1.30-1.63	1.49	0.17
8	Kudur	4.65-4.92	4.80	0.14	0.91-1.00	0.94	0.05	0.90-1.50	1.26	0.32
9	Chempaka	4.97-5.31	5.12	0.17	0.14-0.16	0.15	0.01	0.91-1.40	1.10	0.26
10	Lubok Itek	6.00-6.50	6.30	0.32	1.50-1.70	1.56	0.11	1.89-2.10	1.96	0.11
11	Lating	5.00-5.84	5.43	0.41	1.10-1.30	1.23	0.11	1.87-1.90	1.88	0.01
12	Batu Hitam	4.26-4.65	4.49	0.21	0.78-0.92	0.87	0.07	1.49-1.51	1.50	0.01
13	Machang	4.34-4.88	4.66	0.28	1.10-1.40	1.23	0.15	1.49-1.57	1.53	0.04

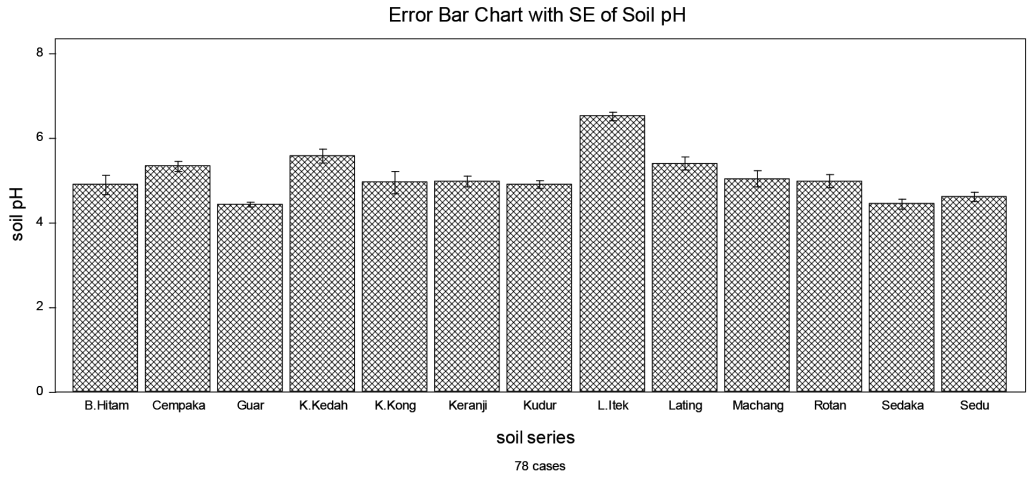

Fig. 2 : Standard error of soil pH selected soil series

TABLE 3
Soil pH, extractable Cu and Zn contents in soils at 15-30 cm

	Soil series	pH			Cu mg kg^{-1}			Zn mg kg^{-1}		
		Range	Mean	SD	Range	Mean	SD	Range	Mean	SD
1	Guar	4.31-4.62	4.49	0.16	0.69-0.73	0.71	0.02	0.98-1.10	1.02	0.06
2	Sedaka	4.15-4.87	4.58	0.37	0.65-0.70	0.67	0.02	1.11-1.21	1.14	0.05
3	Keranji	5.00-5.50	5.17	0.28	0.36-0.45	0.40	0.04	0.98-1.10	1.06	0.06
4	Kuala Kedah	5.50-6.10	5.80	0.30	0.09-0.11	0.10	0.01	0.51-0.56	0.53	0.02
5	Kangkong	5.39-5.67	5.51	0.14	0.58-0.61	0.59	0.01	0.98-1.00	0.96	0.05
6	Sedu	4.50-5.00	4.77	0.22	0.65-0.71	0.67	0.03	0.90-1.00	0.93	0.05
7	Rotan	5.03-5.56	5.22	0.29	0.67-0.71	0.69	0.02	0.89-1.00	0.93	0.05
8	Kudur	4.70-5.20	5.00	0.26	0.67-0.70	0.68	0.01	0.78-0.88	0.84	0.05
9	Chempaka	5.33-5.67	5.55	0.19	0.12-0.13	0.12	0.05	0.62-0.65	0.63	0.01
10	Lubok Itek	5.21-6.67	6.14	0.80	0.96-1.00	0.97	0.02	1.12-1.25	1.20	0.07
11	Lating	4.88-5.76	5.38	0.45	0.77-0.81	0.79	0.02	1.20-1.30	1.20	0.05
12	Batu Hitam	4.70-5.52	5.31	0.53	0.85-0.89	0.87	0.02	0.93-1.00	0.95	0.03
13	Machang	5.35-5.52	5.42	0.08	0.88-0.92	0.90	0.02	0.91-0.94	0.92	0.01

Chemical changes mainly alter the pH and redox potential in the sub-merged condition of paddy fields and eventually affect nutrient availability (Fageria *et al.*, 2011).

A significant correlation was found between pH and soil available Cu and Zn from the samples that were taken from the surface depth. The results showed a positive correlation between soil pH and extractable Cu, and a significant correlation between soil pH and extractable Zn (Table 4).

TABLE 4
Correlation coefficient (r) among pH and extractable Cu and Zn

	pH	Cu	Zn
pH	1.00	0.74	0.51
Cu	0.74	1.00	0.034
Zn	0.51	0.034	1.00

These results are in accordance with Ghosh *et al.* (2009), who reported higher acidity from the surface of the soil samples than the subsurface soil. Furthermore, they also observed the significant negative correlation (-0.466 to -0.670) between DTPA extractable micronutrients and soil pH in subsurface soil sampling.

Soil Available Cu

Cu and Zn in soils showed low contents of both micronutrients in all the 13 soil series. The average extractable Cu content in soils at 0-15cm varied from 0.11 to 1.56 mg kg^{-1} and 0.10 to 0.90 mg kg^{-1} at 15-30 cm depth. The extractable Cu content, as illustrated in Tables 2 and 3, decreased with the increase in soil depth. The sampling from different depths can specifically help to determine the areas where nutrient applications are

warranted. Results are in accordance with Sharma *et al.* (2000), who testified that as the depth increased, the available Cu content would decrease. According to researchers, depth can only affect available Cu content, but total Cu will remain unfluctuating regardless of depth. Similar results were found by Sharma, Jassal, Sawhney and Sidhu (1999), who analysed total Cu in alluvial soils of Northwest India.

The surface soil samples recorded lower Cu content of marine alluvium soils (Kedah) as compared to riverine alluvium soils (Kelantan). The subsurface soil samples showed lesser content in comparison to the surface soil samples. Standard error estimates the accuracy among available Cu mg kg^{-1} in the selected soil series (Fig.3). It is clear from the figure that the soil series of Lubok Itek (riverine alluvium) has more Cu content as compared to the rest of the soil series. The least content of available Cu was recorded at 0.1 mg kg^{-1} in the soil series of Kuala Kedah (marine alluvium), which falls at the critical level (0.1-1.0 mg kg^{-1}) according to the extracting method Mehlich-I (Jones Jr, 2001). Higher available Cu content was observed in the soils of the riverine alluvium belt, except for the Chempaka soil series. The higher content of Cu in these soils may be due to the predominance of the higher content of Cu in the parent material. Spatial variability (variation across the distance) of soil available micronutrients is particularly due to the soil parent material, soil texture and specifically the clay contents in soil (Eriksson *et al.*, 2010), all these parameters influence the micronutrient distribution. The positive correlations between DTPA-Cu and OC, coarse clay and exchangeable K were also noticed; it was observed that available copper had inverse relationships with clay content, very fine sand and total Cu (Sharma *et al.*, 2006). Therefore, the available Cu contents in this study were found to be lower in the marine alluvium soils due to their clay content. Overall, the comparison done through Tukey's test showed that the means with the similar letters for the Cu content were not significantly different, except for the Kuala Kedah and Chempaka soil series, as presented in Table 5. The evidence for the critical values of Cu and Zn content in these soils is mainly due to continuous cropping without any particular micronutrient application.

TABLE 5
Mean comparison of available Cu mg kg-1 among thirteen soil series

Soil series	Cu mg kg^{-1}	Groups
	Mean	
Lubok Itek	1.27	a
Machang	1.06	ab
Lating	1.01	ab
Batu Hitam	0.87	bc
Kudur	0.81	bc
Rotan	0.79	bc
Sedu	0.78	bc
Guar	0.76	bc
Kangkong	0.66	c
Keranji	0.65	c
Sedaka	0.63	c
Chempaka	0.13	d
Kuala Kedah	0.10	d

Means followed by the same letters are not significantly different at p>0. 05 by HSD

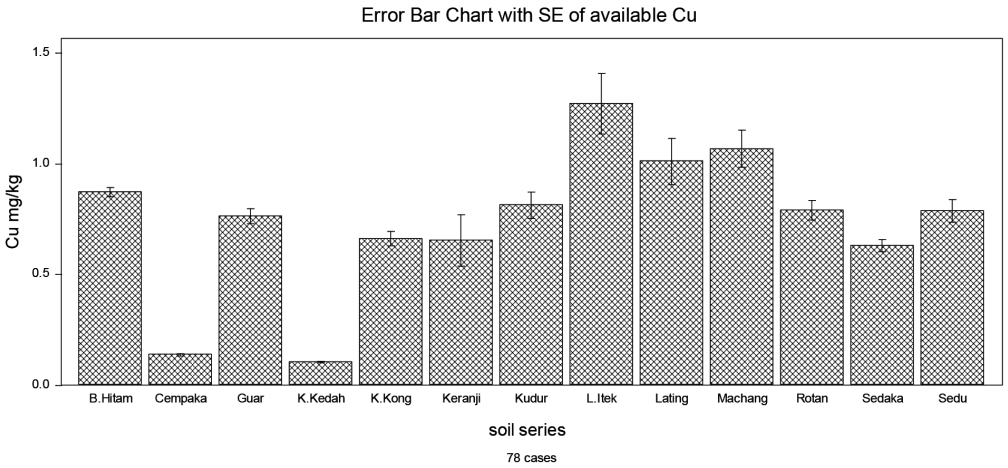

Fig.3 : Standard error of available Cu mg kg^{-1} in selected soil series.

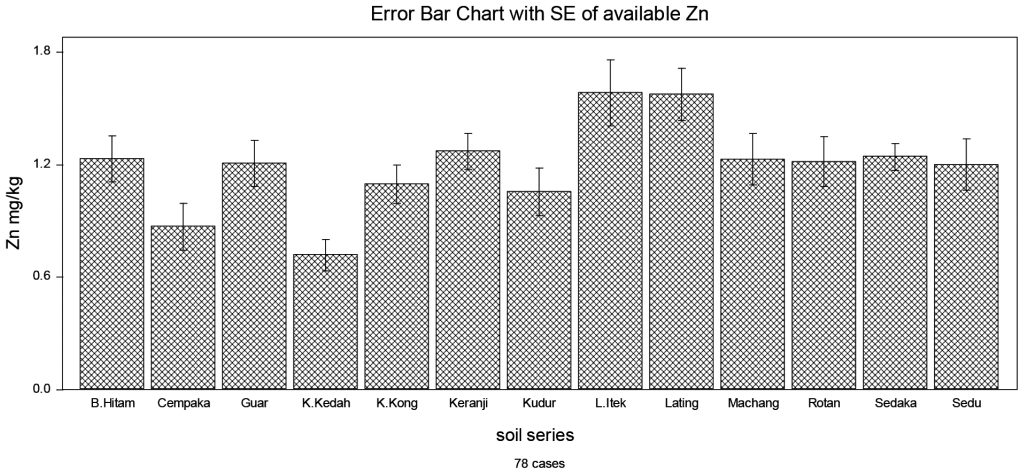

Fig. 4 : Standard error of available Zn mg kg^{-1} in selected soil series.

Soil Available Zn

The amount of Zn extracted by Mehlich-I ranged from 0.90-1.96 mg kg^{-1} and 0.53-1.20 mg kg^{-1} from the surface and subsurface soil samples, respectively. The results showed lower Zn content in the marine alluvium soils of Kedah; however, the Chempaka soil series from the riverine alluvium of Kelantan also showed almost similar values of marine alluvium soils. Overall, the Zn content in the riverine alluvium soils is higher as compared to the marine alluvium soils, except for the Chempaka soil series (Fig.4). On average, a higher amount of Zn (1.65 mg kg^{-1}) was recorded from the soil series of Lubok Itek.

The results of the Zn content revealed the decreasing amount of Zn with subsurface soil sampling (Table 3). This is in line with the finding of Jobbágy and Jackson (2001), who studied the distribution of nutrients with depths and observed the fluctuating nature of nutrient content with depths, whereby they recorded that the subsurface horizon of soil initiated the deterioration in nutrient availability. It is manifested from the data that the average mean of the Zn content from the riverine alluvium soil recorded as slightly elevated compared to the marine alluvium soil. Meanwhile, the Kuala Kedah soil series showed the least amount of Zn as compared to the rest of the 12 soil series and was found to be significantly different ($p<0.05$) (Table 6).

TABLE 6
Comparison of the mean micronutrient Zn mg kg^{-1} in 13 soil series

Soil series	Zn mg kg^{-1}	Groups
	Mean	
Lubok Itek	1.58	a
Lating	1.57	a
Keranji	1.27	ab
Sedaka	1.24	ab
Batu Hitam	1.23	ab
Machang	1.22	ab
Rotan	1.21	ab
Guar	1.20	ab
Sedu	1.20	ab
Kangkong	1.09	ab
Kudur	1.05	ab
Chempaka	0.87	b
Kuala Kedah	0.71	b

Means followed with the same letters are not significantly different at $p>0.05$ by HSD

The effect of pH on the micronutrient availability was also noted, whereby the strong acidic soil series of marine alluvium (Sedaka) with a pH value of 4.31 contained Cu and Zn contents of 0.63 and 1.24 mg kg^{-1}, respectively whereas the soil series of riverine alluvium (LubukItek) with the pH value of 6.50 showed comparatively elevated contents of Cu and Zn 1.56 and 1.96 mg kg^{-1}, respectively. The results also revealed higher Cu and Zn contents in the soils having pH values above 5. A similar finding was also noted by Ghosh, Sarkar and Sahoo (2009), who described the relationship between micronutrient cation and physical and chemical properties of soils in West Bengal. They reported that the surface soils were more acidic compared to subsurface soils; simultaneously, the extractable micronutrients decreased with increasing depth.

There was a positive correlation coefficient (r) between both micronutrients, Cu and Zn (Table 4). However, studies by Sherma *et al.* (2006) indicated a negative correlation for the soil for available Zn and percentage of coarse clay in soil texture. Malaysian soils were found to be deficient in essential micronutrients, as previously reported (Hafeezullah, 2010; Liew *et al.*, 2010; Saleem *et al.*, 2010). According to Dobermann and Fairhurst (2000), the results obtained in this study are also similar to the critical values retrieved from the analytical method Mehlich-I and also from the rice crop growth point of view. There are low contents of Cu and Zn in almost all the rice soils in Kedah and Kelantan. Trace element

availability in acid soils is a constraint for crop productivity. These regions have low rice productivity due to very trace or low availability of these micronutrients in the soils.

It is important to understand the factors inducing the deficiency of such crucial micronutrients; soil pH, organic matter, soil texture accentuated Cu and Zn mining are of vital importance. Soil calcareousness, low soil organic matter, sandy soil texture and most importantly, the mining of Zn by high yielding varieties have been reported (Rashid & Ryan, 2004). The main rice growing areas of Malaysia are continuously cultivated with rice but are not supplied with micronutrient fertiliser. Due to the continuous mining of such nutrients through high yielding varieties, deficiency of these crucial nutrients will eventually occur. Healthier rice production and soil fertility status are possible if these soils are supplied with the required amounts of Cu and Zn, along with the recommended NPK fertilisers and package of management practices.

CONCLUSION

All the soil samples taken from the main rice growing areas of Malaysia were acidic in nature and had low available Cu and Zn contents. The micronutrient contents were found to be different in both of the regions; marine and riverine alluvium. However, the marine alluvium soils had lower micronutrient contents and the lowest amounts of both Cu and Zn were recorded in the soil series of Kuala Kedah (marine alluvium), with the values that are below the critical level of Mehlich-I. Thus, it was perceived that soil depth and pH effects on the micronutrient availability and more contents were observed in the surface soil samples. Soil acidity influences micronutrient content, with slightly acidic soil series containing more Cu and Zn compared to strongly acidic soils. Therefore, there were low yields of rice due to the imbalance in fertiliser practices, which can eventually overcome micronutrient deficiencies. Therefore it is suggested to sample the soil of main rice growing areas of Malaysia for the determination of micronutrients, and as the contents are found to be at critical values, the particular micronutrient should be applied in the form of inorganic fertilisers. Besides fertiliser application, the appropriate agronomic practices should strictly be followed for better crop production.

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Simultaneous Effects of Water and Nitrogen Stress on the Vegetative and Yield Parameters of Choy Sum (*Brassica chinensis* var. *parachinensis*)

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ABSTRACT

Hypothetically, leafy vegetables need water and nitrogen (N) simultaneously in their applications for good growth. Therefore, this study was conducted to determine the effects of four watering frequencies (watering once a day, once a week and once in every two weeks and no watering) and five levels of nitrogen (0, 34, 68, 136 and 272 kg N ha^{-1}) on the vegetative and yield parameters of choy sum over a period of four weeks. The choy sum was grown in polyethylene bags under a rain shelter. The experimental design was a split-split plot with four replications. Plant vegetative and yield parameters measured weekly were plant height, leaf number, total leaf area, maximum root length, the various plant part weights and total tissue nitrogen. Water stress detrimentally affected choy sum's leaf growth more than root growth and the effect of water stress was more than nitrogen stress. Higher nitrogen rates in water-stressed condition increasingly reduced the number of leaves and height of the choy sum. Choy sum grown under once-a-day watering and once-a-week treatments did not experience water stress. The optimal soil water content and nitrogen application rate were 0.4 m^3 m^{-3} and 30 to 40 kg N ha^{-1}, respectively. The rate of 34 kg N ha^{-1} and once-a-week watering treatment generally gave the highest values for all the measured vegetative and yield parameters. Under lower and point of sufficient nitrogen rates, nitrogen was used for leaf thickness and weight rather than for intercepting light via leaf area expansion.

Keywords: Brassica, choy sum, drought, nitrogen fertiliser, water deficit, water stress

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INTRODUCTION

Choy sum (*Brassica chinensis* var. *parachinensis*) is one of the main leafy vegetable crops grown in Asia, including Malaysia (Tin *et al*., 2000). This vegetable is rich in vitamins and fibre, and it has a short life cycle that allows it to be harvested in a month (Chin, 1999). This crop grows best in conditions of adequate water (10 to 12 mm of water per day), nitrogen (N) requirement of 68 kg N ha^{-1} and air temperature between 23 and 35 ^{o}C (Vimala & Chan, 2000).

Chin (1999) remarked that choy sum is able to tolerate mild water deficit better than waterlogged conditions. Furthermore, excessive rainfall (>300 mm per month) could damage the leaves of the choy sum and in turn reduce the yield quality (DOA, 1998), but this problem can be mitigated by growing choy sum under a rain shelter. Different Brassica species respond differently to water stress. Caisin (*Brassica rapa* subsp. *parachinensis*), for instance, was observed to be more tolerant of waterlogging and water deficit than Chinese kale (*Brassica oleracea* var. *alboglabra*) (Issarakraisila *et al*., 2007). Nonetheless, both these Brassica species still experienced reduced total leaf area and leaf weight, delayed flowering and increased tissue nitrogen concentration under 14 days of water deficit conditions.

Adequate water and nitrogen supply are important for plant growth and maximum yield (Gutierrez & Whitford, 1987; Laurie *et al*., 2009). Too little or too much water and/or nitrogen can have negative effects on plant growth, which leads to lower yield. The yield of kale, for instance, had increased by 71% when 100 kg N ha^{-1} was applied and the yield declined after 200 kg N ha^{-1} (Hill, 1990).

Nitrogen is important because it is one of the components of chlorophyll and it plays a role in protoplasm formation as it is also one of the building blocks of amino acids (Campbell & Reece, 2002). According to Russell (1988), both soluble amino compounds and protein increase as nitrogen content increases. Nitrogen deficiency occurs when there is a lack of nitrogen to manufacture adequate structural and genetic materials, which ultimately causes stunted plant growth (Laurie *et al*., 2009). Excessive nitrogen, instead, increases the demand for carbon (C), leading to a decrease in the proportion of carbohydrate available for cell-wall material (Russell, 1988). Excessive nitrogen can also cause the plant to be more susceptible to diseases such as soft rot damage on pak choy (*Brassica campestris* var. *chinensis*) (Hill, 1990) and head rot on broccoli (*Brassica oleracea* var. *italic*) (Everaarts, 1994).

Availability of water and nitrogen affect plant growth and yield differently. Pandey *et al*. (2000) indicated that the effect of nitrogen was highly significant under non-limiting water conditions, but nitrogen gave no significant effect when the plant was water-stressed. They also reported that the yield reduction under water deficit conditions was much more severe when nitrogen was applied at high rates. Barraclough *et al*. (1989) found that the grain yield of winter wheat under drought

with low nitrogen supply showed the lowest yield. Wu *et al*. (2008) showed that leaf area ratio, roots-to-shoot ratio and relative water content of pagoda shrub (*Sophora davidii*) increased under severe drought (20% of field capacity) and with low nitrogen (92 mg N kg^{-1} soil). Under the same severe drought stress condition, the height, leaf number, leaf area and biomass of pagoda seedlings decreased with high nitrogen (184 mg N kg^{-1} soil). Ahmadi and Bahrani (2009) reported that the highest nitrogen application rate with adequate irrigation gave the highest value in plant height, number of branches, pods and seeds and oil yield for rapeseed (*Brassica napus* L.).

Although the response of many crops to water and nitrogen stresses have been widely reported, studies specifically on choy sum's tolerance to these two stresses, especially the simultaneous effects of these two stresses on choy sum, remain rare. The Malaysian Research and Development Institute (MARDI) indicated that under Malaysia's growing conditions, the nitrogen requirement for choy sum is 68 kg N ha^{-1}. Vimala and Chan (2000) showed that choy sum can still grow well in the dry season provided irrigation is supplied. However, the degree to which choy sum can tolerate water stress remains uncertain. Hence, this study was carried out to evaluate the simultaneous effects of several watering frequencies and nitrogen rates on the vegetative and yield parameters of choy sum. We hypothesised that water stress would have a larger effect than nitrogen stress on choy sum vegetative and yield parameters and that choy sum's vegetative growth and yield would be detrimentally affected by water stress when the soil water content falls below the critical level.

MATERIALS AND METHODS

Experimental Setup

The experiment was set up as a split-split plot design with four watering frequencies: S1 (once a day), S7 (once a week), S14 (once every two weeks) and SX (without watering) as whole plots; five nitrogen (N) application rate ratios: N0 (0xR), N0.5 (0.5xR), N1 (1xR), N2 (2xR) and N4 (4xR), where R is the recommended rate (68 kg N ha^{-1}) by MARDI (Vimala & Chan, 2000), as sub plots; and five growth stages (time): 0, 7, 14, 21 and 28 days after transplanting (DAT) as sub-sub plots, with four replications.

This experiment was conducted under a rain shelter at Agronomy Research Farm (2° 59.47' N and 101° 42.882' E), Universiti Putra Malaysia, Serdang, Selangor. The experiment started on March 25, 2011 and ended on April 21, 2011.

The soil (Munchong series – Typic Hapludox), taken from the field at soil depth 0-150 mm, was air dried and sieved (2 mm) before being placed into polyethylene bags (5 kg of soil per bag). The soil pH (1:2.5) (Meter-827 pH Lab), electrical conductivity (EC Meter-Lab 960), total carbon (combustion method; LECO-CR 412 Carbon Analyser), total nitrogen (Kjeldahl method; Jones, 1991), particle size analysis (pipette method; Gee & Bauder, 1986), bulk density (core ring method; Blake & Hartge, 1986) and water retention (membrane plate

method; Richards, 1947) were analysed and are summarised in Table 1.

TABLE 1
Soil properties used in this study

Parameters	Value
Soil series	Munchong (Typic Hapludox)
pH	6.8
EC (dS m^{-1})	0.62
Particle size distribution (%)	
Clay (2-50μm)	65.41
Silt (< 2μm)	7.63
Sand (> 50 μm)	26.74
Texture class (USDA)	Clay
Total carbon (%)	0.99
Total nitrogen (%)	0.15
Bulk density (Mg m^{-3})	1.08
Volumetric soil water content (%)	
Saturation	74.97
Field capacity	44.55
Permanent wilting point	25.32

The NPK fertilisers were applied manually with a rate of 68 kg N ha^{-1}, 10 kg P ha^{-1}, and 96 kg K ha^{-1} (Vimala & Chan, 2000), respectively, using straight fertilisers i.e. urea (46% N), triple superphosphate (20% P) and muriate of potash (50% K). The fertilisers were applied only once every two days before transplanting. The choy sum seedlings were transplanted manually after 14 days in the nursery so that each polyethylene bag had four seedlings. Each experimental unit comprised 20 polyethylene bags. All seedlings were watered only in the mornings with 1 L of water per polyethylene bag or 10 mm per poly bag. One polyethylene bag was randomly selected from every experimental unit, and all plants in the selected polyethylene bag were sampled (destructive sampling) for plant analyses.

Plant Parameters

The plant samples were measured for plant height, number of leaves, leaf area (LI-3100 Area Meter), maximum root length and weights of plant part (leaves including petioles, stem and roots). Specific leaf area (SLA) and shoot-to-roots ratio were also calculated. The total nitrogen (wet ashing method: Jones, 1991; Auto-Analyzer, 2000 Series) for tissues sample was also determined.

The analysis of variance (ANOVA) was done by using a package of Statistical Analysis System, SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). No data was transformed prior to ANOVA as the distribution of data did not violate any of the ANOVA assumptions. The mean separations were analysed by Student-Newman-Keuls (SNK) at 5% of significance level.

Soil Water Content

Daily soil volumetric water content (VWC) throughout the experiment is shown in Fig.1. The soil VWC at field capacity (FC) and permanent wilting point (PWP) (determined using the ceramic plate method; Richard, 1947) were 44.6% and 25.3%, respectively. The mean soil volumetric water content (± standard error) under the S1, S7 and S14 watering treatments were 42% (±0.34), 35% (±0.76) and 29% (±1.15), respectively. The mean soil volumetric water content (VWC) under the S14 treatment (watering once every two weeks) was close to PWP, and the

S1 treatment (watering once a day) close to FC. The mean soil VWC under S1 was 17% and 31% more than that under S7 and S14, respectively. The SX treatment (without watering) contained only 3% (±0.30) mean soil VWC (data not shown) that was 76% lower than PWP, while the water level was far too low and held on too tightly by the soil for plant survival.

RESULTS AND DISCUSSION

The choy sum seedlings in the SX treatment (without watering) died soon after transplanting due to severe water stress. Therefore, the ANOVA was based on the three watering treatments (S1, S7 and S14) and the five application levels of nitrogen. The ANOVA results (Table 2) reveal that the significant SxNxT interaction was not observed in most of the measured parameters except for the leaf number and plant height. The NxT interaction was significant on the root length, total leaf area and SLA, while the effect of the SxT interaction was seen on the total dry weight, the individual dry weights of leaves and shoot and total leaf area.

Water stress (S) detrimentally affects leaf growth more than root growth as in maize (Davies, 2006) and oil palm (Sun *et al*., 2011), giving a lower shoot-to-roots ratio in water-stressed conditions. This trend was observed in this study (Table 2 and Fig.2). Also observed in this study was that the shoot dry weight but not the root dry weight was affected by the water stress (S) (Table 2).

The results of this study show that the choy sum's growth was more affected by water stress than by nitrogen stress. The shoot-to-roots ratio was significantly affected by water stress (S) but not by nitrogen stress (N) (Table 2). The S1 and

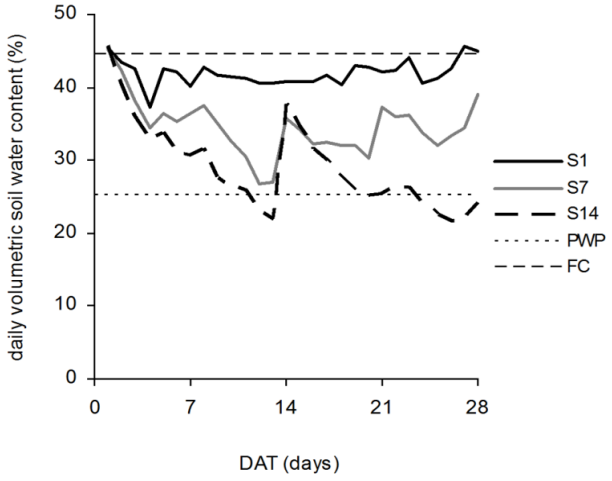

Fig.1: Daily volumetric soil water content for watering frequencies: S1 (once a day), S7 (once a week) and S14 (once every two weeks). Note: FC (field capacity), PWP (permanent wilting point) and DAT (days after transplanting).

S7 watering treatments gave the highest shoot-to-roots ratio, whereas S14 was the lowest (Fig.2). Sun *et al*. (2011) and Yin *et al*. (2009) reported that the plant growth in their studies was more affected by water availability than by nutrient (nitrogen and potassium) availability. Water stress has a larger effect than nitrogen stress on plants because highly water-stressed plants lead to higher plant osmotic stress, and this

TABLE 2
Summary of analysis of variance (ANOVA) indicating Pr > F for Choy Sum's vegetative and yield parameters under effects of watering frequencies (S), nitrogen rate (N) and time (T)

Parameters	S	N	T	SxN	SxT	NxT	SxNxT
Leaf number	0.048*	0.038*	<0.001**	0.211	<0.001**	<0.001**	0.048*
Height	0.011*	0.024*	<0.001**	0.192	<0.001**	<0.001**	<0.001**
Maximum root length	0.836ns	0.364	<0.001**	0.966ns	0.266ns	0.007**	0.592ns
Leaves (dry weight)	0.183	0.096ns	<0.001**	0.380	0.014*	0.137ns	0.527ns
Stem (dry weight)	0.451ns	0.488ns	<0.001**	0.935ns	0.241ns	0.189ns	0.996ns
Roots (dry weight)	0.235ns	0.460ns	<0.001**	0.999ns	0.115ns	0.938ns	0.999ns
Shoot (dry weight)	0.138	0.076ns	<0.001**	0.382ns	0.002**	0.051ns	0.562ns
Total (dry weight)	0.146	0.079ns	<0.001**	0.492	0.002**	0.057ns	0.765ns
Shoot-to-roots ratio	0.030*	0.305ns	<0.001**	0.408ns	0.527ns	0.433ns	0.837ns
Total leaf area	0.201	0.024*	<0.001**	0.283	0.005**	<0.001**	0.470ns
Specific leaf area	0.511ns	0.275	<0.001**	0.357ns	0.165ns	0.049*	0.889ns

Note: ns not significant; * p≤0.05; ** p≤0.01; ns, *, and ** show the applicability of p level.

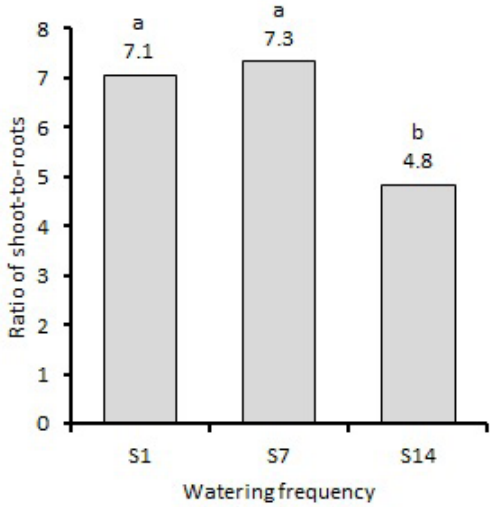

Fig.2: Shoot-to-roots ratio as affected by watering frequencies: S1 (once a day), S7 (once a week) and S14 (once every two weeks). Means with the same letter are not significantly different based on SNK test at p=5% level.

results in increasingly lower nutrient uptake (Mustafa & Abedelmagid, 1982; Pala *et al*., 1996).

Pandey *et al*. (2000) and van den Driessche *et al*. (2003) observed that nitrogen fertilisation under water-stressed conditions would exacerbate the stress experienced by plants, in particular when the nitrogen rate applied is also high. Consequently, in this study, the combination of S14+N4 treatments (highest water stress with highest nitrogen level) gave the lowest leaf number and plant height (Fig.3). In contrast, the S7+N0.5 gave the highest values for these two parameters. Fig.4 additionally shows that the total nitrogen content of the whole plant tissues was the lowest for treatments experiencing the highest water stress level

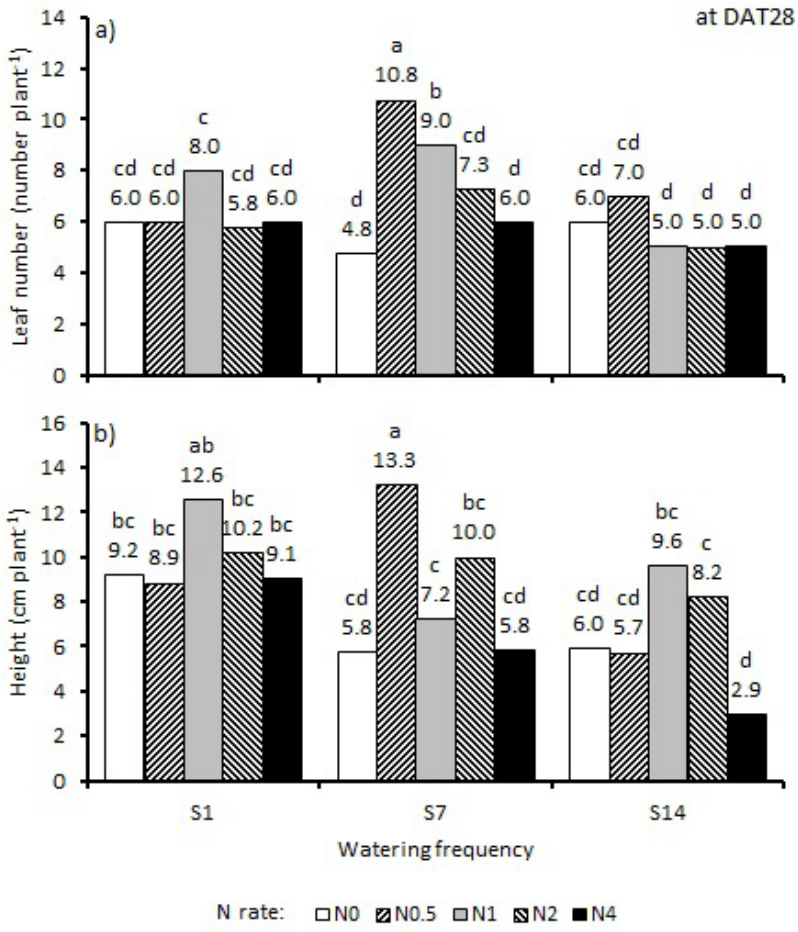

Fig.3: Effect of watering frequencies and N rates on: (a) leaf number and (b) plant height. Means with the same letter are not significantly different based on SNK test at p=5% level (means separations over all treatment combinations). The watering frequencies: S1 (once a day), S7 (once a week), and S14 (once every two weeks) and the nitrogen rates: N0 (0xR), N0.5 (0.5xR), N1 (1xR), N2 (2xR) and N4 (4xR) where R is 68 kg N ha^{-1}. Shown here are the leaf number and plant height at only 28 days after transplanting (DAT 28).

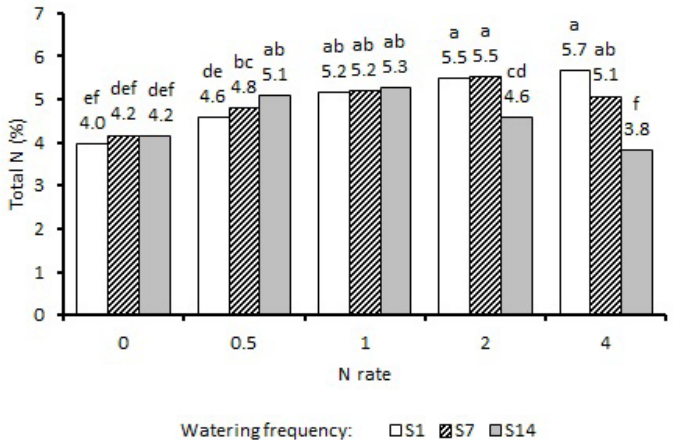

Fig.4: Total N content (at 28 days after transplanting) in choy sum whole plant tissues as affected by watering frequencies: S1 (once a day), S7 (once a week) and S14 (once every two weeks) and nitrogen rates: N0 (0xR), N0.5 (0.5xR), N1 (1xR), N2 (2xR) and N4 (4xR) where R is 68 kg N ha^{-1}. Interaction SxN was highly significant ($F=7.185$; $p<0.01$) and means with the same letter are not significantly different based on SNK test at p=5% level (means separations over all treatment combination).

(S14) and high nitrogen application rates (N2 and N4). This trend is similar to that reported by Sun *et al*. (2011), who observed low nitrogen content in oil palm leaves when the plant experienced high water stress and high nitrogen application rates.

This study showed that the lowest water frequency treatment (S14) had resulted in lower total leaf area by more than 50% at DAT 28 compared to those with more frequent watering treatments of S1 and S7 (Fig.5a and 6a). When the water content in a plant decreases, plant water potential is reduced and the plant cells start to shrink as they lose turgor pressure. The loss of turgor pressure in the cells inhibits turgor-dependent activities such as cell expansion, and this ultimately affects the growth of the whole plant (Campbell & Reece, 2002) such as reduction in plant height, total leaf area, plant mass and yield (Ahmadi & Bahrani, 2009). As a drought tolerance mechanism, plants can lower their total leaf area to reduce their water loss via transpiration (Liu & Stützel, 2004). Fig.6a shows that maximum total leaf area could be achieved at approximately 0.4 m^3 m^{-3} mean soil water content. Note that no function was fitted to this chart as there were only three points. Nonetheless, it is clear that even at a lower mean soil water content of 0.35 m^3 m^{-3}, total leaf area was already close to maximum.

Water stress also caused the total plant dry weight and the individual dry weights of leaves and shoots to decrease as the water stress levels increased, particularly from DAT 14 onwards (Fig.5b-d and 7). The dry weight of choy sum leaves contributed to half of the total plant dry weight. Consequently, any large reduction in the leaves' dry weight, such as due to the S14 treatment, would considerably

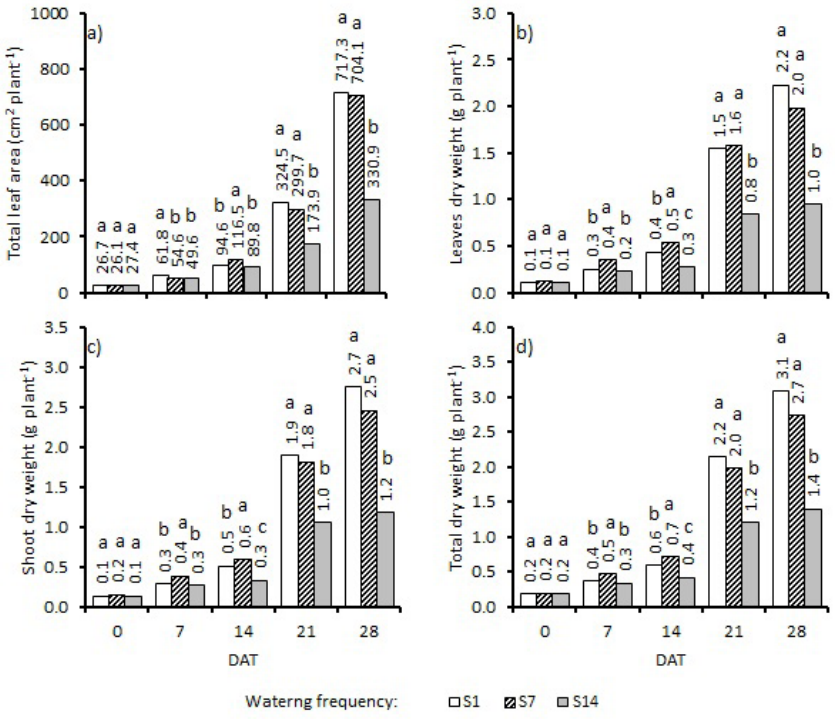

Fig.5: Effect of watering frequencies at each DAT (days after transplanting) on: (a) total leaf area, (b) leaves dry weight, (c) shoot dry weight and (d) total dry weight. At each DAT, means with the same letter are not significantly different based on SNK test at p=5% level. The water frequencies: S1 (once a day), S7 (once a week) and S14 (once every two weeks).

reduce the shoot-to-roots ratio as well (Fig.2). Although no function was fitted to the relationship between shoot dry weight and mean soil water content (Fig.7) for the same reasons as stated earlier, it was clear that maximum shoot dry weight could be achieved at approximately 0.4 m^3 m^{-3} mean soil water content, like the one obtained for the maximum total leaf area (Fig.6a).

The effect of nitrogen on maximum root length was less clear (Fig.8a). This is probably because, as mentioned previously, water stress would affect leaf growth more than root growth and that there was no differences in root dry weight between the treatments (Table 2). A study by Boa *et al*. (2007) also found that the root elongation of *Arabidopsis* (rockcress) was insensitive to increases in nitrogen supply.

From DAT 21 onwards, the total leaf area of choy sum would increase with increasing nitrogen rates until at N0.5, after which the total leaf area would decline with further increases to the nitrogen levels (Fig.8b). Fitting the best function to the relationship between total leaf area and nitrogen rate, it was shown that the maximum total leaf area could be achieved at 28 kg N ha^{-1} (Fig.6b).

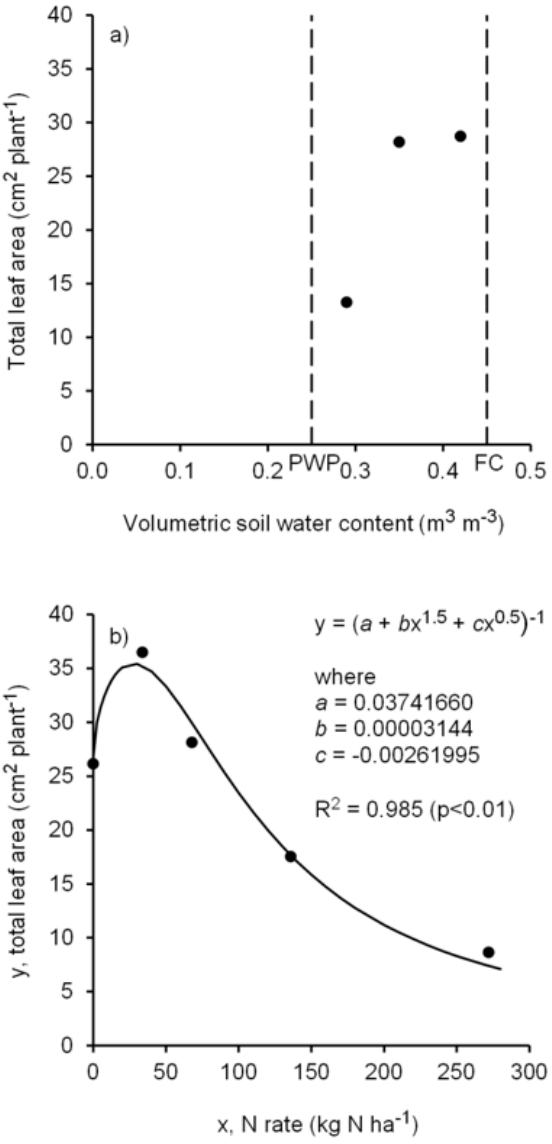

Fig.6: Relationship between choy sum's mean total leaf area at 28 days after transplanting (DAT 28) with (a) mean soil water content and (b) N rates. Note: PWP and FC denote the soil's permanent wilting point and field capacity, respectively. Regression was based on mean values.

These two optimal levels of mean soil water content (0.4 m^3 m^{-3}) and nitrogen rate (28 kg ha^{-1}) were similar to that required for obtaining the maximum number of leaves. Fig.9 shows that the maximum number of leaves can be achieved at approximately 0.35 m^3 m^{-3} mean soil water content and at 30 to 40 kg N ha^{-1}. Consequently, we can

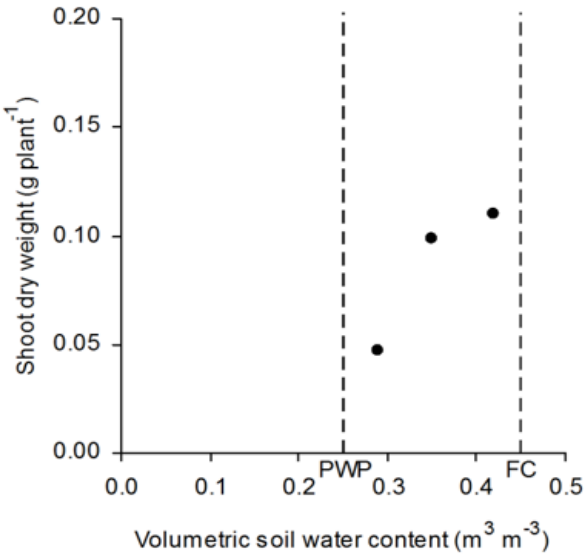

Fig.7: Relationship between choy sum's mean shoot dry weight at 28 days after transplanting (DAT 28) and mean soil water content. Note: PWP and FC denote the soil's permanent wilting point and field capacity, respectively.

generalise that the choy sum grown in this study's conditions would require 0.4 m^3 m^{-3} mean soil water content and 30 to 40 kg N ha^{-1} to obtain maximum yield in terms of the most number of leaves and highest total leaf area and shoot dry weight. The N0.5 treatment in this study had 34 kg N ha^{-1} applied, which is within the optimal 30 to 40 kg N ha^{-1} range.

The optimal 0.4 m^3 m^{-3} soil water content is above the critical water content of the soil used in this study. The critical soil water content (θ_{cr}) is the soil water content below which plants start to experience water stress. It can be determined by Teh's equation (2006):

$$\theta_{cr} = \theta_{PWP} + p\left(\theta_{FC} - \theta_{PWP}\right) \quad [1]$$

where θ_{PWP} and θ_{FC} are the soil's permanent wilting point and field capacity, respectively; and p is typically 0.5 for C3 plants (Doorenbos & Kassam, 1979) such as choy sum. Using Equation [1] and values from Table 1 for θ_{PWP} and θ_{FC} and p = 0.5 meant that the critical soil water content θ_{cr} for this study's soil was calculated to be 0.35 m^3 m^{-3}, which was similar to the mean soil water content under the S7 treatment. This shows that the choy sum growing under the S7 watering frequency treatment did not experience (but was close to experiencing) water stress. This explains the general lack of difference in the measured growth and yield parameters between the S1 and S7 treatments.

The SLA, which is the ratio of leaf area to leaf dry weight, indicates leaf thickness as smaller SLA would indicate thicker leaves (Meziane & Shipley, 2001; Teh *et al*., 2004). The linear decline of SLA with increasing

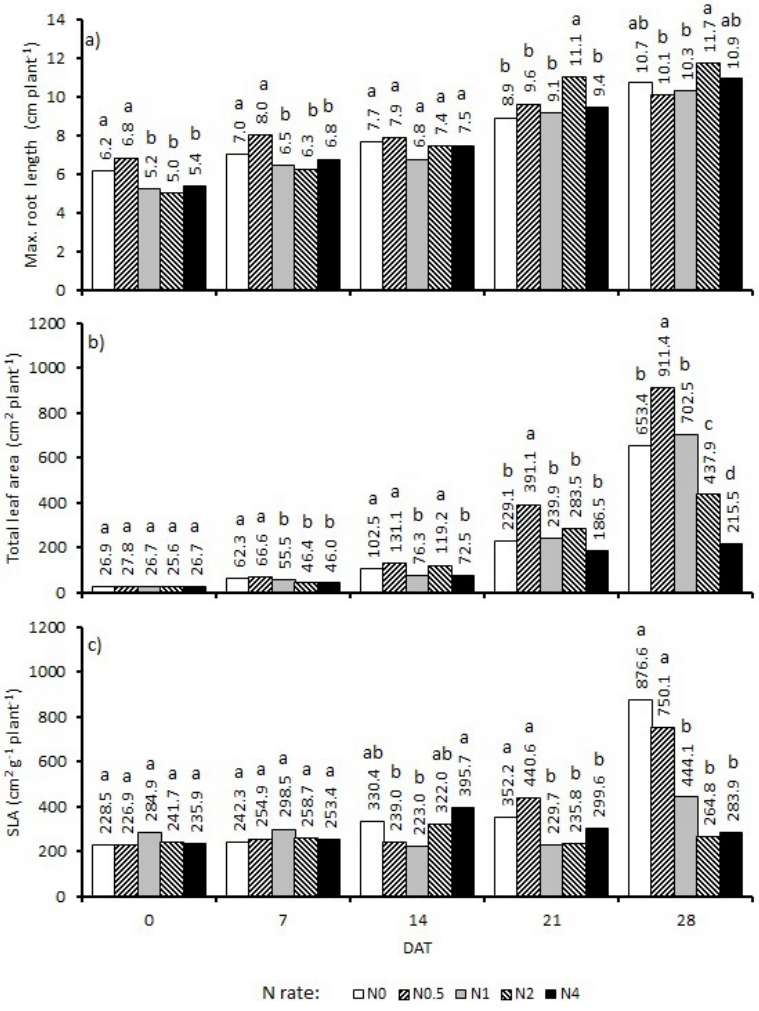

Fig.8: Effects of N rates at each DAT (days after transplanting) on: (a) maximum root length, (b) total leaf area and (c) specific leaf area (SLA). At each DAT, means with the same letter are not significantly different based on SNK test at p=5% level. The N rate levels: N0 (0xR), N0.5 (0.5xR), N1 (1xR), N2 (2xR) and N4 (4xR) where R is 68 kg N ha^{-1}.

nitrogen levels is particularly clear on DAT 28 (Fig.8c). Choy sum treated with N0 level showed the highest SLA, which would decline at a mean rate of 5 cm^2 g^{-1} (kg N ha^{-1})$^{-1}$ (linear regression not shown) until at N2 (136 kg N ha^{-1}), after which the SLA would remain constant. This suggests choy sum invests nitrogen use more for thylakoid stacking in photosynthetic cells and the synthesis of carboxylation enzymes for photosynthesis rather than for increasing the leaf area for light capture (Taiz & Zeiger, 2009). In other words, choy sum's preference is to use nitrogen to increase the weight and thickness of its leaves rather than to expand the area of the leaves. Similarly,

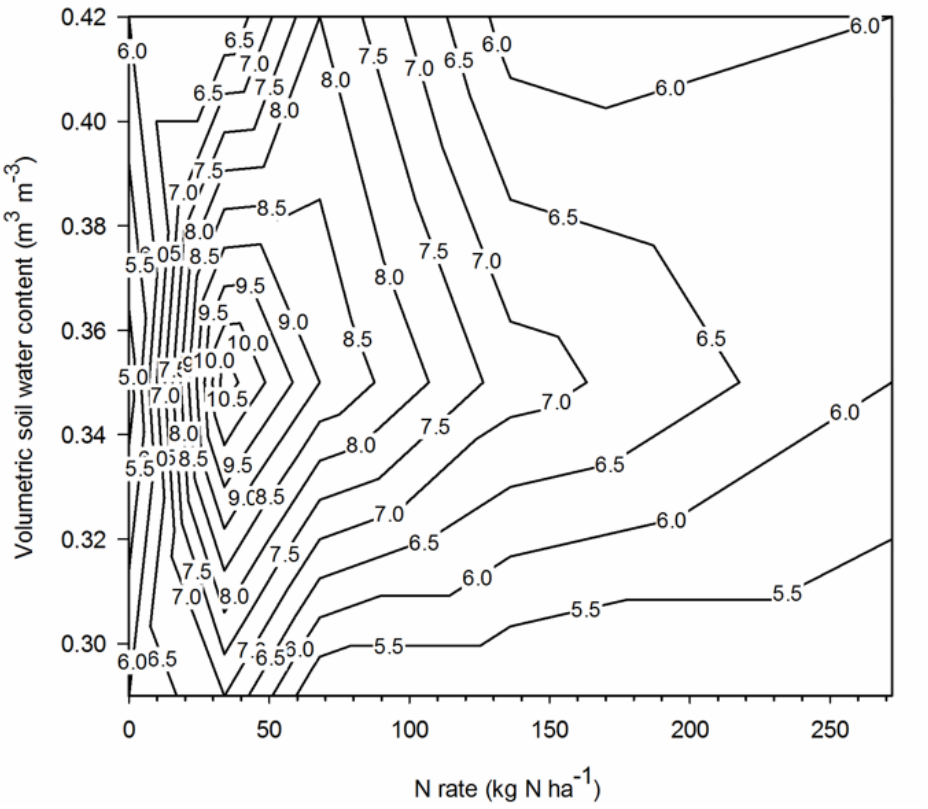

Fig.9: Relationship between choy sum's mean leaf number at 28 days after transplanting (DAT 28) with mean soil water content and N rate applied.

the SLA of Massai grass (*Panicum maximum* cv. *Massai*) also decreased with increasing nitrogen levels (Lopes *et al*., 2011).

CONCLUSION

Water stress detrimentally affected choy sum's leaf growth more than its root growth. Water stress also detrimentally affected choy sum's growth more than nitrogen stress, and increasing nitrogen application rates in water-stressed conditions significantly reduced the leaf number and plant height of choy sum. The S1 (once-a-day) and S7 (once-a-week) watering treatments generally gave a similar effect to the growth and yield of choy sum because choy sum growing under these two watering frequencies did not experience any water stress. Consequently, watering choy sum more than once a week was not needed as long as the soil water content remained above the critical level of 0.35 m^3 m^{-3}. The optimal soil water content and nitrogen application rate were 0.4 m^3 m^{-3} and 30 to 40 kg N ha^{-1}, respectively, for maximum choy sum yield (i.e. most number of leaves and highest total leaf area and shoot dry weight). This optimal nitrogen rate was 40 to 60% of the recommended

nitrogen application 68 kg N ha^{-1}. The N0.5 (half the recommended nitrogen rate) treatment used in this study was 34 kg N ha^{-1}, and this nitrogen rate together with the S7 watering treatment (watering once a week) generally gave high values for all the measured vegetative and yield parameters. Under lower and point of sufficient nitrogen rates, nitrogen was used for leaf thickness and weight rather than for intercepting light via leaf area expansion.

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Case Report

Standing Frontonasal Flap and Maxillary Sinus Trephination in a Horse with Sinusitis

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ABSTRACT

This report describes use of a standing frontonasal bone flap (SFF) technique to treat a case of primary sinusitis affecting the right frontal and maxillary sinuses in a horse. The diagnosis was made based on a history of prolonged unilateral nasal discharge, endoscopy and radiographic findings. A frontonasal bone flap was created and maxillary sinus trephination was performed while the horse was standing and sedated. Standing sedation throughout the whole procedure was achieved through constant rate infusion (CRI) technique with detomidine hydrochloride. The outcome of this case confirmed that standing frontonasal flap surgery provides an effective method to specifically treat primary sinusitis in a horse and eliminates the risk of general anaesthesia in order to perform such invasive surgery.

Keywords: Horse, sinusitis, frontonasal flap, standing surgery

INTRODUCTION

Paranasal sinuses of the horse are air-filled spaces within the skull. There are six pairs of paranasal sinuses: the dorsal, middle and ventral conchal sinus, the sphenopalatine sinus, the frontal sinus and the caudal and rostral maxillary sinuses. The caudal and rostral maxillary sinuses are separated by the maxillary septum and the rostral sinus opens into the ventral conchal sinus. The caudal maxillary sinus has communication with the frontal sinus and the sphenopalatine sinuses. Diseases that could affect the paranasal sinuses are sinusitis, empyema, paranasal sinus cyst, neoplasia and sinus

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trauma (Nickels, 2006). Clinically, the paranasal sinus becomes important due to its susceptibility to infections extending from the nasal cavity or from the upper root tooth infections leading to primary and secondary sinusitis. Primary sinusitis is usually caused by complications caused by Streptococcus species in acute or chronic respiratory disease (Ruggles *et al.*, 1991). In a simple acute case of sinusitis, a course of antimicrobial therapy with or without sinus trephination and flushing of the paranasal sinuses may be sufficient to resolve the infection. Where there is little drainage from the sinuses with trephination and flushing and no response to treatment, with presence of inspissated purulent material in any sinus compartments, it is very unlikely the sinusitis is able to resolve with only medical management, and surgical treatment becomes necessary (Schumacher & Crossland, 1994; Nickels, 2006; Dixon *et al.*, 2012).

In the past, exploratory surgery of the paranasal sinuses was performed through trephinated openings (Ruggles *et al.,* 1993). Through this sinus portal, endoscopic examinations of the paranasal sinuses, as well as collection of sinus contents for bacteriologic, cytologic and histological examinations are able to be performed. However, adequate exposure for surgical manipulations through this small opening is limited (Ruggles *et al.,* 1991), and examination of the rostral and ventral conchal sinus can be difficult. Hence, surgery of the paranasal sinuses through a large frontonasal bone flap technique is indicated to allow for optimum exposure of most parts of the paranasal sinuses, including the rostral maxillary and ventral conchal sinuses and to provide a larger entrance for surgical manipulation as well as direct assessment of the sinuses.

Frontonasal bone flap surgery and bone trephination can be performed while the horse is under general anaesthesia (Freeman *et al.*, 1990) or performed while the horse is standing and sedated (Schumacher *et al.*, 2000; Schumacher & Perkins, 2005). Advances in techniques and improved agents for equine sedation and analgesia have allowed many procedures to be performed while the horse is standing, including surgery of the paranasal sinuses. The purpose of this report is to share our experience of Standing Frontonasal Flap (SFF) surgery to further investigate and treat primary sinusitis in a horse.

CASE DETAILS

History

Ishmael, a six-year old thoroughbred, gelding, weighing 450 kg, used for pleasure riding, was presented to the Equine Unit, Veterinary Hospital, Universiti Putra Malaysia, for treatment of prolonged unilateral nasal discharge. According to the caretaker of the horse, the nasal discharge was noted two to three weeks before a veterinarian was summoned.

Clinical Findings

On physical examination, the temperature, heart rate and respiratory rate were found to be normal. Purulent malodorous nasal

discharges could be seen draining from the right nostril (Fig.1). Auscultation of the lung and trachea revealed no abnormalities. A facial swelling at the region of the right side of the frontal bone was noted. Palpation of the facial protuberance did not elicit signs of discomfort or pain. However, percussion of the area over the right maxillary and frontal sinus revealed changes in resonance compared to the same region on the left side. Examination of the teeth and soft tissue structures in the oral cavity revealed no abnormal findings. Right-sided nasal endoscopy was then performed while the horse was standing and sedated (Detomidine 10 mg/ml). Purulent exudates draining from the nasomaxillary ostia of the maxillary sinus were observed. The ethmoids were normal. Images from the endoscopic view were not able to be photographed due to technical limitations.

Radiography of the paranasal sinuses was performed. Right lateral, lateral oblique and dorsoventral radiographs of the paranasal sinuses were obtained while the horse was standing and sedated (Detomidine 0.01 mg/kg). On the lateral view (Fig.2), the ethmoid was found to be normal, but there were multiple fluid lines in both the frontal and maxillary sinuses. Thus, this finding supports the diagnosis of primary sinusitis in both sinus compartments. On the dorsoventral view (Fig.3), the fluid lines noted on lateral view were confined to the right side and a sign of soft tissue opacification was observed on the region

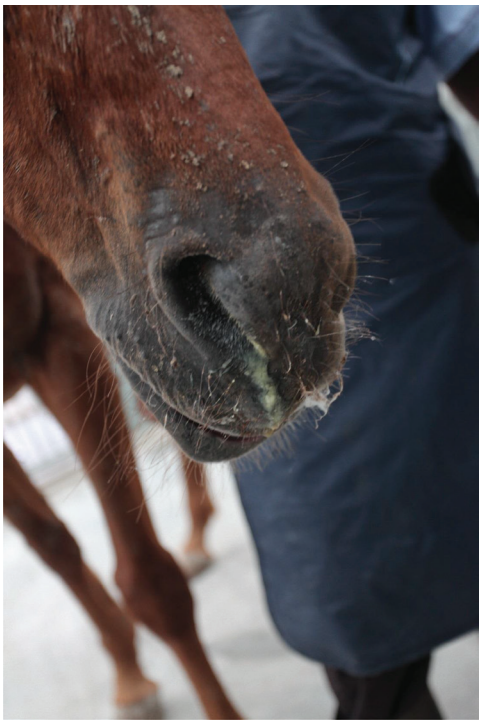

Fig.1: Purulent malodorous nasal discharge can be seen draining from the right nostril.

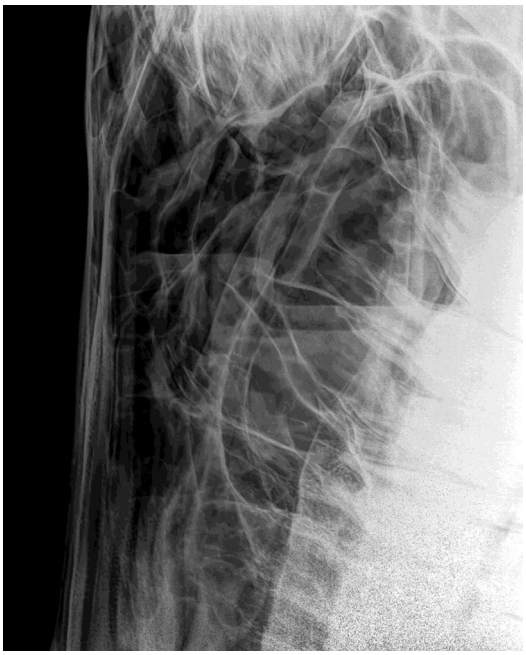

Fig.2: Right lateral view of the paranasal sinuses. The ethmoid region was normal in radiographic appearance, but there are multiple fluid lines in both the frontal and maxillary sinuses.

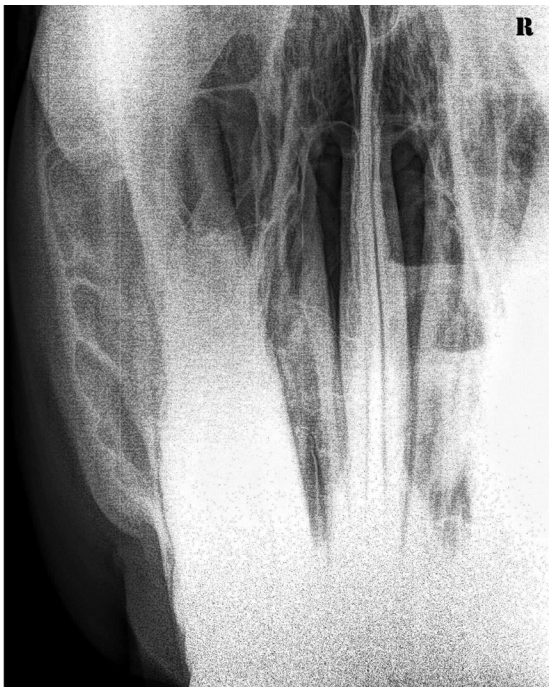

Fig.3: Dorsoventral view of the paranasal sinuses. The fluid lines noted on lateral view were confined to the right side.

of the right maxillary sinus. A diagnosis of primary sinusitis affecting the right frontal and maxillary sinuses was made based on the endoscopy and radiographic findings. All haematological values were within normal limits.

Surgical Protocol

Standing Frontonasal Flap (SFF) surgery was the choice of treatment for this case. The horse was given analgesic drug of flunixin meglumin (2.2 mg/kg, intravenously), broad spectrum antibiotic of penicillin-streptomycin (22,000 U/kg of body weight, intramuscularly) and pethidine hydrochloride (0.5 mg/kg, intramuscularly) preoperatively. The horse was restrained in stocks for the surgical procedure. A 14-gauge intravascular jugular catheter was placed, and the horse was sedated using detomidine infusion. A detomidine constant rate infusion (CRI) was delivered using a 60 drop/ml microdrip administration set. Five ml of NaCl 0.9% was removed from the 500 ml bag and 5 ml of 10 mg/ml detomidine was added into the saline bag to produce 100 µg/ml of detomidine infusion. After intravenous administration of a bolus of detomidine (8.4 µg/kg), the drip rates were adjusted at the following rates: 150 drops/minute for the first 15 minutes, 90 drops/minute for the next 15 minutes and 45 drops/minute thereafter throughout the surgery.

The dorsal aspect of the affected side of the head was clipped and prepared for aseptic surgery. Local anaesthetic (Lidocaine 2%) was infused subcutaneously along the proposed incision line. The dorsal midline of the face served as the base of the flap. The caudal margin of the flap was a perpendicular line from the dorsal midline to a point between supraorbital foramen and medial canthus of the eye. The lateral margin of the flap began from the caudal margin 2.0 to 2.5 cm medial to the medial canthus of the eye, and extended approximately 2/3 the distance from the medial canthus to the infraorbital foramen. For the rostral margin of the flap, the incision line was a perpendicular line from the dorsal midline to the rostral extension of lateral margin (Nickels, 2006) (Fig.4).

Once the boundaries of the surgical incisions were determined, a skin incision, extending through subcutaneous tissue and the periosteum, was made along the rostral, lateral and caudal margin. A small strip (2 mm) of periosteum was reflected from the bone along the incision line. Osteotomies were then performed using a chisel and a mallet along the incised margins. The cut on the bone was bevelled to ensure secure closure when the bone flap was replaced. Once the osteotomy was performed on all the three margins, the flap was elevated using a chisel until the surgeon's fingers could be placed underneath the flap to grab the flap steadily. The bone flap was slowly elevated with a steady, even pressure until it fractured along the dorsal side (base) of the flap that is beneath the intact tissue (Fig.5). Upon opening up of the flap, the frontal sinus and maxillary sinus compartments were exposed. Purulent material and necrotic tissues were observed lining the sinuses mucous membrane. Swab and

Fig.4: Skin incisions, extending through subcutaneous tissue and the periosteum were made along the rostral, lateral and caudal margin for the boundaries of the frontonasal bone flap.

samples of the necrotic materials from the sinus compartments were taken for further diagnostic testing. Samples were submitted for bacterial culture and sensitivity testing and cytological examination. Cytology was requested to rule out the presence of neoplastic cells. The paranasal sinus compartments were then explored and all necrotic tissues and purulent material were removed using fingers, currette and haemostat. Copious lavage using a hose with tap water was directed into the sinus opening to thoroughly dislodge any inspissated pus, necrotic tissue and haemorrhage. This procedure was tolerated well by the horse and provided us with an opportunity to thoroughly clean all the sinus compartments. For closure, the bone flap was replaced, and the subcutaneous tissue was closed with a simple continuous pattern using 2-0 Vicryl. The skin was closed with horizontal mattress pattern using 2-0 Ethilon (Fig.6).

It was noted that during lavage of the sinus compartments, there was still some purulent material left in the base of the caudal maxillary sinus despite attempts to curette it out. Due to its deeper anatomical location (more ventrally), we were not able to properly curette out purulent material from this compartment, and fenestration of the ventral conchal bulla was not performed. With that consideration, trephination of the caudal maxillary sinus was performed using a 5-cm trephine to create a portal for daily flushing of the sinus. A male Foley catheter size 8Fr was placed and secured into the

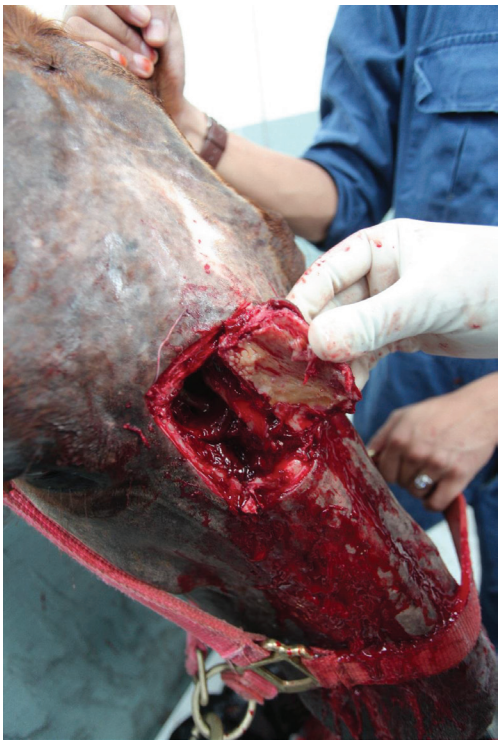

Fig.5: The frontonasal bone flap was created, exposing the frontal sinus and maxillary sinus compartments.

trephinated hole for this purpose.

Then both operative sites were covered with a layer of Op-site that was spread with Solcoceryl gel to create a moist environment for the wound to heal. A second layer of gauze was lined on top of the Op-site, followed by an elastic adhesive bandage roll around the operative site and the horse head (Fig.7). The free end of the Foley catheter was left hanging out for the purpose of daily flushing.

Post-Operative Care and Outcome

The right maxillary and frontal sinus compartments were lavaged twice daily via the Foley catheter for four days using physiologic saline solution (0.9% NaCl). Anti-inflammatory, flunixin (1.1 mg/ml, IV, once daily) and oral phenylbutazone (2 sachet of 2 gram phenylbutazone, once daily) were given for the first three days post-operatively. Penicillin-Streptomycin (22,000 U/kg, IM, every 12 hours) was administered for five days. The cutaneous portion of the bone flap and the trephine site were bandaged and left to heal by first intention. However, on day 5, it was noticed that fly larvae had infested the trephinated site due to movement of the bandage that had exposed this wound. The wound was then properly debrided and all maggots were removed. Due to continuous presence of flies, the wound was kept in bandage the whole time to prevent further maggot

Fig.7: The surgical site was bandaged post-operatively.

infestation. Bandage was changed every three to five days. By the third day of daily sinus lavage, nasal discharge from the nostril had stopped. Thirty days from the day of the operation, the wounds were progressively healing on both surgical sites without any complication.

DISCUSSION

Surgery of the paranasal sinuses in this horse was performed safely through a large frontonasal bone flap while the horse was under sedation and standing. The surgical site was not draped so that the horse's reactions to the procedure could be easily monitored. Performing this procedure while the horse was standing allowed elevation of the head and might have resulted in lower blood pressure or caused less venous congestion in the structures of the paranasal sinuses than if the horse was recumbent under general anaesthesia (Schumacher & Perkins, 2005). The creation of this bone flap provided direct access to the frontal, dorsal choncal, caudal and rostral maxillary and sphenopalatine sinuses with minimal haemorrhage, even with manipulations within the sinuses. Inspissated exudates and necrotic tissues were easily removed through the large flap and close inspection of other regions of the paranasal sinuses was not restricted, compared to the conventional

method of using trephination hole of sinus compartment (Schumacher & Crossland, 1994). Combination of frontanasal bone flap and fenestration of the ventral conchal bulla would be beneficial to gain further access to the rostral maxillary and the ventral conchal sinuses (Perkins *et al.*, 2009) in order to remove inspissated material within these regions. Standing frontonasal sinus flap (SFF) is recommended as a diagnostic and therapeutic measure where clinical signs fail to respond to non-surgical treatment with antibiotics, mucolytics, steam and volatile inhalation or minor surgery through a trephine portal created in a sinus compartment (Lane, 1993).

Throughout the frontonasal bone flap surgery, the detomidine infusion resulted in an excellent level of sedation, in which the surgical manipulation, removal of diffuse and localised lesions in the affected paranasal sinuses and copious lavage of the sinus opening were all well tolerated by the horse. For this surgery, the horse's head needs to be supported by a stand and padding or alternatively, the horse's head needs to be cross-tied from the halter to the stocks to raise the head for the surgeon to perform the surgery and to avoid iatrogenic facial nerve paralysis. A constant rate infusion technique using detomidine hydrochloride provides a safe and effective method to achieve a consistent standing sedation for prolonged standing surgical procedures for horses. Despite the excellent sedation and analgesic effects, a sedated horse is still conscious and must be handled with caution as it may still be able to kick accurately and unpredictably (England *et al.,* 1992).

The outcome of this case confirmed that standing frontonasal flap surgery provides an effective method to specifically treat primary sinusitis in a horse, and also as a treatment of many other cases of paranasal disease. This procedure could be performed safely while the horse is standing, thus it is more convenient and it eliminates the risk of general anaesthesia. In order to perform this procedure, a sound knowledge of the anatomy of the area is required. The most important part is the post-operative care of the bone flap. In a tropical country, the presence of flies is unavoidable, unless the horse is placed in an indoor stable. Thus, intact bandaging at all time is mandatory to prevent maggot infestation until the wound has healed. In this case, it took about 30 days of bandaging.

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Case Report

Management of Lower Urinary Tract Obstructive Disease using Bladder Tube Cystotomy in a Saanen Buck

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ABSTRACT

This report describes a procedure of bladder tube cystotomy to relieve progressing bladder distension in a goat diagnosed to have obstructive urolithiasis. This one-year old Saanen buck was presented with a complaint of not passing urine and being in discomfort for almost three days. On physical examination, pulsation of the urethra in the perineal region could be felt and swelling and pain along the prepuce and perineum were indicated on deep palpation. Amputation of the urethral orifice and attempt to catheterise the urethra failed to relieve bladder distension; thus, bladder tube cystotomy procedure was performed. Following this procedure, all vital parameters returned to normal and normograde cystourethrogram was performed to radiographically assess the lower urinary tract of the goat. The outcome of this case supports that the bladder tube cystotomy technique provides an effective method to allow immediate relief of a distended bladder, allowing the urethra to heal as the urine outflow is diverted through the catheter and allows normograde cystourethrography to be performed.

Keywords: Obstructive urolithiasis, bladder tube cystotomy, cystourethrogram, goat

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INTRODUCTION

Obstructive urolithiasis is a condition where insoluble mineral and salt precipitate and obstruct urine flow anywhere from

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the renal pelvis to the distal urethra. The most common site for obstruction is at the sigmoid flexure of the urethra and at the urethral process (Tibary & Van Metre, 2004; Ewoldt *et al*., 2008; Jones *et al*., 2012). The clinical signs of urinary obstruction are dependent upon the degree of obstruction, location of obstruction and duration of disease, and the signs include persistent straining to urinate, restlessness, tail flagging, vocalisation, anorexia and azotemia. Without any relief of obstruction, this condition will eventually lead to urinary bladder or urethra rupture and result in abdominal distension (Jones *et al*., 2012). Surgical intervention such as amputation of the urethral process, urethrotomy, perineal urethrostomy, cystotomy, tube cystotomy or bladder marsupialisation is necessary to relieve the obstruction either by removal of the urolith or by passing the urine flow pass the obstruction (Van Metre & Fubini, 2006; Ewoldt *et al*., 2008). The purpose of this report is to describe a case of obstructive urolithiasis in a Saanen goat managed with bladder tube cystotomy procedure followed by normograde contrast cystourethrography performed at the Universiti Veterinary Hospital, Universiti Putra Malaysia.

CASE DETAILS

History

A one-year old Saanen male goat with intact testicles was presented to the University Veterinary Hospital (UVH) with a complaint of not passing urine and being in discomfort for almost three days. The goat was managed intensively and served as breeding stock. According to the owner, two weeks before the problem occurred, the goat's diet was changed to a self-mixed feed ration. The owner noticed that the goat was straining to urinate and treated it with ammonium chloride for three days prior to presentation to the hospital given that urine was not passed.

Clinical Findings

Upon physical examination, the animal was bright and alert with a good body condition score (4 out of 5). The body temperature was within normal range, but the respiratory and heart rates were increased. Pulsation of the urethra in the perineal region could be felt, and swelling and pain along the prepuce and perineum were indicated on deep palpation. Based on the history and the presentation of clinical signs, the case was tentatively diagnosed to be of obstructive urolithiasis. Lateral and dorsoventral abdominal radiography to view the urinary bladder were performed. However, the outline of the bladder could not be appreciated due to the superimposition of the rumen and its content. Then a decision was made to amputate the urethral process as the first step to relieve any obstruction. Sedation was achieved with acepromazine (Calmivet Injectable Solution 0.05 mg/kg, Vetoquinol S.A.) given intramuscularly. The penis was exteriorised from the sheath, and the urethral process was amputated using sterile surgical blade (size 20). Several spherical small stones (diameter of less than 0.5 cm) were successfully removed with this procedure. However, amputation of the urethral process

could not re-establish urethral patency despite efforts to urinate. Then urinary catheterisation was attempted using a large dog urinary catheter [BUSTER Disposable Dog Catheter [2.0 x 500 mm; 2.6 x 500 mm) by Kruuse] to relieve the distended bladder (Fig.1). The catheter could be passed along the straight urethra, but resistance was felt as the catheter reached the distal part of the sigmoid flexure of the urethra. Due to concern of urethral trauma or rupture, this technique was not continued.

Ultrasound guided cystocentesis with a 7.5 MHz linear probe (Veterinary ultrasound model SIUI - CTS - 900V) was performed and about 230 mls of urine were aspirated (Fig.2). An ultrasound examination of the bladder revealed that the bladder was still intact but was very much distended with thickened wall. The urethral lining was also scanned to identify any obstruction that could be picked up by the ultrasound probe. However, no uroliths were present in either the urethra or the bladder. The sigmoid flexure of the urethra was difficult to be scanned as the goat was restless and in pain whenever the probe was placed at that region.

On the first day of hospitalisation, the serum biochemistry result revealed azotemia, hypophosphatemia, hypocloraemia and lymphopenia. Urinalysis results revealed that the goat was having proteinuria, and presence of inflammatory cells with bacteria (1+), which is suggestive of lower urinary tract infection. The goat was observed throughout the night in case of a bladder rupture, as surgery could not be

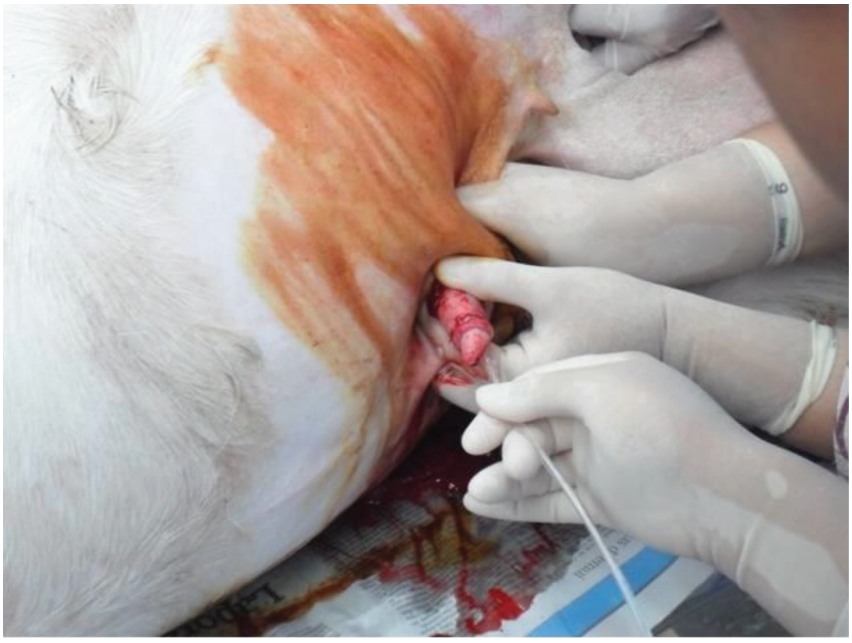

Fig.1: Attempt to catheterise the urinary bladder using a large dog urinary catheter to relieve the distended bladder.

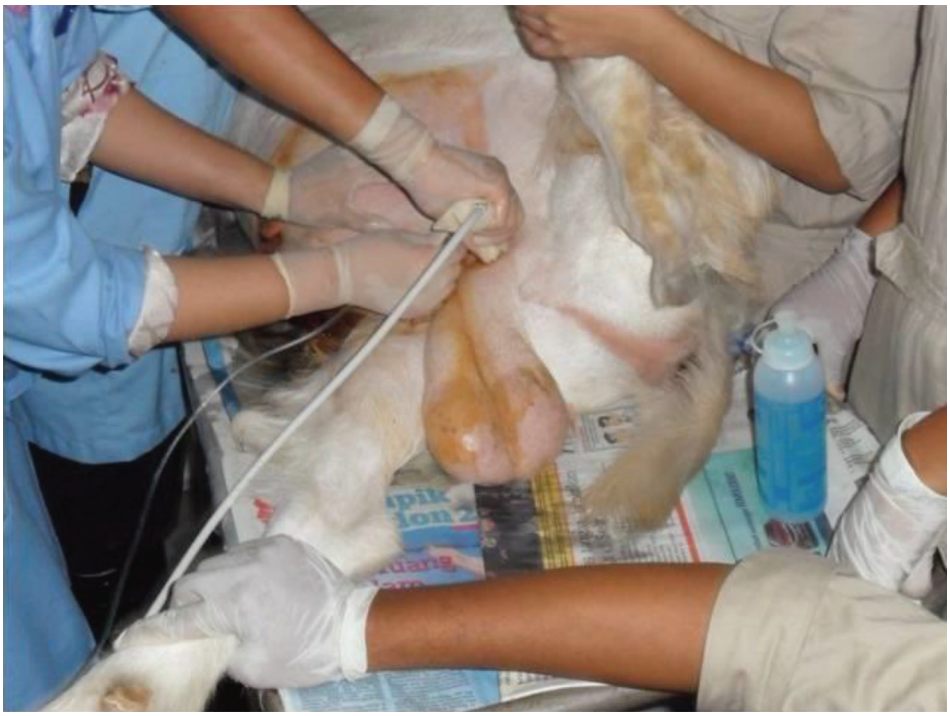

Fig.2: Ultrasound-guided cystocentesis was performed and about 230 mls of urine was aspirated out.

performed on that day. By late evening, it had started panting, tachycardic (112 beats per minute), having high rectal temperature (42°C) and anuric (despite the effort to urinate). Analgesic drug of flunixin meglumin (Flunixin 50 mg/ml, 2.2 mg/kg, intramuscularly, Norbrook Laboratories (GB) Limited) and broad spectrum antibiotic of penicillin (Benacillin 1 ml per 25 kg, intramuscularly, Troy Laboratories Pty. Limited) were administered. By midnight, the goat's strenuous straining efforts had only produced dribbling urine, and no more urination was observed after that.

Surgical Procedure

Bladder tube cystotomy was planned and performed on the second day of hospitalisation. Anaesthesia was induced with xylazine (Ilium-xylazil-20, 0.11 mg/kg, intravenously, Troy Laboratories Pty. Limited) and was maintained with combination of xylazine (0.11 mg/kg, intravenously) and ketamine (Narketan-10, 2 mg/kg, intravenously, Vetoquinol UK Limited). Normal saline at maintenance rate was administered intravenously throughout the surgical procedure. For intraoperative anaesthesia, the goat received epidural anaesthesia using lidocaine hydrochloride (Xylocaine 2%, 0.2 mg/kg, Astrazeneca).

The goat was positioned on right lateral recumbency with the left hindlimb abducted. The ventral abdomen and inguinal region were clipped and surgically prepared. Local anaesthetic drug (lidocaine hydrochloride)

was instilled along the proposed incision line. A ventrolateral abdominal incision of about 5-10 cm was made at the caudal abdomen. The external rectus sheath was incised, and the underlying muscle was undermined with Mayo scissors, followed by incision of the internal rectus sheath and the peritoneum. Then the bladder was identified by deep palpation of the abdominal cavity and was carefully exteriorised. Stay sutures were placed on the wall of the bladder to ease handling and prevent slipping of the bladder into abdominal cavity. An 18-gauge indwelling catheter connected to an extension tube was inserted into the bladder to allow urinary drainage. Approximately 500 mls of urine were removed.

Then a purse string suture pattern using an absorbable suture material 2-0 Vicryl (Coated Vicryl [Polyglactin 910] Suture Undyed Braided, Ethicon LLC) was placed on the ventral surface of the bladder wall. A stab incision was made in the mid region of the purse string pattern, and the tip of the Foley catheter (size 8Fr) including the deflated balloon, was inserted into this small bladder opening. Once the catheter had been placed in the bladder, the purse string suture was tightened to secure the catheter, and then the balloon on the catheter was inflated with air. The abdomen was lavaged with warm sterile saline, followed by suctioning out of the fluid. Then the peritoneum and muscle layers were closed with continuous suture pattern (2-0 Vicryl), followed by

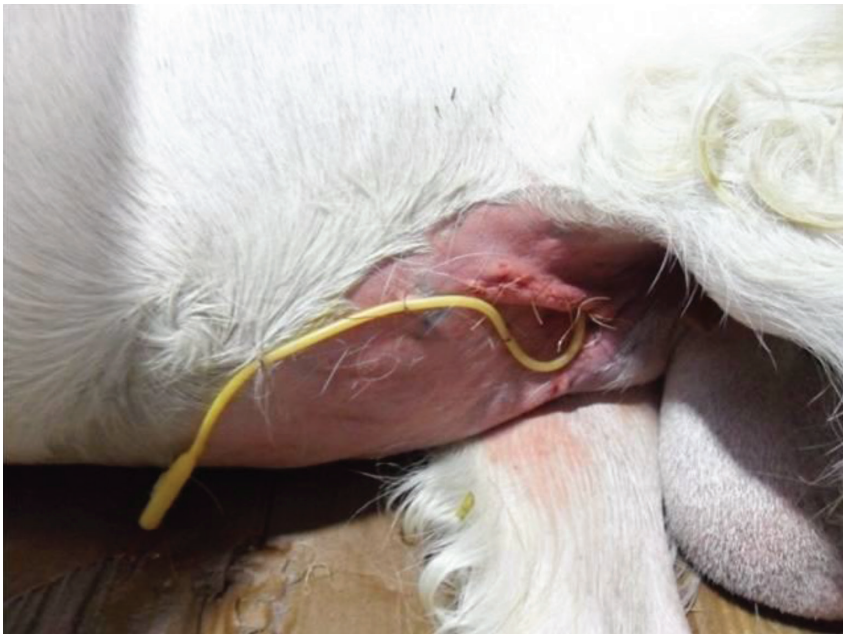

Fig.3: Post bladder tube cystotomy procedure where the end of the Foley catheter was hung outside the abdomen.

modified Cushing pattern for subcutaneous layer (2-0 Vicryl). The skin was closed with interrupted suture pattern using 1-0 Ethilon (Ethilon Polyamide 6, Ethicon LLC). The other end of the Foley catheter was hung outside the abdomen through a small, separate stab incision. The catheter end opening was closed with a clamp, and the catheter was secured to the abdominal wall with Chinese finger trap sutures (Fig.3).

Post-Operative Care

The goat was given 0.9 % NaCl fluids at replacement rate post-operatively. Antibiotic of penicillin streptomycin (Pen & Strep 1 ml per 25 kg, Norbrook Laboratories Limited) was administered for five days, followed by trimethoprim-sulfadiazine (Norodine 1 ml per 16 kg, Norbrook Laboratories Limited) for another three days. Flunixin meglumin (2.2 mg/kg) as analgesic and anti-inflammatory was given for three days, twice a day, followed by another three days, once daily. Aspiration of urine from the bladder tube was performed hourly, and about 3000 mls of urine was removed over 24 hours post-operatively. An Elizabethan collar was applied on the animal to prevent chewing or dislodging the Foley catheter. Twenty-four hours post-surgery, all vital parameters had returned to normal and the goat appeared bright and alert, and started to eat and drink on its own.

Further Diagnostic Workout

On the third day of hospitalisation, a day after the bladder tube cystotomy procedure, a cysto-urethrogram was performed to diagnose if any urolith was present in the bladder and the urethra, as well as to assess the lower urinary tract structures in this goat. For this procedure, the goat was sedated with xylazine and was placed on lateral recumbency. At first, catheterisation of the urethra was attempted in order to infuse the urethra with 35 mls of positive contrast medium (Omnipaque [Iohexol], 0.5 ml/kg, GE Healthcare). However, this technique failed to opacify the outline of the urethra in the radiograph. Then the same contrast medium was infused into the bladder via the Foley catheter, followed by a lateral and dorsoventral view of the bladder and urethra a few minutes later. Radiographic findings revealed that the bladder was intact, the entire urethral lining was clear and the lumen was patent with no evidence of urolith present in either the bladder or urethra (Fig.4). At the end of the procedure, the urine and contrast medium cystocentesis was performed through the surgical cystotomy tube.

Progress Post Surgery

Post cysto-urethrogram, it was noted that the goat was having hematuric dribbling urine, which was likely due to mucosal injury following previous urinary catheterisation. By the fifth day, it had started to dribble clear yellow urine from the urethra. By the seventh day of hospitalisation, urine aspiration was performed twice daily, only to encourage spontaneous micturition. However, at day 10 post-surgery, the dribbling urine was still persisting. Urine sample at day-10 post cystotomy was

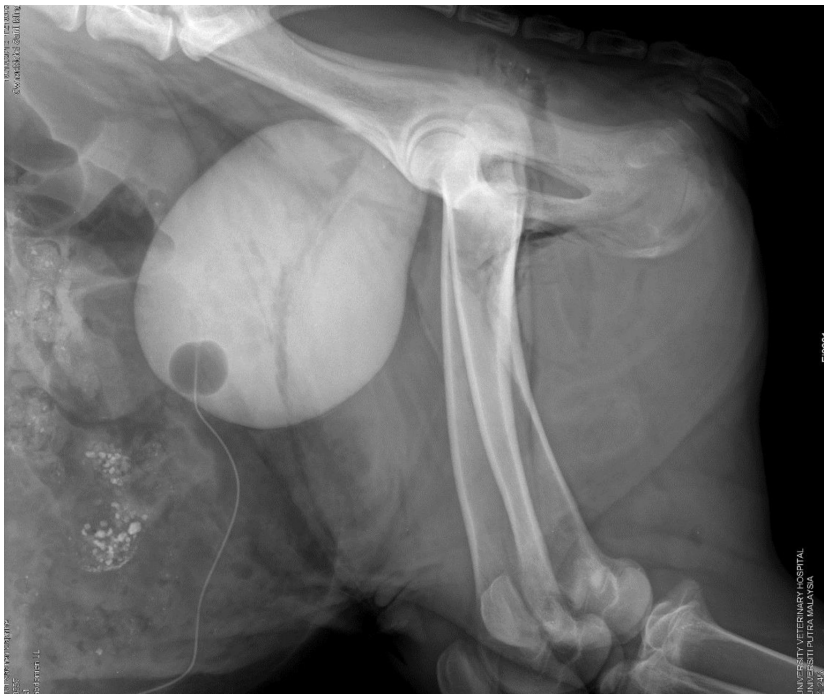

Fig.4: Cystourethrogram of the lower urinary structure of the goat. The bladder was intact, the entire urethral lining was clear and the lumen was patent with no evidence of urolith present in either the bladder or urethra.

taken and sent for bacterial culture and a sensitivity test. The result revealed high counts of bacteria and *Escherichia coli* was isolated from the urine sample. Antibiotic sensitivity test showed resistance towards amoxicillin, enrofloxacin, norodine and oxytetracycline but sensitivity towards gentamicin. Thus, gentamicin (Gentam 100, 6.6 mg/kg, Troy Laboratories Pty. Limited) was administered for another three days. On day 14 post-surgery, the urine flow had improved. The surgical cystotomy tube was then removed by deflating the catheter tip balloon, and immediate removal of the whole catheter from the animal. The catheter exit wound was examined and left open to allow drainage.

The goat was discharged on the 17th day of hospitalisation, and the owner was advised to provide plenty of water and fresh grass, and allow the animal to graze in a grass paddock if possible. Mating was only advised after two weeks of normal urination and penis function observed by the owner. To prevent any other cases of urolithisis in the same or other animals in the farm, the owner was also advised to get the self-mixed feed concentrates to be analysed at the government nutrition laboratory at Salak Tinggi, Selangor. After a week, the owner reported that the urine quality and flow was back to normal.

DISCUSSION

In male goats, the urolith's most common site for obstruction is at the sigmoid flexure of the urethra and at the urethral process (Haven *et al.,* 1993; Tibary & Van Metre, 2004; Ewoldt *et al.,* 2008; Jones et al., 2012). Catheterisation of the urethra is difficult in this case due to the anatomy of the penile urethra, which forms a sigmoid flexure, and the catheter failed to pass through. Perhaps the catheter used in this case is not flexible enough to be bent along this flexure. Male goats possess a urethral diverticulum or recess that communicates with the urethra and contains the ducts of the bulbourethral glands (Jones *et al*., 2012). Even if the catheter passed the sigmoid flexure, the presence of this urethral diverticulum will divert the catheter into this structure instead of entering the bladder, thus preventing catheterisation of the urinary bladder.

Thus, for obstructive urolithiasis in animals that are intended for breeding, surgical bladder tube cystotomy is the preferred technique to relieve a distended bladder and retain breeding capacity (Haven *et al.,* 1993; Rakestraw *et al.,* 1995; Ewoldt *et al.,* 2006; Van Metre & Fubini, 2006). This technique allows immediate relief of a distended bladder, allows the urethra to heal as the urine outflow is diverted through the catheter and allows normograde cystourethrography to be performed. A healing period of approximately 14 days was taken into consideration before removal of the catheter as reported by Rakestraw *et al.* (1995). Periodic occlusion of the tube catheter was practised to monitor micturition ability of the goat.

Based on our experience, urine flow patency occurred only after day 14 post-surgery. Dysuria or dribbling urine in goats with a history of obstructive urolithiasis can be due to several causes: post-trauma healing of the urethra, cystitis or partial bladder atony from prolonged bladder distension. Furthermore, cystitis is one of the potential complications from urethral obstruction that causes dribbling of the urine (Ewoldt *et al*., 2008). In this case, the dribbling urine discontinued after the administration of an antibiotic that the causative organism was sensitive to. Furthermore, urethral stricture due to trauma from the calculi was also considered as one of the important complications where the healing process may take longer to establish patent urethral flow of urine (Todhunter *et al*., 1996).

Normograde cystourethrogram was made possible via the tube cystotomy, which allowed us to evaluate the presence of urolith and patency of the urethra. The contrast study performed here suggested that there was a possibility that the obstructive urolith had dislodged and was no longer obstructing the urethra; thus, patency of the urethra had been restored. Based on the radiographic finding, which ruled out the presence of urolith or urethral stricture, it could be suggested that the dribbling urine post-surgery might have been due to inflammation of the urethra and was not caused by the obstruction or stricture of

the lower urinary tract. Positive contrast normograde cystourethrography through the bladder tube csytotomy as in this case, provides the best visualisation of the lower urinary tract structures (Palmer *et al*., 1998) and aids in the diagnosis of the problem.

The use of gentamicin antibiotic in food animals is not regulated in this country. In this case, it was only used as a last resort, where the cultured organism was resistant to all other antibiotics available and sensitive to gentamicin. It is important to note that the use of gentamicin in food animals is not allowed in certain countries like Australia and the United States (Australia, 1999). The reason for prohibition of gentamicin use in food animals is because the residual effects of this drug are extremely long and the withholding period is measured in years. The use of gentamicin in food-producing animals may increase the risk of bacterial resistance towards this drug that may be subsequently harmful to human medicines upon consumption of dairy or meat product of the treated animal (Australia, 1999).

Despite publications detailing the technique of bladder tube cystotomy in small ruminants, up to this point, there are no local case reports of successful surgical treatment using this technique in the management of urolithiasis in small ruminants in Malaysia. Local veterinarians would have the option to attempt this surgical procedure to relieve bladder distension and correct systemic imbalances during urinary obstruction, as an alternative to slaughter or euthanasia. It has to be taken into consideration that this procedure has the disadvantage of having longer hospitalisation and increased intensity of case management.

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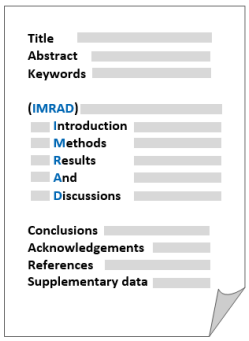

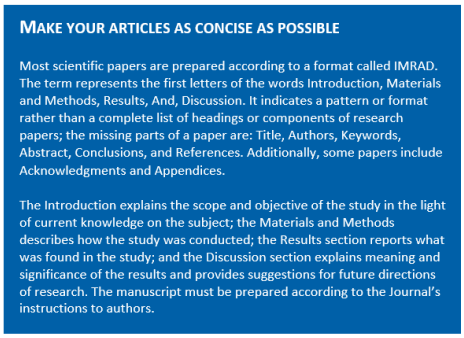

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