Journal of Tropical Agricultural Science

About the Journal

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

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

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

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

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

Editorial Statement

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

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Editorial[i]

Seeds That Changed the World

After over half a century of service in Universiti Putra Malaysia, I feel honoured to be invited to write the Editorial of Pertanika – Journal of Tropical Agricultural Science, particularly when I was one of the foundation members of the Editorial Board in 1978-82 and subsequently the chief Editor from 1983 to 1996. There are many things for me to reminisce, while there are observations and experiences to share from the past era. Looking back in time, I remember the seeds of change have already been sown as far back as 1961. Before the birth of Pertanika, research publications were in the form of an annual college magazine, with contributions from students and staff members. In 1977, the University of Agriculture felt the need for its own University Press. Thus in 1978, the first research journal Pertanika was launched. Within two decades, the university's seeds of change have multiplied and grown beyond recognition, with increases in the number of faculties, courses, students and staff resulted in many changes in Pertanika. Pertanika was the Journal of Tropical Agricultural Science (JTAS), and in 1992, it was split into three journals to meet the needs of the university.

Now, there are three journals to include; Journal of Science and Technology (JST) and the Journal of Social Sciences and Humanities (JSSH). I am glad now that the scope of the original has changed and greatly enlarged in specific fields for the three journals. Each journal has its own national and international editorial board to ensure high standards and to meet an international ISI journal. Pertanika publishes papers from all over the world following the double blind peer-reviewed system and also the code of ethics by the contributors, editors and reviewers. The four issues of each journal are to be published and delivered on time as scheduled, which is a pre-requisite of a good and reputable journal. In this digital age with great advances in information technology, publications of all types and forms have raised the public awareness of all subjects which have significantly changed the world over the 20th Century. I dream and hope that the seeds of knowledge in Pertanika will germinate and help to change the world for the better in the future. Even ideas and speeches have done the same – changing the world.

Similarly, following Nature's footsteps of the great diversity of seeds – the source of life and food for mankind and animals have indeed changed the world. In this editorial, I attempt to show and illustrate two tropical plant species which have indeed been responsible in changing the world for the survival and advancement of mankind. They are rubber *Hevea brasiliensis* and oil palm *Elaeis guineensis* seeds.

The planet earth has been orbiting round the sun for millions of years with precision and regularity. However, the world that we live in on earth has been changing slowly over the years until the 19th century. By the 20th century and the beginning of the new Millennium, changes take place very rapidly day by day, especially with the onset of the digital revolution. Man with his new ideas has changed the world and even his speeches have done the same. Similarly, the question arises, can seeds change the world? If seeds and plants can speak, they will definitely say yes, with the help of mankind's ideas, innovation invention and discovery.

Seeds, as defined by various people, are given as follows: seed is the source of life and it is described as the capsule of life. In his poem, Gailbraith attributed that the seed is the awesome vessel of power. Man has realized the power and the importance of seeds paid their tribute by the United Nations in designating 1961 as the World Seed Year. The importance of seeds is spelt out clearly in four words, "All food is seed". Man and animals are dependent on them for their survival all over the world. In the Green Revolution, mankind was saved from famine by the hybrid wheat and corn. There is a saying 'He who controls the seeds controls the world'. Therefore, it is not surprising they can bring about changes the world over.

One of the most outstanding and striking example is the species *Hevea brasiliensis* or rubber. It is one species of plant which within a century greatly changed the lifestyle of the people in the 20th Century. Now, let us ask ourselves, what life will be like without rubber, surely it would not be as pleasant and comfortable as it is today. The humble beginning of the rubber bouncing balls, erasers, rubber bands and waterproof boots, shoes and gloves have changed the lifestyle of mankind. Today, rubber bearings are used as anti-earthquake structures for skyscrapers like the recently launched tower in Tokyo called the Sky Tree. The invention of rubber tyres has changed our mode of travel with the bicycle, motor vehicles including planes in aviation. Meanwhile, the uses of rubber in health science, as gloves and condoms, play very significant roles in population control. If not for the latter, the world's population would exceed much above the seven billion mark.

The other plant species, oil palm - *Elaeis guineensis*, is one with a shorter history that has also changed the world for the past century. Oil palm is the most versatile plant in which every part of the plant can be utilized for food, fibre, health supplements and

many other industrial uses. It is another crop that everybody in the world is most likely to be in contact with, i.e. palm oil. Unlike rubber, the most valuable parts of the plant are from the fruits and seeds kernel which produce the oil. Today, oil palm is at the top of the world's list of vegetable oil producers. There are more than 10 million farmers in plantations of the equatorial belt of the world, supplying oil that feeds the billions of people, especially in developing nations to ensure food security. Increased demand is anticipated according to FAO; oil production is in a region of 184 million tonnes for the hungry world in developing countries which are heavily reliant on palm oil as a source of nutrition. In fact, palm oil is the most consumed edible oil in the world. Over a hundred products are derived from oil palm, for food, non-food, fertilizers and industrial use.

Rubber and oil palm are not natives to Malaysia; they originated from Amazon in South America and western-central Africa, respectively. They became plantation crops in the early 20th Century, and then became the world's largest producers, thus contributing significantly to the nation's economy. The products from them have changed the lifestyle and standard of living of the people. They have also led to new and unexpected applications in modern living and a host of new industries.

Further in the olden days after rubber had been domesticated, the white latex from the bark is respected as the white blood of the Gods. Lately, oil palm in Malaysia is known as the golden crop producing liquid gold. These two most important crops in Malaysia still have great potentials in the future. One day, we may find and discover new germplasm in the forests for breeding new clones. Alternatively, plant breeders and biotechnologists may design an ideal plant for their needs. In future, more species of plants may be discovered in the forests or some being genetically modified, they will further change the world for the betterment of mankind.

CARE with the SEEDS, JOY with the HARVEST.

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Professor H. F. Chin has served Universiti Putra Malaysia for over half a century. He obtained his four degrees in Agricultural Science from the University of Melbourne, Australia; B. Agric. Sc. (1961), M. Agric. Sc. (1971), Ph.D (1974), D. Agric. Sc. (1994) Honorary. Prof. Chin's main interest is in seed science and technology and his field of research is on seed storage, particularly the recalcitrant species. He has published three books on seeds, namely, *"Agricultural and Horticultural Seeds of Malaysia*", "*Seed Technology in the Tropics*" and *"Recalcitrant Crop Seeds*." Besides his research work, he also has interests in gardening and photography. With these interests and skills, he has also written and edited ten other books, including "*Malaysian Flowers in Colour", "Malaysian Fruits in Colour", "Malaysian Trees in Colour", "Hibiscus – Queen of Tropical Flowers", and "Malaysian Vegetables in Colour*."

Prof. Chin has been active nationally and internationally, as chairman of a number of National Committees. Internationally, he was the Chairman of Technical Committee on Seed Storage of International Seed Testing Association (ISTA) and member of the Advisory Committee on Seed Storage of the International Board of Plant Genetic Resources (IBPGR). He also served as a member of the board of Trustee of IBPGR for 6 years. In 2011, Prof Chin was appointed as a foundation member of the National Seed Council (NSC) by the Minister of Agriculture and Agro-Based Industry, Malaysia.

For his long service and contributions, he was appointed Professor Emeritus in 1995 at Universiti Putra Malaysia (UPM) and Foundation Fellow of the Academy of Sciences Malaysia FASc. (1995) by the Minister of Science, Technology and Environment. He was also awarded an Honorary degree of Doctor of Agricultural Science by the University of Melbourne and appointed the Honorary Research Fellow of the International Plant Genetic Resources Institute (IPGRI), which is now Bioversity International. In June 1990, he was awarded the Johan Setia Mahkota (J.S.M) Order of Chivalry title on the occasion of the birthday of His Majesty, the Yang di-Pertuan Agong (King) of Malaysia.

Prof. Chin was the Foundation member of the Editorial Board of Pertanika (1978-1982) who was then appointed as its Chief Editor (1983-1996).

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[i] DISCLAIMER
The views expressed in this article are those of the author and do not necessarily represent the views of, and should not be attributed to, the Pertanika Journal or the Pertanika Editorial Board.

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Contents

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A Tropical Harpacticoid Copepod, *Nitocra affinis californica* Lang As an Effective Live Feed for Black Tiger Shrimp Larvae *Penaeus monodon* Fabricius

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ABSTRACT

Survival and specific growth rates of *Penaeus monodon* larvae (post-larval stages 1-15), fed with different live feeds and artificial diets, were evaluated using three different treatments, namely: i) *Nitocra affinis*, ii) combination of *Artemia* nauplii + *N. affinis* and iii) artificial diet. The experiment was carried out in 10-L aquaria with 30% daily water exchange for a period of 16 days. The survival rate (61%) and specific growth rate (16.7 %day^{-1}) were highest ($p<0.05$) in the treatment with shrimp larvae fed with *N. affinis*. Likewise, the protein contents of *N. affinis* was found to be the highest ($p<0.05$) among all the diets used. The fatty acids of *N. affinis* was dominated by polyunsaturated fatty acids (PUFA) (22:6n3) forming 19.5% of the total PUFA identified. In fact, *N. affinis* contained the highest ($p<0.05$) amount of PUFA and the highest ($p<0.05$) n-3/n-6 ratio amongst the three diets. Analysis of the copepod fed shrimp showed significantly higher ($p<0.05$) amount of long chain PUFA, both of the n-3 and n-6 series fatty acids, when compared to the artificial diet fed larvae. The results of this study showed that *N. affinis* has the potential to be used as an effective live feed for *P. monodon* due to their high PUFA contents and broad size range (nauplii to adults) to cater for different shrimp post-larval stages.

Keywords: Harpacticoid copepod, Live feed, *Nitocra affinis californica, Penaeus monodon*, Shrimp larvae

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INTRODUCTION

Today, the larviculture of marine decapod crustaceans is still regarded as one of the major bottlenecks impairing the production of several commercially important species (Calado *et al*., 2008). Meanwhile, live food has been considered to be a limiting factor in the commercial larval production of many fish and crustacean species (Kovalenko *et al*., 2002), and it forms an important factor in overall production cost. For penaeid larvae, animal protein is the most important component of the diet (Anderson & De Silva, 2003; Perera *et al*., 2005), and this is generally supplied by live feeds such as rotifers and brine shrimp, *Artemia*. Although live feeds have been proven successful as animal protein source in raising the larvae of many species, inherent problems still remain. The problems include variable nutrient composition and availability, potential introduction of pathogens into the culture system, and in the case of rotifers, the cultures are prone to crashes (Jones *et al*., 1993; Kovalenko *et al*., 2002). Adequate production of quality live food microorganisms, at a lowest possible cost, is essential for the successful development of commercial fish or shrimp hatchery since artificial diets developed have so far not been fully adequate, and generally, the use of live food has yielded better results (Tseng & Hsu, 1984; Watanabe & Kiron, 1994; Calado *et al*., 2008).

The increased global demand for seed-stock experienced by shrimp farming industry, specifically *P. monodon*, during the last two decades has prompted extensive improvement and refinement of the basic larval rearing techniques (Jory, 1997; Martin *et al*., 2006). The objective of this study was to obtain predictable production of high quality hatchery reared penaeid post-larvae by improving feeding regimes evaluated using three different treatments (Wilkenfed *et al*., 1981, 1984; Kuban *et al*., 1987; Barros & Valenti, 2003).

The harpacticoid, *Nitocra affinis*, is a widely distributed species found commonly in coastal areas, living on fine sand or mud, in shell sand among stones and algae, in mangrove sea, in salty wells, tidal pools and shore reefs (Lang, 1948; 1965; Kunz, 1975). They are epibenthic and euryhaline with a salinity tolerance well beyond the range of most natural salinity fluctuations. Their food requirement is flexible, as they can live and thrive on a variety of food such as algae, bacteria, yeast and artificial food sources (Hicks & Coull, 1983; Gee, 1989; Weiss *et al*., 1996). Thus, *N. affinis* is a desirable organism for experimental work and a good candidate for mass cultivation. The present study was undertaken to evaluate the survival and relative growth rate of *P. monodon* post-larvae fed with *N. affinis* solely, and in combination with the traditionally used *Artemia* nauplii and artificial shrimp diet for a period of 16 days.

MATERIALS AND METHODS

Treatments

Three treatments were employed in this experiment. *Penaeus monodon* (post-larvae stages 1-15) were fed with i) *N. affinis* (size range: 50-400 µm), ii) a combination of

Artemia nauplii (size range: 410-430µm) and *N. affinis*, and iii) artificial diet (ground freeze-dried form, size range: 17-22 µm). For each treatment, three replicates were randomly assigned. The experiment was run for 16 days. The use of *Artemia*, as sole live food source, was excluded in this study due to the fact that *Artemia* is in no doubt still the prime live feed for the larviculture industry (Jones *et al*., 1989; Abelin *et al*., 1991; Samocha *et al*., 1999; Wouters & Van Horenbeeck, 2003; Robinson *et al*., 2005). In addition, it is well established that the use of Artemia can yield from 50% up to 75% survival of shrimp post larvae in commercial hatcheries throughout the world (Jones *et al*., 1989; Abelin *et al*., 1991; Samocha *et al*., 1999; Wouters & Van Horenbeeck, 2003; Robinson *et al*., 2005).

Species Isolation

Nitocra affinis californica was isolated from the coastal waters of Peninsular Malaysia. The copepods were collected using Schindler Patalas trap, along the sandy beach with water level reaching 0.35 m. The copepods were transferred into a 1-L beaker with filtered (0.20 µm filter) seawater, and provided with 24 hour-aeration for conditioning. Then, each copepod carrying egg sacs was separately transferred into a new container filled with filtered (0.20 µm filter) seawater, while mixed algae (*Chaetoceros calcitrans, Nannochloropsis oculata and Tetraselmis tetrathele*) were provided as food sources. To ensure a monospecies stock, the culture was started with one gravid female grown continuously for several generations in the laboratory.

Feed Preparation

Small scale mass production of *N. affinis* was started by transferring 150 gravid female copepods (from the original stock) in 2-L of filtered aged seawater to each culture vessel. The 3-L culture vessels were cylindrical basins (semi transparent and lightly coloured plastics with smooth surface on the inside and out) of polypropylene material, with a base area equals to 177 cm^2, a mouth area of 416 cm^2, and a height of 10 cm. The aged seawater (seawater stocked for a period of time; chlorine free and with stable water chemistry) was filtered in a 0.45 µm membrane filter paper before use. The culture was maintained at salinity 30 $g{\cdot}L^{-1}$ and at temperature levels between 25ºC to 35ºC (seawater at room temperature changed from a minimum of 25ºC, occurring in the early morning while a maximum of 35ºC usually in the mid afternoon). When salinity increased, fresh water was added to the culture vessel until the desired salinity was achieved. The cultures were exposed to natural lighting condition (ranging from 25-40 µmol m^2s^{-1}) and photoperiod (12h light:12h dark cycles). All the above parameters provided for in this study were determined to provide the maximum population growth of *N. affinis* (Matias-Peralta *et al*., 2005). Maintenance of copepod culture involves siphoning of waste water, replenishing the vessels with fresh aged water and feeding (Matias-Peralta *et*

al., 2011). In other words, copepods were harvested daily, in such a way that gravid females were separated from nauplii and copepodids (Matias-Peralta *et al*., 2011). The procedure described above was able to produce 14.9 x 10^3 copepod L^{-1} for a period of 14 days, with a total of 75 gravid females L^{-1} as inoculum (Matias-Peralta *et al*., 2011).

The *Artemia* nauplii were produced by incubating cysts for 18 hours in seawater under optimal conditions of temperature (28-30ºC), salinity (30-32 $g{\cdot}L^{-1}$), pH (7.5-8.0) and continuous illumination using the Philips white light tubes. The cysts were incubated in a 1-L capacity conical shaped container at a density of 2 g cysts L^{-1} of seawater. The production of *Artemia* nauplii without enrichment followed the standard procedure adapted by shrimp hatcheries in Malaysia (Shrimp hatchery operators in Negeri Sembilan, Malacca and Johor, Malaysia, pers. com.).

Artificial feed was prepared by weighing the recommended amount of feed according to the number of shrimp larvae used in the experiment. This procedure was done according to the manufacturer's recommendation.

Source of P. monodon Post larvae (PL)

The shrimp larvae were purchased from a private hatchery located in Port Dickson, Negeri Sembilan (approximately 100 km from the laboratory). Shrimps at their first mysis stage were transported from the hatchery to the Aquatic Animal Health Unit for acclimation and further rearing until molting to PL1 (11 days old). The mysis were fed ad libitum four times daily, with a diatom, *Skeletonema* sp., until they were stocked into the experimental tanks.

Tank Preparation

Twelve 10 L capacity plastic aquaria (made of transparent acrylic plastic) with the dimension of 31.5 cm x 16.5 cm x 24 cm were used in the experiment. The tanks were thoroughly washed with bactericidal soap, soaked in 100 mg L^{-1} chlorine, rinsed and dried to prevent any disease transfer during the culture. Then, the tanks were filled with 4 L of chlorinated (aerated under high pressure air bubbles for at least three days before use), filtered (1 µm) seawater a day before stocking. A few pieces of stones and 20 mm diameter plastic pipes cut into a length of 40 mm (which were soaked in 100 mg L^{-1} chlorine for 24 hours, washed, rinsed and dried) were placed inside the tanks to serve as refuge for the weak shrimp during the molting period. Each tank was provided with individual aeration of low pressure air bubbles.

Stocking

The shrimp post larvae (PL1), with an average initial weight of 34.24±1.02 mg, were hauled from the acclimation tank using soft net into a basin with fresh seawater. The larvae were collected from the basin using a wide mouth plastic bottle with attached soft net, counted and transferred into a tared beaker with seawater and weighed in a Vibra (Shinko Denshi) digital tuning fork scale. These procedures were done quickly and carefully so as to avoid stress that might

lead to shrimp mortality. Individual weight was calculated from the bulk weight over the total number of individuals. Each tank was stocked at a density of 45 PLs L^{-1} of seawater.

Feeding

Larval live feeds (*Artemia* nauplii, Superior 90 Brine Shrimp Eggs, USA; and *N. affinis*) and artificial shrimp diet (Mixed Feed for *P. monodon*, Higashimaru Co. Ltd.) were given ad libitum four times a day at six hours interval (0600, 1200, 1800 and 2400 hours, respectively). Live feeds were counted, while artificial diet was weighed prior to feeding. Live feeds were also washed with filtered seawater upon harvest from their respective culture containers before counting. Live feed counts were done by determining the number of individuals/ml in three 1 ml samples using graduated pipette. The counts were done using 1 ml gridded counting chamber under Leitz Diavert inverted microscope. The counts were also done daily before feeding. The dry weights of the live food were also determined to be compared with that of the artificial diet (Table 3).

Water Quality

Water parameters, such as salinity, pH, dissolved oxygen, and temperature, were measured *in situ* twice (morning and noon) daily. Salinity and temperature readings were done using YSI 30 Salinity/ Conductivity/Temperature meter, while YSI 52 Dissolved Oxygen meter and ORION portable pH meter were used for dissolved oxygen and pH reading, respectively. On the other hand, water samples for total ammonia nitrogen and nitrite nitrogen were collected (before cleaning the tanks) and analyzed daily in the laboratory following the procedures by Parsons *et al*. (1984).

Meanwhile, daily water exchange was carried out at 30%. Uneaten food and faeces were siphoned out using a 1 mm flexible hose. Using this hose, the water level was gradually reduced to provide minimal disturbance to the shrimp larvae and also to prevent the larvae from being siphoned out. Feeding was commenced as soon as the desired amount of seawater had been removed and the refilling was begun.

Feeding was stopped eight hours before harvesting. Upon harvest, all the shrimp larvae were collected, weighed and measured. All the collected samples were washed thoroughly with double distilled water to remove salt before freeze drying (prior to analysis). Protein was determined following the method described by Meyer and Walther (1988). Fatty acid methyl esters (FAME) were prepared according to the direct methylation techniques by Divakaran and Ostrowski (1989). The freeze dried samples (100 mg) were refluxed at 100ºC for 10 minutes with 10 mL of 2% methanolic NaOH. The samples were further refluxed with 6.25 mL 14% Boron Triflouride and Heptane. Then, 2 mL of saturated NaCl was added to make the FAME float, and 1 g of anhydrous Na_2SO_4 was added to absorb the remaining water in the FAME for best recovery.

The FAME of the samples were analyzed using gas liquid chromatograph (Shimadzu GC-8A) equipped with a FID and BPX-70 (SGE) or Supelco 2330 capillary column. Individual peaks of FAME were identified by comparing them with the retention times of known standards obtained from Sigma Chemicals Company and using cod liver oil as a secondary standard. A Chromatopac (SHIMADZU C-R3A) quantified the magnitude of the peaks of each chromatographic reading.

Data Analysis

The specific growth rate (SGR) was calculated from the body weight using the following formula:

$$SGR = 100\ [(expG)-1]$$

Where:

$$G = \frac{\ln(wf) - \ln(wi)}{\Delta t}$$

where:

ln(wf) is the natural logarithm of the weight at time (t),

and ln (wi) is the natural logarithm of the initial wt of the shrimp larvae.

The collected data were analyzed using one-way analysis of variance (ANOVA). Significant differences among the individual treatment effects were determined using Tukey's honestly significant different test (T-HSD) at 0.05 level of probability. The data (whenever appropriate) were arcsine-transformed to satisfy the condition of homogeneity of variance (Gomez & Gomez, 1983; Zar, 1984). Statistical analyses were done using the Statistical Analysis System (SAS Inc. 1992) computer package (version 6.07).

RESULTS

Shrimp larvae fed with *N. affinis* alone achieved the highest ($p<0.05$) survival rate (60.6%), whereas those fed with the combination of *Artemia* + *N. affinis* had the lowest survival rate (24.1%) (see Table 1). Similarly, the highest ($p<0.05$) specific growth rate (SGR) was achieved by the shrimps fed with *N. affinis* alone, while those fed with the combination of *Artemia* and harpacticoid copepod have the lowest ($p<0.05$) growth rate (14.3%) (see Table 1).

TABLE 1

Mean (± S.E.) survival, specific growth rate of shrimp larvae and total weight of different diets fed to *Penaeus monodon* larvae for a period of 15 days. Means in the rows with the same superscript are not significant ($p<0.05$).

Parameters	Treatment		
	I	II	Control
Survival (%)	$60.6^{a}\pm6.8$	$24.1^{c}\pm1.0$	$43.0^{b}\pm3.2$
Specific growth rate (% d-1)	$16.7^{a}\pm0.2$	$14.3^{c}\pm0.2$	$15.8^{b}\pm0.2$
Total weight of feed used (mg dry weight)	$94.9^{b}\pm2.4$	$53.0^{c}\pm0.6$	$109.8^{a}\pm4.5$

Note: Treatment I = fed *N. affinis* (size range 50-400 µm)
Treatment II = combination of *Artemia* nauplii (size range 410-430µm) and *N. affinis*
Treatment III = artificial diet (grounded freeze-dried form, size range 17-22 µm).

Meanwhile, significant differences in the total amount of feed consumed were found among the treatments (Table 1). The shrimp larvae consumed significantly ($p<0.05$) higher amount of artificial diet compared to the live feed used.

The results also revealed that the highest ($p<0.05$) level of protein in the copepod tissue (52% = 52.32±2.13) compared to the two other diets used (namely, *Artemia* nauplii = 34% or 33.82±0.35 and artificial diet = 40% or 39.82±0.39). The fatty acids of *N. affinis* were dominated by polyunsaturated fatty acids (PUFA), 22:6n-3 accounting 19.5% of the total PUFA identified (see Table 2). On the other hand, *Artemia* nauplii and artificial diet were dominated by the monounsaturated fatty acids (MUFA), 18:1n-9 contributing 31.3% and 21.3% of the total MUFA identified, respectively. Among the three diets, the artificial diet was found to contain the highest ($p<0.05$) amount of saturated fatty acids (SFA), whereas *Artemia* was found to contain the highest ($p<0.05$) MUFA, and *N. affinis* contained the highest ($p<0.05$) amount of PUFA (Table 2). The results also showed that *N. affinis* contained the highest ($p<0.05$) n-3/n-6 ratio compared to the other feed used.

The copepod fed shrimp showed significantly higher ($p<0.05$) amount of long chain PUFA, both of the n-3 and n-6 series fatty acids (Table 3), as compared to the artificial diet fed larvae. The fatty acid of the artificial diet fed shrimp was dominated by MUFA, specifically 18:1 and 16:1. However, the MUFA content of shrimp larvae fed with either copepod or artificial diet did not show any significant difference (Table 3).

No significant difference ($p>0.05$) was detected in the water with regards to salinity, pH, dissolved oxygen and temperature from all the treatments (Table 4). Nonetheless, significantly high ($p<0.05$) total ammonia nitrogen (TAN) and nitrite nitrogen (NO_2-N) were detected from the tanks containing the artificial feed (control) (Table 4). On the other hand, the tanks with *N. affinis* (treatment II) was found to contain the lowest ($p<0.05$) concentrations of TAN and NO_2-N compared to the other treatments (Table 4).

DISCUSSION

This study illustrated that *N. affinis* was the preferred food by the *P. monodon* larvae. Meanwhile, the *Nitocra affinis* fed shrimp larvae achieved the highest survival rate and SGR compared to those fed with other food items. According to Mock *et al*. (1980), food organism in an intermediate size between algae (≤ 8-14µm) and freshly hatched *Artemia* nauplii (≤ 430µm) could increase shrimp survival and growth rates. In addition, Motoh (1981) suggested that a gradual change from algal diet during acclimatization to a diet of *Artemia* nauplii, which was fairly large (≤ 430µm) for small shrimp larvae with carapace length ranging from 0.5-2.2 mm with intermediate sized food items could reduce stress and mortality. In this study, harpacticoid copepod from copepodid to adult (ranging from 50 to 400µm) served as an appropriate sized prey

TABLE 2
Means (± S.E.) of different fatty acids (% of total fatty acids) of food sources for the post larvae *Penaeus monodon* Means in the rows with the same superscript are not significant ($p<0.05$).

Fatty acids	*Nitocra affinis*	*Artemia* nauplii	Artificial diet
C12:0	nd	nd	11.76^{a}±1.13
C14:0	0.65^{b}±0.02	0.51^{c}±0.00	1.47^{a}±0.02
C14:1	0.60^{b}±0.01	0.65^{b}±0.00	1.37^{a}±0.29
C14:2	6.65^{a}±1.14	nd	nd
C15:0	0.65^{b}±0.11	0.46^{b}±0.00	14.39^{a}±3.83
C16:0	11.83^{b}±0.16	12.55^{a}±0.00	5.06^{c}±1.31
C16:1	3.26^{a}±0.27	nd	1.14^{b}±0.21
C16:1n – 7	1.31^{b}±0.03	13.24^{a}±0.00	nd
C18:0	nd	5.57^{a}±0.00	1.54^{b}±0.27
C18:1	nd	nd	4.61^{a}±1.58
C18:1n – 9	14.10^{c}±0.10	31.30^{a}±0.00	21.26^{b}±2.99
C18:2n – 6	9.73^{a}±0.08	6.23^{a}±0.00	
C18:3n – 3	3.55^{b}±0.15	8.08^{a}±0.00	0.48^{c}±0.00
C18:3n – 6	0.37^{a}±0.15	nd	0.13^{b}±0.00
C18:4	0.76^{a} ±0.05	nd	0.37^{b}±0.00
C18:4n – 3	0.86^{b}±0.00	5.09^{a}±0.00	nd
C20:1n-9	0.15^{b}±0.02	nd	3.73^{a}±1.79
C20:2	2.32^{a}±0.26	nd	0.30^{b}±0.11
C20:4n – 6	2.32^{b}±0.26	1.41^{c}±0.00	5.13^{a}±0.06
C20:5n – 3	11.26^{a}±0.73	nd	0.64^{b}±0.19
C22:1n - 11	0.88^{b}±0.18	0.62^{a}±0.00	nd
22:5n - 3	2.57^{a}±0.23	nd	nd
C22:6n - 3	19.50^{a}±0.29	nd	8.07^{b}±1.87
Total SFA	13.13^{c}±0.14	19.09^{b}±0.00	28.73^{a}±6.11
Total MUFA	20.30^{c}±0.26	45.81^{a}±0.00	32.10^{b}±6.87
Total PUFA	59.89^{a}±1.27	20.81^{b}±0.00	15.11^{c}±1.63
Total n - 3	37.74^{a}±0.62	13.17^{b}±0.00	9.19^{c}±1.68
Total n - 6	12.42^{a}±0.80	7.64^{b}±0.00	5.26^{c}±0.06
n - 3/n - 6	3.04^{a}±0.22	1.72^{b}±0.00	1.75^{b}±0.30

nd = not detectable; SFA = saturated fatty acids;
MUFA = monounsaturated fatty acids;
PUFA = polyunsaturated fatty acids.

TABLE 3
Mean (± S.E.) fatty acid composition (% of total fatty acids) of post larvae (PL 15) *Penaeus monodon* fed with different diets. Means in the rows with the same letters are not significant ($p<0.05$).

Fatty acids	*Nitocra affinis* fed larvae	Artificial diet fed larvae
C14:0	3.25^{a}±0.00	2.24^{b}±0.12
C14:1	0.05^{b}±0.00	1.80^{a}±1.08
C15:0	0.29^{a}±0.00	Nd
C16:0	11.25^{a}±0.35	10.00^{a}±0.99
C16:1	1.23^{b}±0.00	4.10^{a}±0.46
C16:1*n* – 7	3.57^{a}±0.47	Nd
C18:0	4.90^{b}±0.00	8.05^{a}±0.46
C18:1	Nd	13.62^{a}±2.49
C18:1*n* – 9	17.43^{b}±1.54	21.17^{a}±0.21
C18:2n – 6	2.60^{a}±0.42	Nd
C18:3*n* – 3	1.80^{a}±0.00	0.95^{b}±0.03
C18:3*n* – 6	Nd	0.38^{a}±0.21
C18:4	Nd	0.47^{a}±0.01
C18:4*n* – 3	2.15^{a}±0.14	Nd
C20:1*n*-9	4.30^{a}±0.00	3.04^{b}±0.35
C20:2	Nd	0.98^{a}±0.25
C20:4*n* – 6	4.71^{a}±0.01	2.86^{b}±0.76
C20:5*n* – 3	15.62^{a}±0.47	1.08^{b}±0.11
C22:1*n* – 11	1.13^{b}±0.00	3.57^{a}±0.11
C22:5*n* – 3	1.80^{a}±0.00	Nd
C22:6*n* – 3	16.59^{a}±0.02	16.33^{a}±0.59
Total SFA	19.69^{a}±0.35	19.78^{a}±2.28
Total MUFA	27.71^{a}±2.02	28.28^{a}±1.19
Total PUFA	45.26^{a}±0.16	23.04^{b}±0.28
Total *n* – 3	37.95^{a}±0.59	18.36^{b}±0.45
Total *n* – 6	7.31^{a}±0.43	3.24^{b}±0.97

nd = not detectable; SFA = saturated fatty acids;
MUFA = monounsaturated fatty acids;
PUFA = polyunsaturated fatty acids.

TABLE 4
Mean (±S.E.) of different water quality parameters measured. Means in the rows with the same superscript are not significant ($p<0.05$).

Parameters	Treatment		
	I	II	Control
Salinity (gL-1)	30.14^{a}±0.09	30.14^{a}±0.11	30.04^{a}±0.10
Temperature (ºC)	27.51^{a}±0.10	27.52^{a}±0.04	27.51^{a}±0.10
pH (range)	7.90-8.31	7.90-8.31	7.92-8.30
Dissolved Oxygen (mg L-1)	5.85^{a}±0.05	5.88^{a}±0.04	6.20^{a}±0.08
Total Ammonia-N (µgL-1)	18.4^{c}±0.06	$19.\ 5^{a}$±0.08	19.2^{b}±0.08
Nitrite-N (µgL-1)	24.3^{c}±0.15	29.1^{a}±0.15	28.3^{b}±0.07

Note: Treatment I = fed *N. affinis* (size range 50-400 µm)
Treatment II = combination of *Artemia* nauplii (size range 410-430µm) and *N. affinis*
Treatment III = artificial diet (ground freeze-dried form, size range 17-22 µm).

for the shrimp larvae as they did not have to spend excess energy manipulating big sized prey.

Harpacticoid copepods have been widely used as a live prey for hatchery reared fish and crustaceans (Kahan *et al*., 1982; Watanabe *et al*., 1983; Szyper, 1989; Kraul *et al*., 1992; 1993; Nanton & Castell, 1998; Shields *et al*., 1999) because they have relatively high caloric content per unit weight and superior nutritional value (Kahan *et al*., 1982; Volk *et al*., 1984; Chandler, 1986; Gee, 1989). Although the absolute nutritional requirements of penaeid larvae are not fully known (Biendenbach *et al*., 1989), Colvin and Brand (1977) reported that a relatively high level of protein is important for the growth and survival of the early post-larval stages of many important penaeid species. In fact, they further suggested that larval protein requirements must be equal to or greater than 44%. In this case, the copepod *N. affinis* has provided the much needed protein requirement (52%) for the shrimp larvae. In addition, Leger *et al*. (1986), Norsker and Støttrup (1994), and McEvoy *et al*. (1995) have reported that harpacticoids are richer in essential fatty acids, most notably 22:6n-3 and 20:5n-3 and higher DHA:EPA ratio compared to *Artemia* (even after they have been fed with diet rich in DHA) and rotifer *Brachionus*. Meanwhile, several studies (e.g. Millamena *et al*., 1988; Ozkizilcik & Chu, 1994; Sorgeloos & Leger, 1992) have shown that the content of long-chain PUFA, in particular the n-3 series, is mainly responsible for the nutritional value of live feed for marine animal larvae. In this study, compared to *Artemia* (13.17 % total fatty acid) and the artificial feed (9.19 % total fatty acid) used, *N. affinis* (37.74 % total fatty acid) was found to contain significantly higher amount of long-chain PUFA, particularly of the n-3 series. According to Jones *et al*. (1979), highly unsaturated fatty acids (HUFA), specifically EPA and docosahexaenoic (DHA, 22:6n-

3), play an important role in penaeid larval nutrition and are required in larval diets. In addition, Kreeger *et al*. (1991) and Gonzalez-Felix *et al*. (2002) suggested the importance of EPA and DHA for the survival and growth of many important marine crustacean species. In the present study, it was evident that *N. affinis* provided adequate nutritional requirements for fatty acids, as reflected by the high survival and growth of the shrimp. Diets containing higher level of n-3 HUFA would improve growth and survival of penaeid larvae, since DHA is essential for penaeid larval growth (Gonzalez-Felix *et al*., 2002).

According to D'Abramo and Sheen (1993), the combined percentage of at least 15% of C-20 PUFAs of the n-3 and n-6 families (C 20:4 n-6; 20:5 n-3; 22:5 n-3; 22:6 n-3) in the whole body tissues of an animal serves as an indicator that the optimal growth requirement for essential fatty acids has been met. In this study, the combined percentage of these fatty acids for the *N. affinis* fed shrimp was >38%, while those fed with artificial diet had approximately 20%.

Merican and Shim (1996) suggested that linolenic (LNA, 18:3n-3) and DHA have been identified as essential fatty acid for *P. monodon*. Similarly, Glencross and Smith (1999) and Glencross *et al*. (2002) noted that linoleic acid (LOA, 18:2n-6) and EPA also have considerable growth-promoting value for shrimp. In fact, they suggested that additional growth would occur when two or more of the essential fatty acids were provided in an optimal combination. Growth was significantly reduced when either LNA or DHA was not provided in the diet. Moreover, Merican and Shim (1996) also showed that without any HUFA in the feed, the survival of shrimp was reduced to 52%. In this study, shrimp larvae obtained adequate supply of essential fatty acids (Table 2) from *N. affinis*, as illustrated by the significantly higher survival and growth rates compared to those fed with other diets, even with the combined Artemia + *N. affinis*.

In the present study, the tanks containing *Artemia* + *N. affinis* (treatment II) showed the highest ($P<0.05$) concentrations of TAN and NO_2-N as compared to other treatments. However, the levels found were within the range suitable for shrimp/prawn hatcheries. Olivar *et al*. (2000) reported that the unconsumed *Artemia* could have contributed to the higher ammonia levels in hatchery tanks. It is interesting to note that the treatment tanks using the artificial feed had significantly ($p<0.05$) lower levels of TAN and NO_2-N compared to those with *Artemia* + *N. affinis*, although one would have expected artificial feed to produce higher levels of the same. Moreover, artificial diet is easily consumed because of the small particles size appropriate for the requirements of the shrimp larvae with little or no excess. However, the levels of ammonia were primarily contributed by the shrimp fed with artificial diet, as reported by Shishehchian *et al*. (1999).

CONCLUSION

The results of this study suggest that *N. affinis* could be used as a live food source

for culturing penaeid shrimp larvae. This is because the shrimp larvae fed with *N. affinis* showed the highest survival percentage as compared to those fed with artificial diet and the combination of *N. affinis* and *Artemia*. In addition, the relative growth rate of shrimp larvae fed with copepod was significantly higher than that of *Artemia* nauplii combined with *N. affinis*. Furthermore, *N. affinis* was found to contain the highest amounts of protein and PUFA as compared to both *Artemia* and artificial diet. Another benefit of using copepods as shrimp larval feed is the maintenance of good water quality of the culture tanks. It is evident from this study that the amount of nitrogenous substances in the water was kept at significantly lower level compared to other treatments although similar methods of maintenance were applied in all the treatment tanks.

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Distribution and Concentrations of Ni in Tissues of the Gastropod *Nerita lineata* Collected from Intertidal Areas of Peninsular Malaysia

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ABSTRACT

Nickel (Ni) is an essential metal but not a well-studied metal in gastropods. In this study, *Nerita lineata* snails were collected from 20 sites along the western coast of Peninsular Malaysia from December 2005 to December 2010. The concentrations of Ni were determined in the total soft tissues, opercula and shells of the snails. Different patterns of Ni distribution were found in different tissues (shells, opercula and soft tissues) as well as spatial differences and distributions. This finding showed that the distributions of Ni in the shells and total soft tissues of *N. lineata* were significantly different, and this could be due to the different rates of Ni accumulation, excretion and sequestration. Since *N. lineata* can be abundantly found in rocky shores, below jetties and mangrove trees along the intertidal areas of the west coast of Peninsular Malaysia and it can show the ability to accumulate Ni, the snails can therefore act as potential biomonitors of Ni pollution in the western coast of Peninsular Malaysia.

Keywords: Ni, *Nerita lineata*, Peninsular Malaysia, opercula

INTRODUCTION

Aquatic ecosystems may receive anthropogenic pollutants originating from various sources and many pollutants, including heavy metals which are toxic to aquatic organisms and can cause lethal and sublethal deterioration in living organisms (Wang *et al*., 2005). Activities such as fossil fuel burning, emissions from vehicles, disposal of domestic and industrial wastes were some of the sources that contribute to the release of heavy metals (particularly Ni) into the environment (Yusuf *et al*., 2011).

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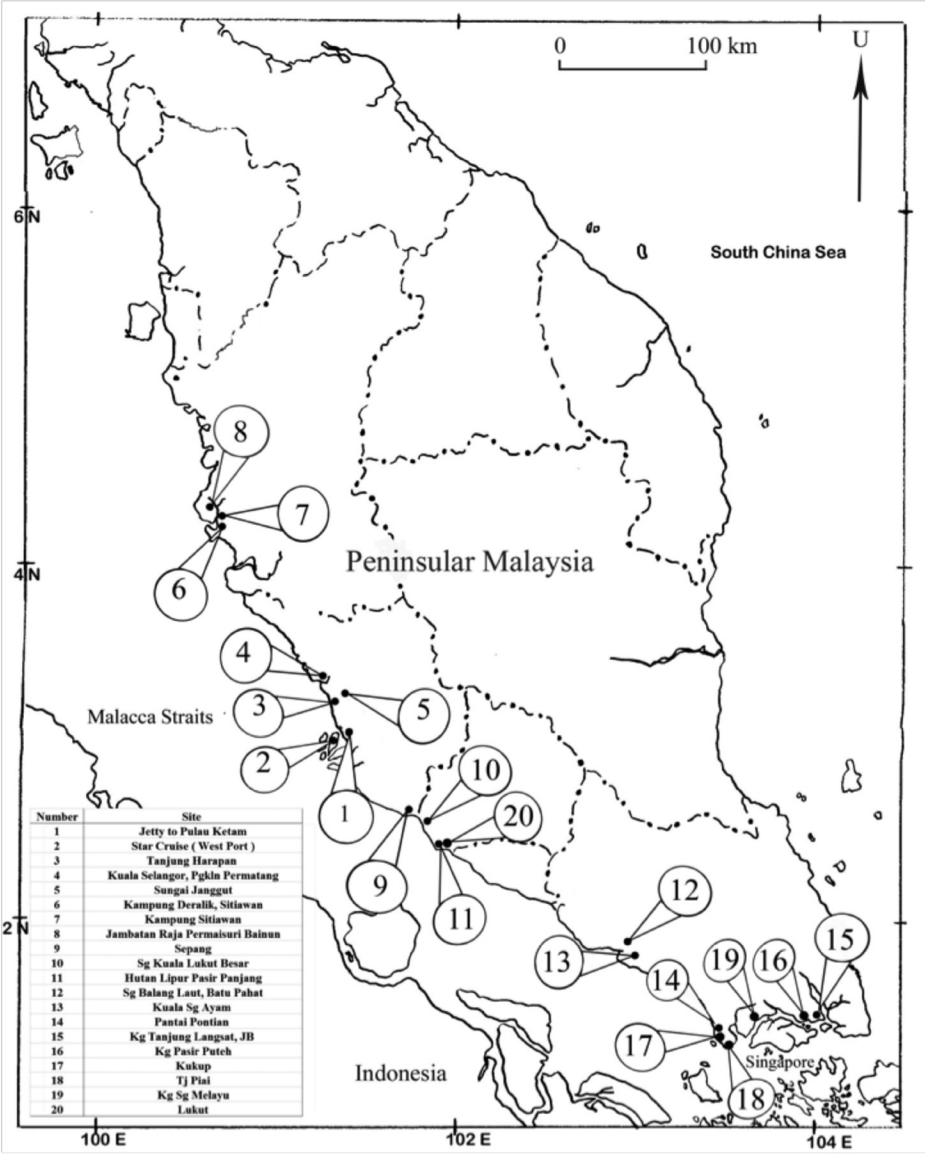
Therefore, biomonitoring studies remain crucial in the field of ecotoxicology. In past studies, bivalve species were applied as biomonitors for heavy metal pollution in global monitoring programmes (Rainbow *et al*., 2000; Wang *et al*., 2005; Yap *et al*., 2002a; Yap *et al*., 2004; Yap *et al*., 2006). Besides marine mussels, heavy metal reports on other intertidal molluscs such as the gastropods are also available in the literature (Foster & Cravo, 2003; Amin *et al*., 2006). Similarly to marine mussels, gastropods meet many of the recommended criteria as a good biomonitor, and these include wide distribution and high abundance, sedentary lifestyle, of relatively high longevity, as well as easily collected and weighed (Blackmore, 2001; Rainbow, 1995).

Nerita snails are dwellers of rocky shores and mangrove areas along the western coast of the peninsular. *N. lineata* is of the Order Archeaogastropoda and the Family Neritidae. They are herbivorous gastropods that graze on micro-algae growing on rocks, shells or larger plants (Hughes, 1986). They are known by the locals as 'siput timba' (bucket snail).

Prior to 1975, according to Boyle and Robinson (1988), Ni was considered to have no essential biological function but it was later discovered to be significant in a number of plant, animal and bacterial systems, although a well-defined biochemical mechanism for the role of Ni is still obscure. Meanwhile, the uptake and metabolism of Ni are very decisive for certain enzymatic activities, depending on the organisms (Yusuf *et al*., 2011). In general in bivalves, many of the toxic responses to Ni involve interferences with Fe metabolism and Ni, like most metals, bound to protein and nucleic acid (Stokes, 1988). In this paper, the focus was on Ni because this metal had been shown to be relevant for ecotoxicological studies. Based on some recent literature, Ni has been a focused metal such as in sediments (Yap & Wong, 2011), mussels (Yap *et al*., 2006), snails (Yap *et al*., 2008) and plants (Ong *et al*., 2011). Therefore, this study aimed to determine the distribution and concentrations of Ni in three different tissues of *N. lineata* collected from 20 sites along the western coast of Peninsular Malaysia.

MATERIALS AND METHODS

Surveys and samplings were done from the northern part to the southern part of Peninsular Malaysia (Fig.1). Out of the 25 surveyed sites, snail samples (*N. lineata*) were collected from 20 sites while sediments were collected from 17 sites along the western coast of Peninsular Malaysia from December 2005 until December 2010 (Table 4). The samples were placed in a cooler box with ice cubes (<10 ºC) during transportation to the laboratory. In this study, the *N. lineata* was not depurated of the contents of their alimentary canal since depuration might lead to contamination (Rainbow & Blackmore, 2001). Most importantly, it was found that there was no significant difference ($P>0.05$) between metal levels before and after depuration based on our preliminary study.



Number	Site
1	Jetty to Pulau Ketam
2	Star Cruise (West Port)
3	Tanjung Harapan
4	Kuala Selangor, Pgkln Permatang
5	Sungai Janggut
6	Kampung Deralik, Sitiawan
7	Kampung Sitiawan
8	Jambatan Raja Permaisuri Bainun
9	Sepang
10	Sg Kuala Lukut Besar
11	Hutan Lipur Pasir Panjang
12	Sg Balang Laut, Batu Pahat
13	Kuala Sg Ayam
14	Pantai Pontian
15	Kg Tanjung Langsat, JB
16	Kg Pasir Puteh
17	Kukup
18	Tj Piai
19	Kg Sg Melayu
20	Lukut

Fig.1: A map showing sampling sites of *Nerita lineata* on the western coast of Peninsular Malaysia

A total of 15 snails with relatively similar sizes were collected from each sampling site and they were dissected and separated into three parts (shells, opercula and total soft tissues). The snails were then dissected and separated into three parts, namely, shells, opercula and total soft tissues. All the samples were dried for 72 hours at 60ºC in an oven to constant dry weights. About 0.5g of homogenized samples (shells, opercula and total soft tissues) was digested in 5ml concentrated nitric acid (AnalaR grade, BDH 69%). They were placed in a digestion block at 40ºC for

1 hour and were later fully digested at 140ºC for 2-3 hours (Yap *et al*., 2002a). For the analyses of the sediment samples, the direct aqua regia method was applied. About 1g of each dried sample was digested in 5ml of a combination of concentrated nitric acid (69%) and perchloric acid (60%) in the ratio of 4:1; first at a low temperature (40ºC) for one hour, and followed by increasing the temperature to 140ºC for three hours. Then, the digested samples were diluted to 20 ml with double distilled water (DDW). After dilution, the samples were filtered with Whatman No. 1 filter paper and stored for further Ni determination.

All the samples stored in the acid-washed pill boxes were analyzed for Ni by using air-acetylene Perkin-Elmer™ flame Atomic Absorption Spectrophotometer (AAS) Model Analyst 800. Standard solution of Ni was prepared from 1000ppm stock solution provided by MERCK Titrisol for Ni (Yap *et al*., 2002a). All the data were presented in µg/g dry weight basis. The data were converted into µg/g by multiplying the data measured (C in mg/L) with dilution factor or total volume (D.F in ml). This was then divided with dry weight of sample (W in gram) in the following equation:

$$\text{Heavy metal concentration in gram sample (µg/g)} = \frac{C \times D.F}{W}$$

To avoid possible contamination, all glassware and equipment used were acid-washed and the accuracy of the analysis was checked against the procedural blank standard addition testing procedure (Yap *et al*., 2004). The percentage of recovery was 101% for Ni.

The Pearson's correlation coefficients were applied to find the correlation of Ni between the different tissues and sediments by using Statistical software (SPSS software version 15.0 for Windows).

RESULTS AND DISCUSSION

Table 1 shows the concentrations (µg/g dry weight) of Ni in the soft tissues, shells and opercula of *N. lineata*. The Ni concentrations in the soft tissues ranged from 0.40 – 10.51. As for opercula, the metal ranges were 10.84 – 34.90. It was found that the Ni concentrations ranges in the shells were 14.41 - 34.98.

In total in the soft tissues, the highest Ni concentration was recorded from the Kukup samples (10.90 ±0.75) while those from Tg. Harapan (2.93 ± 0.38) recorded the lowest level of Ni. Meanwhile, the highest level of Ni was found in the opercula of Kuala Lukut Besar (36.33 ± 0.10), whereas the samples from Tg. Harapan had the lowest level of Ni (11.88 ± 0.52) in the opercula. The shells of the snails were found to contain the highest level of Ni (35.83 ± 0.18) in the samples collected from the Kuala Lukut Besar population. On the other hand, the lowest level of Ni was found in the shells collected in 2010 from the Sg. Ayam (13.53 ± 0.13) population.

As for the samples collected from two different periods of time (namely, 2005 and 2010), the heavy metal concentrations showed some variations. In more specific, the Ni concentrations of all the tissues

TABLE 1
Mean µg/g dry weight, minimum and maximum concentrations of Ni in the soft tissues (ST), opercula and shells of *Nerita lineata* (n=15) and sediments (total) (n=11) collected from the west coast of Peninsular Malaysia.

No	Location		ST			Opercula			Shells			Sediment		
1	Jetty to Pulau Ketam (2005)	mean±se	4.72	±	0.51	15.53	±	0.13	16.37	±	0.12	10.30	±	0.01
		min - max	4.15	-	5.74	15.37	-	15.79	16.16	-	16.56	10.30	-	10.30
	Jetty to Pulau Ketam (2010)	mean±se	3.46	±	0.23	13.39	±	0.87	14.43	±	0.98	10.3	±	0.01
		min - max	3.03	-	3.79	11.82	-	14.84	13.40	-	16.40	10.30	-	10.30
2	Star Cruise	mean±se	6.61	±	0.34	19.21	±	0.45	25.09	±	4.95		na	
		min - max	5.97	-	7.10	18.32	-	19.77	19.89	-	34.98		na	
3	Tj. Harapan (2005)	mean±se	2.93	±	0.38	11.89	±	0.52	15.53	±	0.57		na	
		min - max	2.26	-	3.57	10.84	-	12.44	14.41	-	16.29		na	
	Tj. Harapan (2010)	mean±se	2.69	±	0.27	16.51	±	3.59	15.14	±	0.95	13.59	±	0.15
		min - max	2.15	-	3.00	12.09	-	23.62	13.70	-	16.94	13.32	-	14.08
4	Pengkalan Permatang	mean±se	6.02	±	0.29	25.81	±	0.50	26.67	±	0.21	11.70	±	0.07
		min - max	5.46	-	6.43	25.23	-	26.80	26.28	-	27.01	11.60	-	11.80
5	Sg. Janggut (2005)	mean±se	8.34	±	1.14	23.09	±	0.92	21.08	±	0.93	7.96	±	0.27
		min - max	6.66	-	10.51	21.31	-	24.41	19.71	-	22.84	7.49	-	8.44
	Sg. Janggut (2010)	mean±se	3.08	±	0.57	14.91	±	0.70	18.77	±	0.81	7.19	±	1.05
		min - max	2.16	-	4.13	13.66	-	16.10	17.28	-	20.05	7.14	-	7.41
6	Deralik	mean±se	4.77	±	1.22	21.30	±	0.51	20.09	±	0.06	6.04	±	0.08
		min - max	3.44	-	7.20	20.38	-	22.13	19.98	-	20.20	5.90	-	6.17
7	Kg. Sitiawan	mean±se	7.99	±	0.67	21.57	±	0.55	23.09	±	0.40	12.00	±	0.60
		min - max	6.67	-	8.87	20.95	-	22.67	22.31	-	23.66	11.00	-	13.10
8	J.R.P. Bainun	mean±se	4.92	±	1.15	33.86	±	0.59	33.68	±	0.44	12.80	±	0.11
		min - max	3.49	-	7.20	32.85	-	34.90	33.07	-	34.53	12.60	-	13.00
9	Sg. Sepang Besar (2005)	mean±se	6.58	±	0.67	19.46	±	0.28	19.59	±	0.24	11.20	±	0.19
		min - max	5.31	-	7.57	18.90	-	19.77	19.28	-	20.06	10.90	-	11.50

Table 1 (*continued*)

	Sg. Sepang Besar (2010)	mean±se	8.36	±	1.73	15.04	±	0.63	15.29	±	0.99	7.96	±	0.58
		min - max	0.32	-	0.56	2.62	-	4.08	4.31	-	6.99	5.17	-	5.84
10	Kuala Lukut Besar	mean±se	7.94	±	0.32	36.33	±	0.10	35.83	±	0.18	4.47	±	0.08
		min - max	7.34	-	8.43	36.13	-	36.43	35.62	-	36.19	4.33	-	4.61
11	Pasir Panjang	mean±se	3.62	±	1.62	21.25	±	0.38	22.02	±	0.22	22.60	±	0.93
		min - max	0.40	-	5.53	20.83	-	22.00	21.70	-	22.45	21.00	-	24.20
12	Sg. Balang Laut	mean±se	6.14	±	0.27	24.54	±	0.26	26.09	±	0.26	16.10	±	0.04
		min - max	5.64	-	6.57	24.10	-	24.99	25.76	-	26.59	16.00	-	16.20
13	Sg. Ayam (2006)	mean±se	5.90	±	1.15	24.71	±	0.53	25.65	±	0.14	20.60	±	0.40
		min - max	3.67	-	7.51	23.64	-	25.29	25.48	-	25.92	19.90	-	21.30
	Sg. Ayam (2010)	mean±se	4.52	±	0.34	11.91	±	1.09	13.53	±	0.81	23.16	±	0.43
		min - max	3.89	-	5.06	9.74	-	13.11	12.13		14.94	22.55	-	23.99
14	Pontian	mean±se	5.92	±	0.69	23.76	±	0.84	24.40	±	0.18		na	
		min - max	4.75	-	7.15	22.08	-	24.63	24.06	-	24.66		na	
15	Tj. Langsat (2006)	mean±se	5.32	±	1.57	27.80	±	0.47	27.45	±	0.20		na	
		min - max	2.18	-	6.94	27.00	-	28.64	27.06	-	27.73		na	
	Tj. Langsat (2010)	mean±se	6.00	±	0.42	26.70	±	1.16	19.80	±	0.42	13.76	±	0.73
		min - max	5.49	-	6.84	24.40	-	28.11	19.29	-	20.63	10.63	-	11.91
16	Kg. Pasir Puteh	mean±se	7.56	±	0.92	22.55	±	3.15	28.24	±	1.93	17.30	±	0.39
		min - max	5.71	-	8.49	17.34	-	22.10	24.91	-	31.60	16.86	-	17.53
17	Kukup	mean±se	10.90	±	0.75	22.96	±	1.17	22.26	±	0.97	14.98	±	0.38
		min - max	9.72	-	12.29	20.93	-	24.98	20.33	-	23.41	11.13	-	11.51
18	Tj. Piai	mean±se	7.57	±	2.23	22.32	±	0.81	19.63	±	1.24		na	
		min - max	5.07	-	12.01	21.38	-	23.94	22.02	-	17.90		na	
19	Kg. Sg. Melayu	mean±se	9.17	±	0.91	20.52	±	1.22	15.24	±	1.21	14.75	±	1.15
		min - max	8.74	-	10.92	18.15	-	22.24	13.84	-	17.64	13.40	-	15.38
20	Lukut	mean±se	9.00	±	0.89	16.04	±	0.14	19.94	±	0.86	11.63	±	0.78
		min - max	7.25	-	10.16	15.82	-	16.29	18.38	-	21.34	24.06	-	25.08

Note: na = not available

(soft tissues, opercula and shells) collected from the jetty to Pulau Ketam, Sg. Janggut, Sg. Sepang Besar and Sg. Ayam in 2005 were higher compared to those collected in 2010 (Table 1). Nonetheless, the Ni concentrations from the soft tissues and shells collected from Tj. Harapan did not show much difference between the two periods of sample collections and they only showed an increase in the opercula from 2005 to 2006 (Table 1). As for Tj. Langsat, the Ni concentrations of in the soft tissues and shells were lower in the samples collected in 2005 as compared to those taken in 2010, while the opercula showed a higher Ni concentration in the samples from 2005 compared to those in 2010 (Table 1).

Generally, the pattern of accumulation of Ni, from high to low, was in the sequence of shells≈opercula>soft tissues, as depicted in Table 1. This is similar to the results found by Amin *et al*. (2006b) on this snail. The lower metal concentration of Ni in the soft tissues compared to those in the shells could be due to Ni not being an essential metal for this species and therefore it was regulated downwards in the soft tissues. Meanwhile, the elevated levels of Ni in the soft tissues of marine gastropods were detoxified by making them biologically unavailable as insoluble phosphate salts (Nott & Nicolaidou, 1996). Other marine molluscs, such as *Perna viridis* (Yap *et al*., 2003) and *Telescopium telescopium*

TABLE 2
Correlation coefficient of Ni in the soft tissues, opercula and shells of *Nerita lineata* with those in the SET fractions of sediments collected in 2005 and 2006 (N= 11).

	Ni soft tissues	Ni opercula	Ni shells
Aqua Regia Fraction	-.486	-.205	-.093
EFLE	-.342	-.095	.002
Acid Reducible	-.539	.028	.092
Oxidisable	-.089	.202	.338
Resistant	-.455	-.229	-.154

** Correlation is significant at 0.01 level (2-tailed).
* Correlation is significant at 0.05 level (2-tailed).

TABLE 3
Correlation coefficient of Ni in the soft tissues, opercula and shells of *Nerita lineata* with those in the SET fractions of sediments collected in 2010 (N= 10).

	Ni soft tissues	Ni opercula	Ni shells
Aqua regia Fraction	.353	.295	.267
EFLE	-.110	.104	.233
Acid Reducible	.152	.015	.386*
Oxidisable	-.056	-.229	-.087
Resistant	.094	.157	.047

** Correlation is significant at 0.01 level (2-tailed).
* Correlation is significant at 0.05 level (2-tailed).

TABLE 4
Descriptions of sampling sites and means of shell length (mm), shell width (mm) and shell (mm) height of *Nerita lineata* from the west coast of Peninsular Malaysia (N=30 individuals for each population).

No	Sampling Sites	GPS	Sampling Date	Shell Length (min-max)	Shell Width (min-max)	Shell Height (min-max)	Sites descriptions
1	Jetty to Pulau Ketam	N 03º 01'12''	12-Dec-05	21.9 ± 0.590 (16.5-29.1)	16.3 ± 0.520 (12.7-28.1)	11.9 ± 0.340 (8.75-16.2)	A jetty with shipping activities.
		E 101º 21'42''	18-May-10	26.81 ± 0.34 (25.28 ± 28.34)	18.64 ± 0.17 (17.51 - 19.31)	14.67 ± 0.28 (13.27 - 16.04)	
2	Star Cruise	N 02º 59'12''	12-Dec-05	21.1 ± 0.480 (16.0-26.8)	15.2 ± 0.290 (11.9-18.0)	11.6 ± 0.270 (8.45-14.6)	Shipping activities.
		E 101º 20'42''					
3	Tg. Harapan	N 03º 5'58''	12-Dec-05	22.6 ± 0.620 (12.2-28.7)	16.1 ± 0.360 (9.70-19.1)	12.6 ± 0.370 (6.75-16.3)	Rocky beach, industrial area, a jetties and port area
		E 101º 21'38''	18-May-10	19.33 ± 0.34 (17.71 - 20.75)	14.50 ± 0.22 (13.52 - 15.52)	10.63 ± 0.20 (9.80 - 11.53)	(near to the north port)
4	Pengkalan.	N 03º 21' 24"	24-Feb-06	23.2 ± 0.560 (16.1-28.7)	16.7 ± 0.300 (12.8-19.9)	12.9 ± 0.300 (8.62-15.8)	A fishing village and a jetty.
	Permatang	E 101º 14' 38"					
5	Sg. Janggut	N 04º 8'10''	20-Mac-06	27.3 ± 0.720 (14-14)	19.3 ± 0.360 (11.3-22.0)	15.4 ± 0.400 (7.90-17.8)	River, water irrigation.
		E 101º 22'31''	18-May-10	30.06 ± 0.83 (33.82 - 25.19)	21.07 ± 0.40 (18.93 - 22.47)	16.94 ± 0.39 (14.70 - 18.19)	
6	Deralik	N 04º 14' 53.8"	25-Feb-06	25.5 ± 0.910 (12.1-33.8)	18.6 ± 0.510 (9.62-22.4)	14.4 ± 0.500 (6.47-18.6)	A jetty and fishing village (near Sitiawan) .
		E 100º 42'09"					
7	Kg. Sitiawan	N 04º 14' 44"	25-Feb-06	26.2 ± 0.630 (20.7-33.5)	19.5 ± 0.510 (15.5-28.8)	10.9 ± 18.0 (10.9-18.0)	Recreational park (kayaking).
		E 100º 41' 35"					

Table 4 (*continued*)

8	J.R.P. Bainun	N 04º 16' 46"	27-Feb-06	22.1 ± 0.880 (12.9-31.4)	16.2 ± 0.540 (9.95-21.1)	12.4 ± 0.480 (7.25-17.0)	Near industrial and a highway.
		E 100º 39'50"					
9	Sg. Sepang Besar	N 2° 36'4"	20-Dec-05	22.5 ± 0.670 (15.4-30.6)	16.6 ± 0.440 (11.3-22.2)	12.1 ± 0.400 (8.20-16.5)	An estuary receiving pig-farming effluents in 2000.
		E 101° 42'22"	5-May-10	22.53 ± 0.77 (19.56 - 27.87)	15.97 ± 0.29 (14.56 - 17.68)	11.12 ± 1.21 (0.75 - 14.61)	
10	Kuala Lukut	N 02° 34' 49"	28-Apr-06	23.8 ± 0.710 (15.9-31.1)	17.4 ± 0.440 (12.2-21.0)	13.5 ± 0.410 (8.75-18.7)	Receiving water exchange from a prawn aquacultural
	Besar	E 101° 49' 34"					area.
11	Pasir Panjang	N 02° 24' 55"	28-Apr-06	17.5 ± 0.400 (15.0-25.9)	12.7 ± 0.170 (11.3-14.8)	10.3 ± 0.360 (8.35-18.2)	Receiving domestic wastes.
		E 101° 56' 31"					
12	Sg. Balang Laut	N 01° 52' 21"	29-Apr-06	21.9 ± 0.550 (14.8-26.6)	16.3 ± 0.350 (11.0-18.8)	12.3 ± 0.520 (7.60-21.5)	Receiving water exchange from a prawn aquacultural
		E 102° 44' 16"					area.
13	Sg. Ayam	N 01° 45' 12"	29-Apr-06	23.2 ± 0.860 (15.9-31.3)	17.0 ± 0.550 (12.6-21.9)	12.6 ± 0.490 (8.20-17.3)	A fishing village with a jetty.
		E 102° 55' 45"	18-June-10	26.30 ± 0.56 (23.78 - 30.40)	18.65 ± 0.43 (16.86 - 21.66)	14.21 ± 0.30 (13.18 - 15.94)	
14	Pontian	N 01° 29' 23"	29-Apr-06	20.6 ± 0.790 (15.5-31.0)	15.8 ± 0.500 (12.2-22.2)	11.1 ± 0.450 (7.75-17.1)	A rocky beach and receiving domestic wastes.
		E 103°23' 08"					
15	Tg. Langsat	N 01 °28' 19"	30-Apr-06	19.5 ± 0.780 (11.5-27.5)	14.3 ± 0.530 (7.25-19.2)	11.0 ± 0.550 (5.65-18.2)	Near a port (Tg. Langsat Port).

Table 4 (*continued*)

		E 104 °00' 41"	20-June-10	27.13 ± 0.63 (24.46 - 31.46)	19.22 ± 0.43 (18.11 - 22.80	14.19 ± 0.33 (12.71 - 16.53)	
16	Kg. Pasir Puteh	N 01° 26'08"	20-June-10	25.02 ± 0.85 (22.54 - 30.91)	18.63 ± 0.54 (16.87 - 21.78)	13.44 ± 0.40 (12.01 - 15.88)	A jetty with restaurants, fishing village, shipping and industrial activities.
		E 103° 56'09"					
17	Kukup	N 01° 19.46'	18-June-10	26.04 ± 0.88 (23.08 - 30.82)	18.50 ± 0.56 (16.58 - 21.78)	13.51 ± 0.49 (12.10 - 16.52)	A fishing village and shipping activities.
		E 103° 26'52"					
18	Tj. Piai	N 01° 19' 49'	18-June-10	20.51 ± 0.20 (19.51-21.56)	14.86 ± 0.14 (14.28 - 15.58)	11.26 ± 0.12 (10.55 - 11.78)	It is a reserved area and a tourist attraction site.
		E 103° 26'52"					
19	Kg. Sg. Melayu	N 01° 27'04"	19-June-10	23.28 ± 0.50 (21.05 - 26.44)	16.88 ± 0.31 (15.98 - 18.50)	12.07 ± 0.29 (11.16 - 13.86)	A jetty, fishing village, shipping activities and mussel aquaculture.
		E 103° 41'69"					
20	Lukut	N 02° 34.511'	5-Dec-10	21.88 ± 0.48 (19.56 - 24.40)	15.52 ± 0.22 (14.24 -16.31)	11.99 ± 0.26 (10.54 - 13.09)	Industrial and urban area and a fishing village.
		E 101°47.529"					

(Amin *et al*., 2006a), also recorded higher concentrations of essential metals (Cu and Zn) over non-essential metals (Cd and Pb) in the soft tissues.

As for shells, Amin *et al.* (2006b) reported that the shells of *N. lineata* collected from Dumai had higher affinity for Ni compared to that of the soft tissues. Minor elements and trace metals might be incorporated into the carbonate crystalline lattices of the shells by replacing calcium ions in calcite or aragonite (Foster & Cravo, 2003; Yap *et al*., 2003). Once the metals were incorporated into the crystalline lattices in the shell matrices they would not be affected by the reproductive and physiological states of the organism (Yap *et al*., 2003). The shells might also act as a biodeposition site of unwanted chemical species (Bertine & Goldberg, 1972; Yap *et al*., 2003), and this could be the reason for the higher concentrations of Ni in the shells. This pattern of accumulation was found to be similar with that of *Telescopium telescopium* (Yap *et al*., 2008), where the Ni concentration in the shells was found to be higher than in the remainder soft tissues.

However, no significant correlations ($P>0.05$) were found between the sediments with soft tissues, opercula and shells of the *N. lineata* collected in 2005 and 2006 (Table 2). The samples collected from 2010 also showed no significant correlations ($P>0.05$) between the sediments and all the tissues, except for the shells and the acid reducible fraction ($P<0.05$), as shown in Table 3. This finding indicates that *N. lineata* is not reflecting the Ni contamination of the sampling sites although they serve as the biomonitors of the Ni bioavailability of these sites (Rainbows, 2004). It should be highlighted here that future genetic studies on the genetic similarity of the different geographical populations of this particular snail should be conducted since a good biomonitor should be a single species (Yap *et al*., 2002b) with genetically similar population in different locations.

CONCLUSION

The results showed that the *N. lineata* was able to accumulate metal such as Ni from the environment. However, further studies on the correlation of metal levels between the snails and the environmental samples are needed in order to validate its usefulness as a good biomonitor of the Ni pollution.

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Floral Biology, Flowering Behaviour and Fruit Set Development of *Jatropha curcas* L. in Malaysia

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ABSTRACT

This paper describes the flowering behaviour of *Jatropha curcas* cultivated under Malaysian tropical climate. Investigation was carried out by observing the floral morphology, flowering sequence of pistillates, floral anthesis time, flower daily anthesis, flowering and fruiting plant behaviour, flower sex and fruit set ratio. Floral reproductive organs were examined using Scanning Electron Microscope (SEM). Jatropha is monoecious and it produces individual flowers in a dichasial cyme. Each Jatropha inflorescence has at least six compound cymes. The male flower anthesis started the earliest at 12.00 am and once again at 6.10 am to 6.46 am. The female flower anthesis commenced at 6.35 am to 8.25 am. The male flowers opened for a period of 8 to 11 days, while the female flowers opened for only 3 to 4 days. The reading of the male to the female flower ratio was taken twice, 22:1 in December 2008 and 27:1 in April 2009. The flower to fruit ratios were 6:5 (January 2009) and 2:1 (May 2009). Numerically, 0-10 female flowers and 25-215 male flowers are produced in the same inflorescence. In this study, the terminal stem of Jatropha bore fruits profusely in January, May and August 2009. Meanwhile, the development of the floral meristem consists of three stages which include a vegetative stage, a transition from vegetative to floral stage and development of flower parts. The meristem was in the transition stage at day 6. Although all sepals and a petal were developed at day 18, the presence of reproductive organs developing at this particular stage was not detected. Flower and fruit development took approximately 3 months to complete the full cycle, i.e. from the initiated floral bud stage until fruit maturity.

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INTRODUCTION

Jatropha curcas L. (Euphorbiaceae) was introduced as a plant in Malaysia and it is valued for its use as an oilseed crop (Heller, 1996; Openshaw, 2000). Due to concerns over depletion of fossil fuel in the recent years, this plant has attracted attention because of its potential to partially replace fossil fuel as biodiesel. Currently, 40 million tonnes of diesel are annually consumed in India, and for this reason, India is now growing approximately 7.4 million hectares of Jatropha, making it the largest Jatropha producing nation. In Malaysia, Jatropha planting has been initiated at a trial scale. From this initial start, the biggest constraint on Jatropha identified to date has been the small number of fruits produced per inflorescence and also the differential ripening time of fruits on the same inflorescence. Bhattacharya *et al.* (2005) reported that only 50% of the female flowers set to fruit in Lucknow, India. Fruiting behaviour and pollination ecology of *Jatropha curcas* have also been studied by Raju and Ezradanam (2002). Their studies were carried out in the Eastern Ghats, India, at an altitude of 900 m where the climate is tropical monsoonal with an average rainfall of 1000 to 1600 mm annually and the mean temperatures varying from 20 to 25ºC in the winter and 30 to 32ºC in the summer (Murthy *et al.,* 1982).

Although there have been reports on Jathropa cultivation, most work has been carried out in India and due to the different climatic and soil conditions, information on the flowering and fruit set of Jatropha in Malaysia is apparently required. Malaysia is a country that enjoys a tropical, equatorial climate, with temperatures varying from 20 to 36ºC and an average annual rainfall of 2300 mm. In order to further understand the flowering and fruit characteristics of Jatropha, a floral and fruit development timeline is needed to address the identified problems with the small number of fruit produced. One approach to address this particular problem is to study the floral biology, floral ontogenesis, floral anthesis characteristic, as well as pollination ecology and pollen-style interactions. The objectives of the present study were to describe the floral biology and flowering behaviour of *Jatropha curcas* and to determine the timeline of the floral and fruit development in Malaysia.

MATERIAL AND METHODS

Field observations were carried out from November 2008 to June 2009 on three- to four-year old plants at Field 2, Universiti Putra Malaysia in Serdang (03°00.512N, 101°42.101E). Twenty four-year old plants were selected randomly and used for flower data collection and observation. Flowers were observed for their floral morphology, flowering sequence of pistillates, floral anthesis time, flower daily anthesis, flowering and fruiting plant behaviour, flower sex and fruit set ratio. These data were used to construct a timeline for flower development. In addition, vegetative shoots were tagged and observed for their developmental changes up to fruiting stage. Floral structures were observed using

Scanning Electron Microscopy (SEM). The samples were collected and fixed in 70% formalin acetic acid (FAA) and dehydrated to the critical point using osmium tetraoxide. Dehydrated samples were then mounted on aluminium stubs and sputter coated with gold and viewed under a JEOL JSM-5610LV scanning electron microscope at an accelerating voltage of 15 kv (Spence, 2001). The male to female flower ratio and flower to fruit ratio were also recorded based on 10 inflorescences which were randomly selected from 20 plants. Observations on the order of male and female anthesis were carried out to determine their protandry or protogyny characteristics.

RESULTS AND DISCUSSION

Floral Biology and Flowering Behaviour

Jatropha curcas is monoecious and its flowers are unisexual. The plant produces individual male and female flowers in a compound dichasium cyme pattern. Several dichasial cymes are clustered at the main inflorescence. Inflorescences are formed at the terminal of branches. Jatropha flowers are pale green in colour (see Fig.1a and Fig.1b), with a pedicel measuring 0.6cm to 1.0cm in length. There are five petals and the male flowers (Fig.1a) measure around 0.75cm to 0.9cm in length and 0.3cm to 0.4cm in width, while the female flowers (Fig.1b) measure about 0.7cm to 0.9cm in length and 0.3cm to 0.4cm in width. Flowers have five sepals; with each sepal ranging from 0.40cm to 0.60cm in length and 0.20cm to 0.30cm width in the male flower (Fig.1a), and approximately 0.45cm to 0.75cm in length and 0.20cm to 0.40cm in width in the female flower (Fig.1b).

Staminate flowers have ten functional stamens which are varying from 0.6cm to 0.7cm in length, and are arranged in two distinct whorls of five each in a single

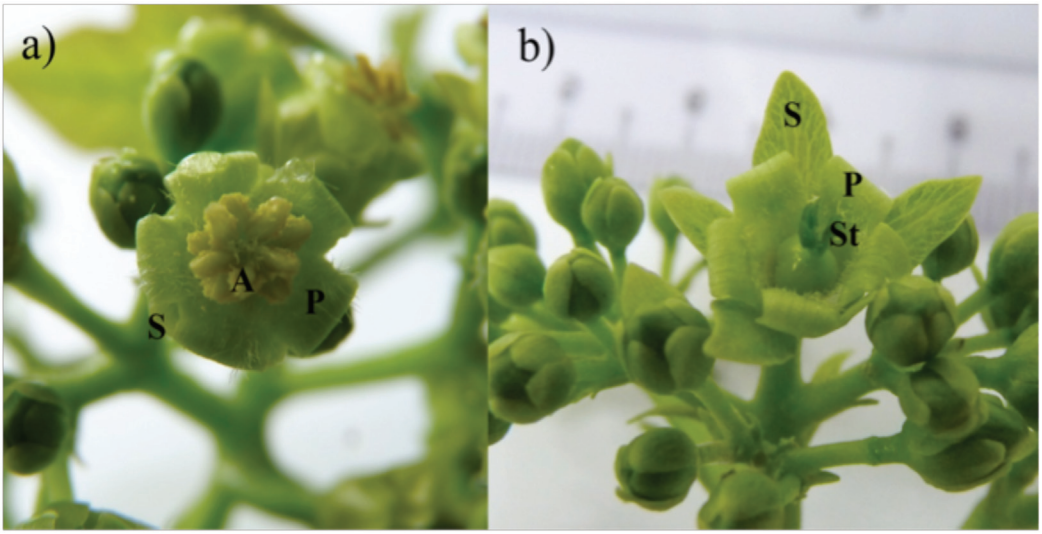

Fig.1: *Jatropha curcas;* (a) male and (b) female flower. Abbreviations: P, petal; S, sepal; A, anther and St, stigma

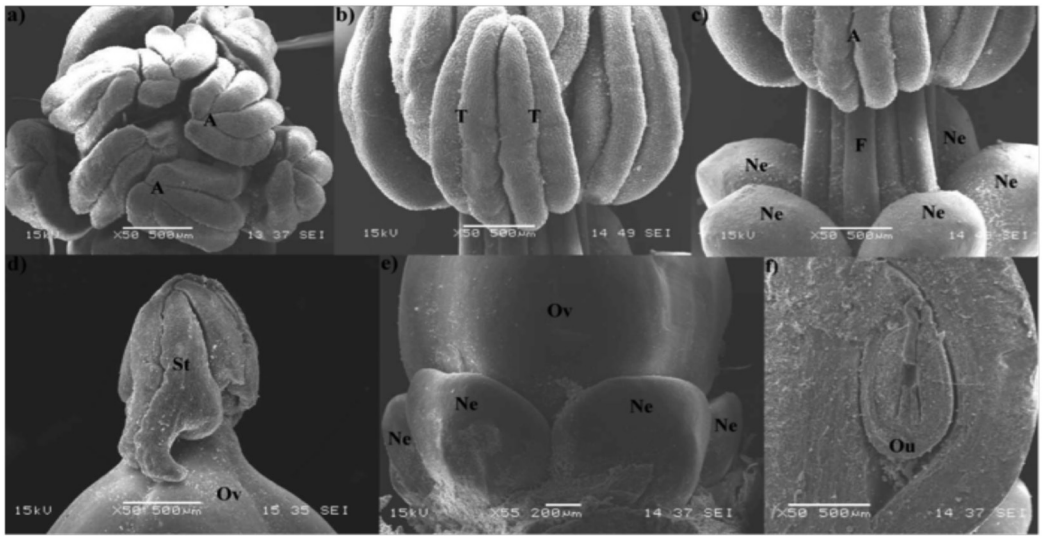

Fig.2: Scanning electron micrograph of floral reproductive organs of *Jatropha curcas* (x50). a-c. Staminate; anther with two theca and stamens are closed with each other. d-f. Pistillate; three-lobed stigma, ovoid ovary and apical- axile placenta. Abbreviations: T, theca; F, filament; A, anther; Ne, nectaries; St, stigma; Ov, ovary; Ou, ovule

column and adjacent to each other (Fig.2a). The anther is dithecal with longitudinal dehisced pollen (Fig.2b). The ovary is completely absent in staminate flowers but has five nectaries (Fig.2c). The pistillate flower was devoid of stamens and the style arose at the ovary apex (Fig.2d), with a distinct ovoid ovary terminating in a three-lobed stigma and surrounded by five nectaries (Fig.2e). The placenta was present in the apical-axile position with 3 placentae at the top of a septate ovary (Fig.2f). The pistil measured around 0.45cm to 0.68cm in length and 0.3cm to 0.35cm in width.

The compound dichasium cyme is composed of few individual simple cymes. Generally in the simple cyme, the female flowers are produced at the centre surrounded by the male flowers (Fig.3). In some cases, however, the expected female flower positions are replaced by the male flowers, making the ratio of the female florets lower than that of the male florets. The arrangement of the individual flowers grouped together into inflorescences also promotes attraction and foraging rate by foragers (Solomon & Ezradanam, 2002). Large number of flowers tends to increase the attraction of pollinators because the emission of chemical attractants is more intense and flowers are more visible (Tcherkez, 2004).

Jatropha inflorescences can either be simple with 6 individual cymes or more complicated with up to 10 individual cymes. Normally, when showing a complicated structure, the secondary inflorescence located at the base of the main inflorescence will have more tertiary inflorescences attached to it (see Fig.4). Based on eight observations, the flowering sequence of the female flowers in *Jatropha curcas* begins at

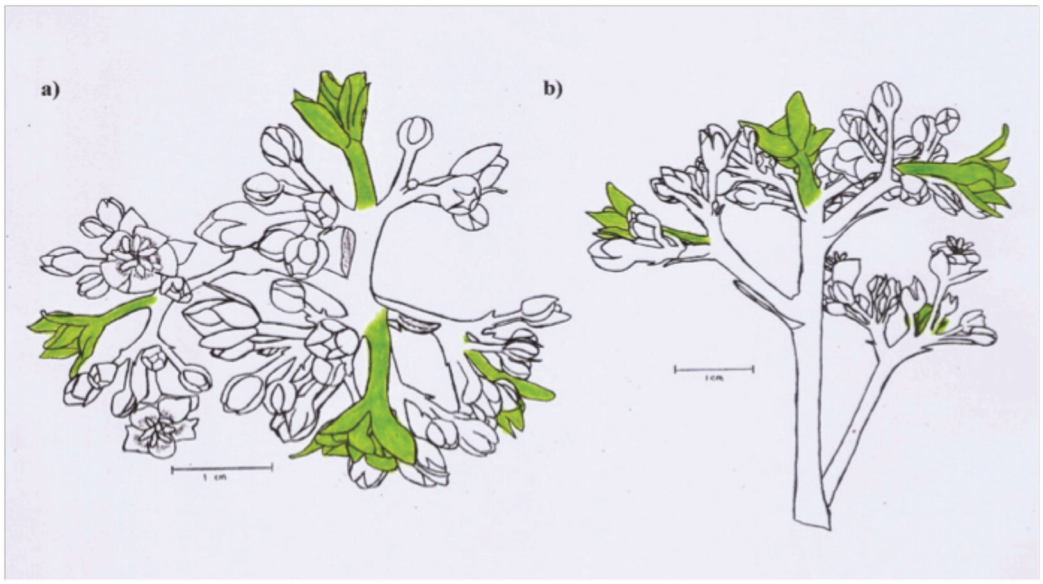

Fig.3: *Jatropha curcas;* inflorescence (a) Upper view (b) Side view. The female flowers were located at the centre of cyme which coloured with green colour. Abbreviations: cm, centimeter

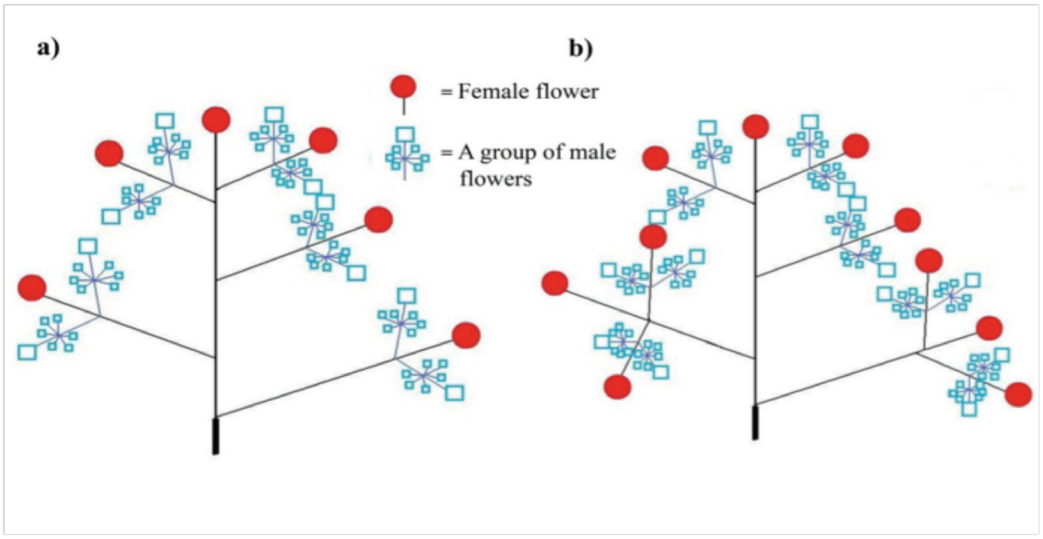

Fig.4: The *Jatropha* inflorescence structure with (a) 6 compound cymes (b) 10 compound cymes

the upper most terminal of the inflorescence (F) and simultaneously on the upper most terminal of the lowest cyme tier (A) and this is followed by B, D, C, A1 and E for the second day of flowering (Fig.5). This sequence creates only mature fruit on each bunch. Mature fruit are present at the upper most terminal of each inflorescence (F) and the lowest cyme tier (A), with green fruit in the middle of each inflorescence.

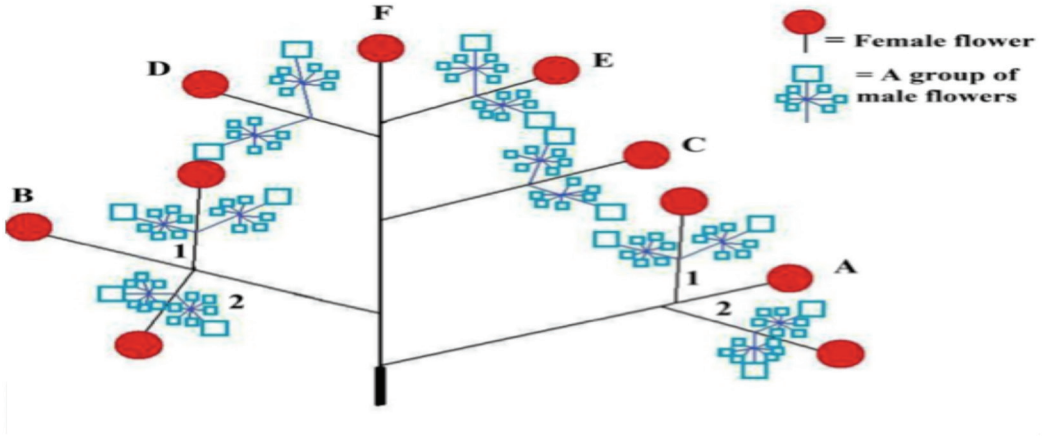

Fig.5: The flowering sequence of female flowers of *Jatropha curcas* begins at upper most terminal floret (F) in simultaneously with upper most terminal floret in the lowest cyme (A) and followed by B, D, C, A1 and E for the second day of flowering

The timing of the floral anthesis for the male flowers resulted in two distinct peaks following seven observations. The male flower anthesis was initiated at 12.00 am (24-25ºC) and at 6.10 am to 6.46 am (22-23ºC), while the female flower anthesis commenced at 6.35 am to 8.25 am (22-24ºC), as shown in Table 1. The male flowers take about 30 to 45 minutes from the initiation of a small opening to full bloom and the subsequent pollen release, while the female flower takes approximately 40 to 55 minutes from the initial opening to full bloom. From all the observations, the plants showed a protandrous pattern of opening, with male flowers opening before the female flowers. This observation contrasts with that of Heller (1996) and Sunder (2006), but supports that of Solomon and Ezradanam (2002). This mechanism promotes cross pollination either via geitonogamy or xenogamy but allogamy is also possible given the fact that pollen is released from the male flowers and the anthesis of the female flower occurs simultaneously. Nonetheless, studies on the Jatropha breeding system, pollination ecology and crop nutrition are suggested to further elucidate this mechanism.

TABLE 1
Floral anthesis time of the male and female flowers

Number of Observation	Flower Anthesis Time	
	Male	Female
1	12.05 am	8.25 am
2	6.29 am	7.30am
3	6.15 am	6.43am
4	6.25 am	6.40am
5	6.10 am	6.35 am
6	6.27 am	6.39 am
7	6.46 am	7.45 am

The male flowers open for a period of 8 to 11 days, while the female flowers open for only 3 to 4 days (Table 2). From the four inflorescences observed everyday over the period of flowering, the ratio of the male to

TABLE 2
Flower daily anthesis for four inflorescence

Day of Anthesis	Inflorescence 1		Inflorescence 2		Inflorescence 3		Inflorescence 4	
	Male	Female	Male	Female	Male	Female	Male	Female
Day 1	6	-	7	2	1	-	5	2
Day 2	8	2	32	6	6	-	10	2
Day 3	29	5	26	2	14	3	20	4
Day 4	23	1	43		24	2	20	
Day 5	17	0	23		17	1	24	
Day 6	8	0	21		29		29	
Day 7	7	1	22		10		14	
Day 8	9		3		9		24	
Day 9			1		3		30	
Day10			3				5	
Day 11							2	

the female flowers opened on the same day was sufficient enough to ensure successful pollination (Table 2). The peak time for the female flower opening is on days 2 to 3 of the flowering period (Table 2). However, the flowering pattern of the female flowers was not consistent throughout the flowering period. On other occasions, the flowering of the female flowers terminated in the middle of the flowering period and then resumed the following day. There was a situation at Inflorescence 1 where no female flowers were open on days 5 and day 6, but then the plants recommenced flowering on day 7 (Table 2).

Cymose inflorescences lack a main axis. The main shoot terminates in a flower, while growth continues through lateral axes produced below the terminal flower. These lateral axes again form terminal flowers and this process is repeated several times. The basal flower matured first with subsequent maturation occurring from apex to base (Simpson, 2006). This pattern will cause flower maturity to occur at different times and lead to a discrete period of flower opening for both the male and female flowers.

Meanwhile, the ratios of the male to female flowers were 22:1 (December 2008) and 27:1 (April 2009) from the data on ten inflorescences. The flower to fruit ratios were 6:5 (January 2008) and 2:1 (May 2009) (Table 3), respectively. Numerically, 0-10 female flowers and 25-215 male flowers were produced in the same inflorescence. The initial fruit set for Jatropha reached as high as 92% for the pistillate flowers. This indicates that individuals do not suffer from under-pollination. The production of pistillate flowers is low and each is surrounded by a large number of staminate

TABLE 3
Mean and number of staminate and pistillate flowers in an inflorescence and the number of fruits produced from the pistillate flowers for two observations

Inflorescence no.	No. of staminate		No. of pistillate		No. of fruits	
	Obs. 1	Obs.2	Obs. 1	Obs.2	Obs. 1	Obs.2
1	78	80	3	4	3	4
2	98	38	4	0	4	0
3	183	25	5	0	4	0
4	105	36	8	0	7	0
5	112	48	6	3	6	2
6	95	39	8	0	8	0
7	109	69	3	0	3	0
8	215	39	10	0	10	0
9	155	96	3	5	2	4
10	143	67	6	3	6	3
Mean	129	54	6	2	5	1

Notes: (Obs.) Observation

flowers with a male to female flower ratios of 22:1 to 27:1 that promote effective pollination maximally. This result is similar to that achieved by Bhattacharya *et al*. (2005) who recorded a 29:1 male to female flower ratio in their studies.

Floral and Fruit Development

Jatropha trees produce many leaves when they are in flowering period (Fig.6a). Trees then drop their leaves after fruit has set (Fig.6b). In the current study, the terminal stems of Jatropha profusely bore fruit in January, May and August 2009. In March, June, and October, flowering took place after the vegetative stage. It then took about a month from the vegetative flush to the initiation of visible flower buds. From the observations carried out, the development of the floral meristem consisted of at least three stages which included a vegetative stage (Fig.7a), a transition stage (Fig.7b) and the development of the flower parts (Fig.7c and Fig.7d). During the initial day of the sampling (day 0), the meristem showed a vegetative dome shape that measured around 150μm (Fig.7a). At day 6, the meristem was in the transition stage where it started to rise and was ready to differentiate into organs (Fig.7b). At day 18, all the sepals and petal were developed, but there was no presence of reproductive organs developing at this particular stage (Fig.7c and Fig.7d). Meanwhile, the floral bud became visible after 24 days from the first day of observation (Fig.8a and Fig.8b). It then took approximately 26 days from the day of visible floral bud to floral anthesis (Fig.8c). Once the floral anthesis had began, flowers then opened daily. Flowering lasted approximately 8 days (Fig.8d) and this was followed by 33 days for the fruit to

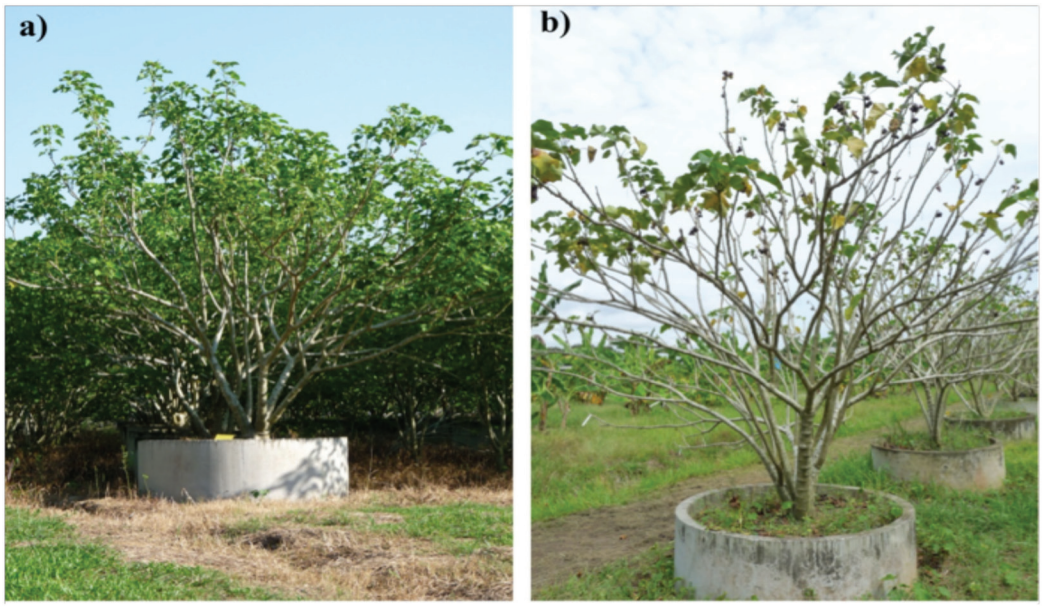

Fig.6: Jatropha trees approximately three to four years old are (a) full of leaves when it was in flowering period, (b) drop their leaves abundantly when it was in the fruiting period

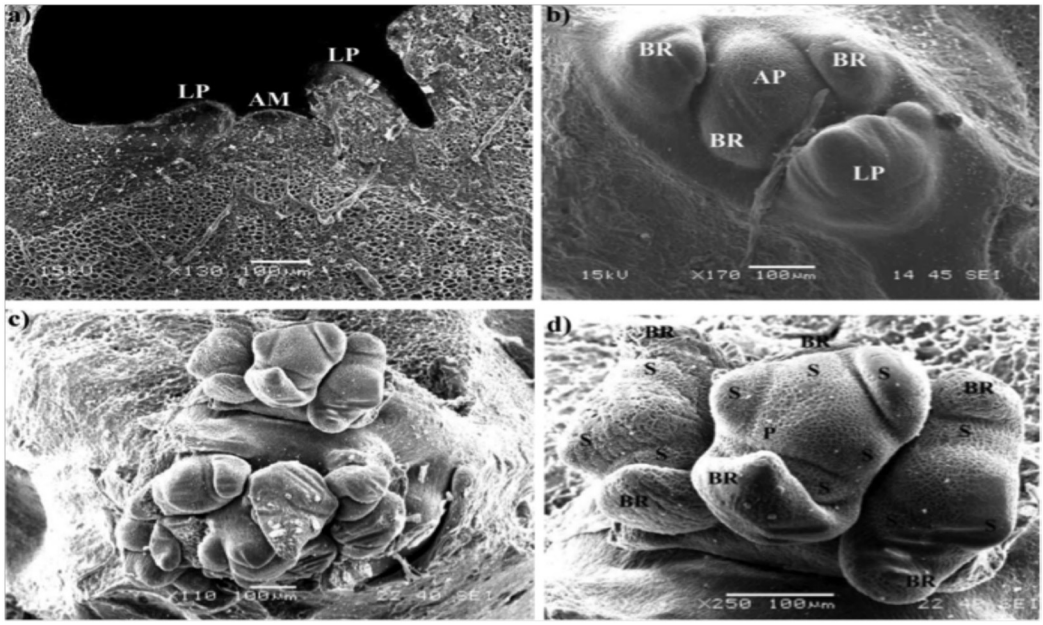

Fig.7: Micrographs of the vegetative, transition and early floral stages of *Jatropha curcas*. (a) Vegetative dome shape meristem that measures around 150 µm was found on day 0. (b) At day 6, shoot meristem started to rise and ready to differentiate into organs. (c) Two clear branch of inflorescence are presence at day 18. (d) Focus image of day 18 showed the sepals and petal have been developed at this stage. Abbreviations: LP, Leaf Primordia; AM, Apical meristem; AP, Apex; BR, Bract; P, Petal; S, Sepal

mature (Fig.8e). Fruit senescence occurred seven days after fruit maturity (Fig.8f). A complete cycle of fruit set and development required approximately 100 days (Fig.9).

Jatropha in Malaysia shows a characteristic year round free bearing habit combined with multiple cyclical fruiting peaks. Natural peaks can be altered by weather conditions and by culture manipulations in plantations (Milan, 2008). Flowering is usually triggered after a dry and a dormant period and it is induced by prolonged periods of raised soil water availability (Jongschaap *et al*., 2007). Flower formation could be influenced by the weather conditions at the time of bud differentiation. Dry weather induces flower bud formation and heavy rainfall promotes formation of vegetative buds (Heller, 1996).

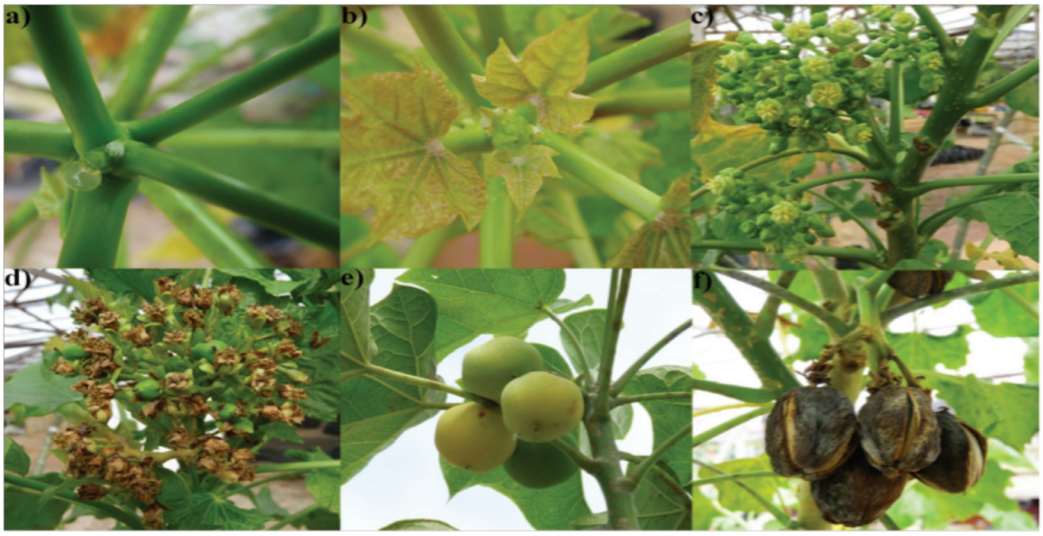

Fig.8: Macrophotographs of flower and fruit development showing (a) Vegetative stage at Day 0, (b) First visible flower bud at Day 24, (c) Anthesis at Day 50, (d) Flower senescence and fruiting at Day 58, (e) Mature fruit at Day 93 and (f) Fruit senescence at 100 days.

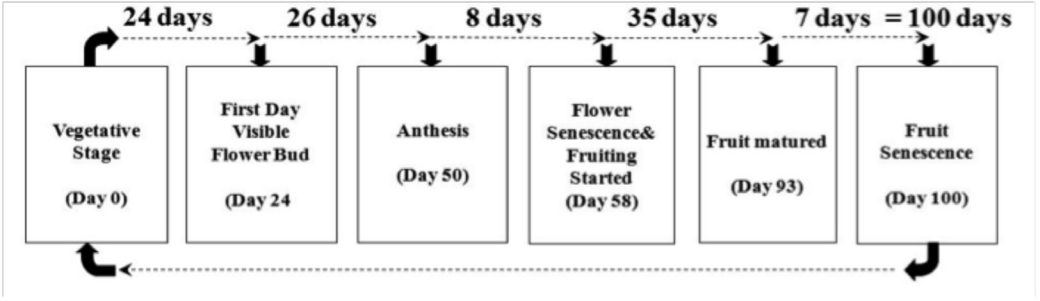

Fig.9: Timeline of flowering and fruit set showing *Jatropha curcas* took about 3 months to complete a fruiting cycle

CONCLUSION

The study revealed that the female flowers of Jatropha opened for a period of three to four days, while the male flowers opened for longer periods varying in duration from eight to eleven days. Continuous flowering and the incidents where flowering terminated in the middle of the flowering period were factors believed to have caused the wide range of fruit ripening times recorded. The initial fruit set of Jatropha was high, i.e. as much as 92% of the pistillate flowers set fruit. The low fruit set problems in *Jatropha curcas* is mainly caused by a small number of pistillate flowers present in each inflorescence that range from 0 to 10 flowers in the same inflorescence. Details of the flower structure and understanding their individual functions during the process of fruit setting will assist cultivar improvement and can optimise yields and synchronize fruit maturity.

Floral and fruit development takes approximately 3 months to complete the cycle from the initiated floral bud stage until fruit maturity. This indicates that Jatropha could have two to four cyclical fruiting peaks in Malaysia, depending on the weather conditions and cultivation practices.

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A Retrospective Study on Post-arrival Mortality Rate of Australian Boer Goats in a Breeder Farm in Malaysia

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ABSTRACT

Post-arrival mortality pattern and the causes of those mortalities were studied in a Boer goat breeding farm in Malaysia. The farm was established in October 2005, following an importation of 597 breeder Boer goats from Australia. Further importations of 534 Boer goats were made in July 2007, and 166 goats in March 2008. Farm records covering the period between October 2005 and December 2008 were analyzed for monthly mortality pattern with special attention on the post-arrival weeks. Upon arrival, goats were provided with vitamins, anti-stress and antibiotic cover. They were fed with cut grasses and supplemented with goat pellets at 350g/goat/day. Drinking water was also available *ad libitum*. During the study period of 2005 to 2008, there were significantly ($p<0.05$) higher rates of annual mortality during rainy months (7%-14%) as compared to dry months (2%-5%). Meanwhile, the post-arrival mortality showed an average of 27%, ranging between 13% and 43%, of the Boer goats died during the first 6 weeks of post-arrival. In particular, the goats arriving in the rainy months of October 2005 and March 2008 showed higher post-arrival mortality than those arriving in the dry month of July 2007. The post-arrival mortality pattern revealed a gradual but significant ($p<0.05$) increase as early as week 1, with an average of 5% mortality to reach peak at week 3 with 35% mortality before it gradually decreased to 6% at week 6 and 3% at week 7. The major causes of post-arrival mortalities were pneumonic mannheimiosis and helminthiasis, which were associated with the stresses of handling, loading and unloading during shipment.

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INTRODUCTION

The goat industry in Malaysia, with approximately 200,000 goats that are kept

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mainly by smallholders, is rather small-scaled as it is supplying only 8% of the local demands for chevon (Aziz, 2007). Therefore, Malaysia has spent approximately RM5.8 million (USD1.7 million) annually to import livestock products, including goats and its products to fulfil this demand (Aziz, 2007). In trying to reduce the cost of importation of livestock products, the Malaysian government decided to enhance its livestock industry in 2005 (Ibrahim *et al.,* 2006). The first step taken towards enhancing the industry was to increase the cattle and goat populations to approximately 1 million heads respectively by 2010 through the breeding programme (Ibrahim *et al.,* 2006*)*. Thus, importation of goats to increase the number of breeders, particularly Boer goats was started in 2006 from various goat-producing countries, particularly from Australia (Aziz, 2007). Currently, there are a total of 360,000 heads of goats in Malaysia with 56,000 Boer goats (DVS, 2008). This paper reports the post-arrival mortality pattern and the major causes of the mortality observed among the newly arrived Boer goats at a breeder farm in Malaysia.

MATERIALS AND METHODS

Study Population

This study was conducted at a Boer goat breeder farm that was established in 2005, following an importation of 597 Boer goats from Australia. The study was carried out for a period of 39 months, i.e. between October 2005 and December 2008, within which the period Boer goat importations took place 3 times, and it involved a total of 1,297 heads of breeder Boer goats. All the imported goats were subjected to disease status and quarantine as required by the authority. At the end of the study period in December 2008, the farm consisted of approximately 1,500 goats of various ages that were kept in 9 slatted-flooring houses for tropical goat rearing (Jansen & van den Burg, 2004). Each house was able to keep between 100 and 300 goats with floor space of approximately $12ft^2$/goat. Water was available *ad libitum*, while feeding regime consisted of cut grasses at the rate of 2kg/goat/day and supplemented with goat pellet at the rate of 300-400g/goat/day. During non-raining months, the goats were allowed to graze between 11 a.m. and 3 p.m.

Study Protocol

The study was started by analyzing the farm records for the period between October 2005 and December 2008 to obtain the monthly mortality pattern. Special attention was given to the post-arrival mortality pattern of the newly arrived Boer goats which arrived in October 2005 (597 goats), July 2007 (534 goats) and March 2008 (166 goats). The post-arrival mortality study period was 7 weeks, i.e. the period when mortality was observed before it returned to pre-arrival rate.

Meanwhile, post-mortem examinations were carried out on all dead goats. Appropriate samples were collected and processed for identification of the agents, particularly bacteria and parasite. The causes of the post-arrival mortalities for all cases were also noted and analyzed.

Statistical Analysis

The mean rates of mortality at different times of shipment were compared using the analysis of non-variance and LSD All-Pair-wise Comparison Test (Statistix 9, USA). Pearson's correlation (Statistix 9, USA) was also selected to determine the correlation between the rainy and dry months. All the data were considered as significant at $p<0.05$.

RESULTS

Mortality Pattern

Fig.1 reveals the general monthly mortality pattern among the goats in the farm during the study period of 2005 to 2008. It revealed significantly ($p<0.05$) higher rates of mortality (i.e. between 8% and 11%) during the rainy months of March to June and in October to December (i.e. between 7% and 14%) each year. The average monthly mortality in 2005 was significantly ($p<0.05$) higher at 10.1% compared to only 3.8%, 3.9% and 2.5% respectively in 2006, 2007 and 2008 (see Fig.1).

An average of 27% (ranging between 13% and 43%) of the newly arrived Boer goats was found to die within the first 6 weeks post-arrival. Meanwhile, the arrivals during the rainy months in 2005 and 2008 showed higher post-arrival mortality at 43% and 24%, respectively, as compared to merely 13% mortality following the arrival during the dry month in 2007. Following the analysis of the three shipments, an average of 5% newly arrived goats died in week 1, 6% in week 2, 35% in week 3, 31% in week 4, 18% in week 5 and 6% in week 6. These mortalities were significantly ($p<0.05$) higher than that of the pre-arrival period of week O (2.1%) (see Fig.2).

Causes of Mortality

The post-arrival mortalities were due to several major causes. In more specific, about 30% were due to pneumonia associated with infection by *Mannheimia haemolytica,*

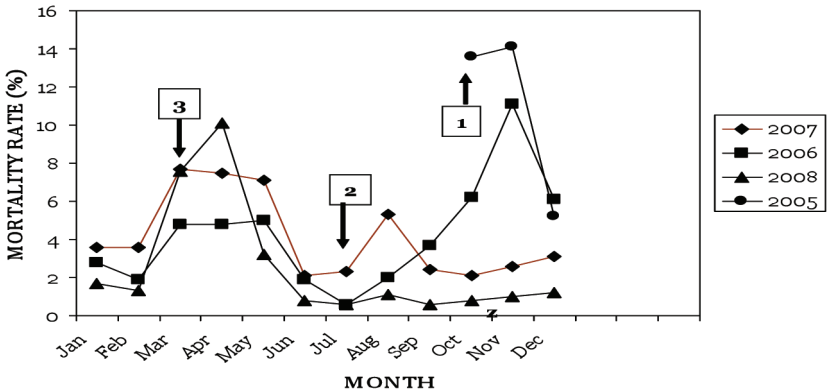

Fig.1: The monthly mortality rate of Boer goats between October 2005 and December 2008. There were significantly ($p<0.05$) higher rates during the rainy months of April to June and October to December each year. 1= first importation in October 2005, 2= second importation in July 2007 and 3= third importation in March 2008

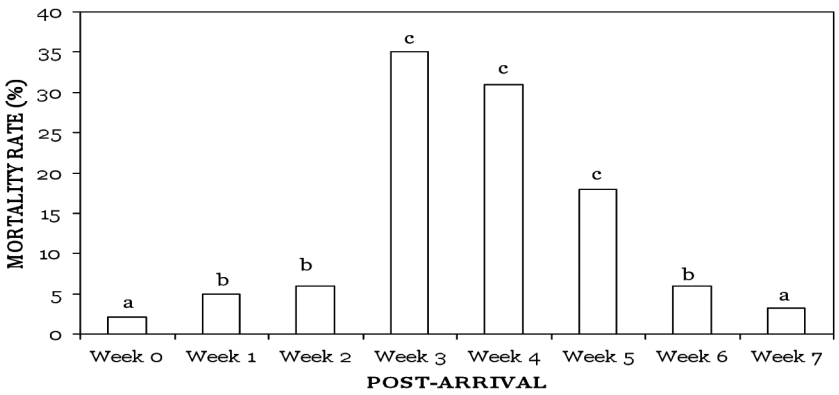

Fig.2: The weekly pre- and post-arrival mortality of goats in Malaysia. Mortality started as early as week 1 post-arrival and peaked in week 3 post-arrival before it slowly declined to pre-arrival rate in week 7 post-arrival. Different letters represent significant ($p<0.05$) differences.

29% were due to haemonchosis, 25% were due to complications associated with non-infective abortions, 10% were due to general weakness as a result of malnutrition, 4% were due to bloat following changes in diet and 2% were due to urinary calculi.

DISCUSSION

This study revealed a high average monthly mortality in 2005 compared to 2006, 2007 and 2008. This is believed to be due to the inexperience in handling the newly arrived goats at the farm, which was established in 2005. There were evidences of poor feeding regime and the absence of basic disease control protocol.

A literature search on the pattern of post-arrival mortality among the imported goats had been futile. Nevertheless, it is well accepted that adaptation period leads to high mortality (Alexandre & Mandonnet, 2005). The post-arrival mortality pattern observed in this study is believed to be associated with the stress due to handling, loading and transportation. This is in agreement with Minka *et al.* (2009), who found that handling was the most stressful time as compared to loading and unloading, particularly post-transportation. The stressful effects were further influenced by the arrivals during the rainy months (Scott, 2011). Although mortality was significantly higher as early as 1 week post-arrival, the majority of mortalities were observed in weeks 3 to 5 of post-arrival, before the mortality was reduced significantly in week 6 and back to almost pre-arrival rate in week 7. The average of 27% mortality within the first 6 weeks post-arrival is similar as the 25% mortality of the imported Dorset Horn sheep into Malaysia (Fatimah *et al.,* 1985). It seemed that during acclimatization, the energy expenditures by goats were affected, particularly during the first 8 weeks (Patra *et al.,* 2008) and this led to severe shipping stress (Kannan *et al.,* 2000) that further caused higher rate of mortality. Therefore, the mortality pattern observed in this study

should be noted and the timing of arrival that avoids rainy months attempts to reduce handling stress (Dass *et al.,* 2001) and herd health programme (Huttner *et al.,* 2001) upon arrival is important to minimize mortality, considering the fact that Boer goats are fairly resistant to many diseases (Erasmus *et al.,* 2000).

The respiratory tract infection and helminthiasis observed among the newly arrived Boer goats in this study have also been recognized as the major causes of mortality among goats (Kusiluka *et al.,* 1998). In fact, helmithiasis and pneumonic mannheiniosis have been recognized as two major farmed goat and sheep diseases in Malaysia (see Fatimah *et al.,* 1985; Jasni *et al.,* 1991) and these incidences have been reported to be higher during rainy months than during dry months (Kusiluka *et al.,* 1998). Similarly, significant increases in the incidences of pneumonia and haemonchosis in adult goats during rainy months, as observed in this study, have led to the high rates of mortality (Mellado *et al.,* 1991). Nevertheless, the post-arrival mortality pattern observed in this study, which peaked at week 3 and returned to almost normal rate at week 7, was mainly due to post-arrival exhaustion and inability to eat properly, causing the animals to become stressed and leading to diseases. Therefore, it is extremely important for the newly arrived animals to be given anti-stress, vaccinated against pneumonic mannhemiosis and treated with anthelmintic to reduce mortality.

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Evaluation of Nitrogen Uptake Efficiency of Different Oil Palm Genotypes Using ^{15}N Isotope Labelling Method

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ABSTRACT

High demands for palm oil world wide induce the expansion of oil palm plantations in Malaysia. Malaysian soils which are highly weathered require high nitrogen fertilizer input in order to maintain high yield output, resulting in increase in production cost as well as inducing negative impacts to the environment. It is crucial to understand the performance of different oil palm genotypes in taking up nitrogen to increase nitrogen use efficiency, minimize environmental pollution caused by leached nitrate and maximize plantation profit, while maintaining sustainable agriculture practices. ^{15}N labelling method was utilized in a greenhouse study to quantify the nitrogen up-take performance of nine oil palm genotypes at 6 and 9 months after planting. Measurements of total dry matter, total N, and percentage N derived from fertilizer (%NdFF) were carried out during the study. At 6 month old, oil palms of different genotypes did not show any difference in nitrogen uptake with and without P fertilizer applications. However, 9 months old oil palms demonstrated significant differences between the genotypes in total dry matter production and total N taken up, hence, resulting in significant differences in N derived from fertilizer among genotypes. Oil palms at 9 months old also showed significant effects in the N uptake as affected by P fertilizer application. Genotype A (14/34 x 2367/17) demonstrated significantly higher nitrogen uptake compared to other genotypes, except for genotype F (9/103 x 2318/17). Thus, the ^{15}N labelling technique could serve as a useful assessment to the nitrogen uptake abilities of oil palm genotypes.

Keywords: Oil palm, nitrogen fertilizer, nitrogen uptake, ^{15}N isotope labelling

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INTRODUCTION

High demands for palm oil world wide have made oil palm (*Elaeis quineenis*) an important plantation crop in Malaysia, which contributes to high National Gross Export (Azman *et al.*, 2004). The total oil palm area in 2009 was 4.69 million hectares, i.e. with an increase of 4.5% as compared to the previous year. Meanwhile, total export of oil palm products, consisting of palm oil, palm kernel oil, palm kernel cake, oleochemicals, biodiesel and finished products increased by 2.9% or 0.64 million tonnes to 22.40 million tonnes in 2009 from 21.76 million tonnes recorded in 2008 (Mohd. Basri, 2010). This increase has further induced the expansion of oil palm plantations in Malaysia. Thus, more lands have been converted into oil palm plantation as a result of the increases in oil palm hectarage. However, most soils planted with oil palm in Malaysia are of the order Ultisols and Oxisols, which are low in fertility status (Goh *et al.*, 2003), and planting oil palm in these soils could cause further soil degradation (Tessens & Shamshuddin, 1983). Hence, applying additional fertilizers to compensate nutrient removal from soils by plant seems to be a direct answer to this situation.

It is important to highlight that excessive applications of fertilizers, especially nitrogen, have harmful consequences on the environments. Nitrogen applied as inorganic chemical fertilizer, especially nitrates, could easily experience leaching by rainfall due to its negative charge, and as much as half of the nitrogen fertilizers applied could be loss at the end of the planting season (Sukreeyapongse *et al.*, 2001). Therefore, increasing fertilizer rates without any proper precaution will often lead to intense environmental pollution. As fertilizers are the most expensive inputs (Sabri, 2009) and the largest variable cost item in oil palm production (Goh *et al.*, 2003), leaching of N from chemical fertilizer will not only bring negative influences to the environment but also represent a significant economic loss. In 2008 alone, the industrial plantation in Malaysia consumed 1.29 million tons of N fertilizers. A study by Goh (2005) also indicated that over-application of as much as 0.25 kg ammonium nitrate/palm/year would result in an extra cost of RM117.25 million per year to the oil palm industry in Malaysia. Hence, overcoming nutrient loss from soils via increase in fertilizer application was apparently and economically unfavourable. Nevertheless, insufficient nutrient supply to meet the requirement of oil palm will result in a significant drop in the productivity of oil palm for the subsequent years (Mohd Tayeb & Tarmizi, 2001). Mohd Noor *et al.* (2005) reported that oil palm demonstrated a slow recovery of productivity, following a decrease in fertilizer application, and the slow recovery of oil palm productivity would affect the profitability in the subsequent years. Goh (2005) indicated that under-application of fertilizers might result in the loss of oil palm yields. Hence, increasing oil palm nitrogen use has efficiency become crucial in optimizing nitrogen use, reduce fertilizer cost, and avoid N fertilizer pollutions.

Oil palm growth performance can vary due to oil palm genetics (Soh, 2004; Rafii *et al.*, 2002; Kushairi *et al.*, 1999). Research workers observed that variation of oil palm genotypes contributes in producing variable genetic expression (Norziha *et al.*, 2008), such as different heights, sizes of canopy, sizes of bunches, amounts of mesocarp, kernel contents (Kushairi *et al.*, 1999), and different vigorous levels (Soh, 2004). Meanwhile, a high level of variability in yield has been observed from oil palm progenies (Norziha *et al.*, 2008; Soh, 2004). Despite all the efforts done in this area of research, the exploitation of genotypic performance and site yield potential of oil palm planting materials are still at its infancy (Goh *et al.*, 1994). Hence, in order to maintain high oil palm yields with low chemical fertilizer inputs, it is essential to screen oil palm for the genotypes that have superior nitrogen uptake abilities. Thus, the primary objective of this study was to evaluate the ability of the oil palm genotypes for nitrogen acquisition ability at the nursery stage.

MATERIALS AND METHODS

The experiment was carried out in a glass house (with an average temperature of 29°C and a relative humidity of 96%) at the Faculty of Agriculture, Universiti Putra Malaysia. The oil palm seedlings used in this study were obtained from Sime Darby Research Sendirian Berhad (Table 1). The experiment was conducted as a 9 X 2 factorial laid out in Randomized Complete Block Design (RCBD) with four replications. The treatments comprised of nine genotypes of three-month old oil palm seedlings (as shown in Table 1) and two P levels (with P, 6.70 g $palm^{-1}$; and without P, 0 g $palm^{-1}$). A total of 72 three-month old oil palm seedlings were planted in black polythene bags containing 30 kg of Serdang series soil (*Typic paleudult*). The soil chemical properties are shown in Table 2. At planting, nutrients were added according to the fertilizer programme for oil palm seedlings (Gillbanks, 2003), namely, 4.90g nitrogen, 6.70g phosphorus, 5.00 g potassium and 3.90 g magnesium per palm. Ammonium sulphate (AS), labelled with 5% atom excess (a.e) ^{15}N, was used as a source of nitrogen. This was split-applied at day 1 and day 45 after planting to minimize the leaching of nitrogen. Each application of ^{15}N labelled AS was applied in solution formed by dissolving 11.67g of 5% a.e. AS with distilled water. Phosphorus fertilizer in the form of Gafsa phosphate rock (14% P) was applied to only 36 bags of the oil palms seedlings, which were subjected to P fertilizer, while the remaining 36 bags of oil palms seedlings would rely on the available P in the soil as a source of phosphorus without any addition of P fertilizer to reflect the P fertilizer synergistic effect to N uptakes of oil palm. Potassium and magnesium were applied in the form of Muriate of Potash and Kieserite. 20g of AJIB© was applied for micronutrients. Weeding and irrigation were carried out manually for all the pots. Using the same methods, another set of experiment was conducted separately using six-month old oil palm seedlings. Both sets of oil

palm seedlings were left to grow for three months to allow the uptake of nutrient and ^{15}N labelled fertilizer. At the end of three months, destructive sampling was carried out for both sets of oil palm seedlings. Plant samples were separated into rachis and leaves, which were then oven dried at 70˚C until constant weight was achieved. Dry weights of the samples were measured using analytical balance. The samples were then ground to pass through 1mm sieve and analyzed for their total nitrogen and ^{15}N enrichment (IAEA, 1983). The percentage of N derived from fertilizer (%NdFF) was calculated using the following equation, based on the isotope dilution technique (IAEA, 1983):

$$\%\,Ndff = \frac{\%N - 15\ a.e.\ plant}{\%\,N - 15\ a.e.\ fertilizer} \times 100 \quad [1]$$

$$N\ yield = Dry\ Matter\ (DM)\ yield\ of\ plant \times \%\ N\ in\ plant = Total\ N\ uptake\ in\ plant\ (g\ plant^{-1}) \quad [2]$$

$$Fertilizer\ N\ yield = \frac{N\ yield \times \%\ Ndff}{100} \quad [3]$$

The ability of the oil palm seedlings to take up the N fertilizer was evaluated from the total N uptake derived from the labelled fertilizer (Fertilizer N Yield). The means were compared by the analysis of variance (ANOVA) using the SAS statistical software version 9.0 (SAS, 2002), and the treatment means were separated by Student-Newman-Keuls Test at 5% probability level.

TABLE 1
Genotypes of Oil Palm (obtained from Sime Darby Research, Banting)

Genotypes	Mother Palm (Dura)	Pollen Palm (Pisifera)
A	14/34 (UR X JL)	2367/17 (AV)
B	2/35 (UR X UR)	2367/17 (AV)
C	2/209 (BD X BD)	2367/17 (AV)
D	19/19 (UR X UR)	2367/17 (AV)
E	25/49 (BD x UR)	2367/17 (AV)
F	9/103 (BD X BD)	2318/17 (AV)
G	23/34 (JL X JL)	2367/17 (AV)
H	1/39 (UR X UR)	2318/17 (AV)
I	33/17 (JL X JL)	2318/17 (AV)

TABLE 2
The Chemical Properties of Serdang Series Soils

Parameters	Value
$pH_{(water)}$	4.5
Total N (g kg^{-1})	1.3
Bray-2 P (mg kg^{-1})	5.4
Organic C (g kg^{-1})	8.5
Cation Exchange Capacity (cmol(+) kg^{-1})	4.3
Exchangeable K (cmol(+)kg^{-1})	0.1
Exchangeable Ca (cmol(+)kg^{-1})	0.8
Exchangeable Mg (cmol(+)kg^{-1})	0.2

RESULTS AND DISCUSSION

Growth Performance

Most of oil palm characters, such as bunch yield, bunch weight and bunch number, which are economically important are controlled by polygene, and they are more susceptible to the influences of environment factors (Ooi *et al.*, 1973; Thomas *et al.*, 1969). Rafii *et al.* (2002) reported that there is a significant influence by environment on genetic variances in D × P progenies, as yield

and bunch quality characters of the progenies would vary from one location to another. Hence, this experiment was conducted in such a way that oil palm seedlings were grown in a controlled environment with uniform fertilizer applications to detect the effect contributed by oil palm genotypes on the nitrogen uptake ability. These oil palm seedlings (Table 3) were produced in the same progeny by crossing or selfed different source of Dura, namely from Ulu Remis (UR), Johore Labis (JL), and Banting (BD), in order to produce mother palms, and these were crossed with Dura mother palm and the siblings of Avros Pisifera (AV) originated from BM 119 (1316) Avros Pisifera. The dry matter ($p = 0.69$) and the total nitrogen content ($p = 0.54$) of oil palm seedlings at 6 months old were not significantly affected by genotypes, and this indicated that the six-month old oil palm seedlings do not possess enough difference in their growth performance to significantly highlight the difference between each genotype. However, when the same experiment was repeated with nine-month old seedlings, oil palm genotypes provoked significant effects to the plant total dry matter ($p < 0.01$) as well as the total accumulated N in plants ($p < 0.01$). As indicated by the total dry matter of oil palm seedlings, genotype A which built up dry weight of 115.82g showed significant superior growth performance compared the most of the genotypes which ranged from 84.25g to 106.34g, except for genotype F (127.78g), while all the other genotypes were not significantly different. Similarly, genotype A also showed a significantly higher total accumulated N content within the plant (2.86g) as compared to the other genotypes, ranging from 1.35g to 2.1 g per plant. Despite the fact that the total dry matter and the total accumulated N in the plant were not significant for the 6 months old oil palm seedlings, a similar trend was observed throughout the data as compared

TABLE 3
The Effect of Genotype on Total Dry Matter and Total Nitrogen of Oil Palms

Genotypes	Total Dry Matter (g plant^{-1})				Total N (g plant^{-1})			
	6 months		9 months		6 months		9 months	
A	71.83	A	155.82	a	1.47	a	2.86	A
B	54.29	A	101.48	b	1.41	a	1.80	B
C	68.66	A	84.25	b	1.46	a	1.54	B
D	61.67	A	84.50	b	1.35	a	1.73	B
E	69.43	A	104.13	b	1.65	a	2.01	B
F	68.97	A	127.78	ab	1.53	a	2.10	B
G	58.38	A	99.76	b	1.18	a	1.35	B
H	67.01	A	106.34	b	1.49	a	1.92	B
I	58.64	A	105.90	b	1.34	a	1.90	B

Note: Values in a column with the same letter(s) are not significantly different according to the Student-Newman-Keuls Test at $P \geq 0.05$

to the 9 months old seedlings, in which genotype A achieved highest in dry matter, followed by genotype F, whereas genotypes A, E, and F recorded the highest total N content in both the 6 and 9 months old oil palm seedlings. These findings suggested that 9 month old oil palm seedlings could be a better indicator for the difference between oil palm genotypic characteristics.

The Effects of P Fertilizer on Plant's Nitrogen Uptake

According to Zin *et al.* (2007), oil palm's response to N fertilizer was severely restricted in the absent of P fertilizer. In this experiment, the interaction between oil palm genotype and P fertilizer was not observed ($P \geq 0.7$) for all the parameters measured. Hence, the discussion will focus on the main effect of P fertilizer. Total dry matter of the oil palm under the two different P levels recorded a similar trend, as shown by the oil palm growth performance (Table 4). This also indicates that P fertilizer does not contribute to significant difference in the growth of seedlings across all the genotypes for the 6-month old oil palm seedlings ($p = 0.51$), but significant effects were seen in 9-month old oil palm seedlings ($p = 0.02$).

It is important to note that the soil used as a planting medium in this experiment contained small amount of phosphorus, and the oil palm seedlings were planted in poly bag containing 30kg of soil. Consequently, due to the less P requirement of young oil palm seedlings, the phosphorus within the soil would minimize the effects of P fertilizer on the growth performance of young seedlings over a short period of time. The results of this study is concurrent with the findings of other researchers who have reported that N and P fertilizers contribute to increase in the dry matter productions in plants (Wilkinson *et al.*, 1999) and P has synergetic effects on nitrogen in term of yield (Zin *et al.*, 2007). However, at both ages of oil palm seedlings, P fertilizer does not contribute to any significant difference for the total N accumulation in the plant ($p = 0.58$ and 0.09). As mentioned earlier, the planting medium used in this experiment contained small amounts of phosphorus, and the seedlings planted in this experiment might not confront with P stress condition, and hence, the P fertilizer's effect would be suppressed as long as P threshold had not been overcome (Fong & Lee, 1998).

TABLE 4
The Effect of P Fertilizer to Total Dry Matter and Total Nitrogen in Oil Palms

	Total Dry Matter (g plant^{-1})		Total N (g Plant^{-1})	
	6 months	9 months	6 months	9 months
With P	65.98 a	117.02 a	1.46 a	2.01 a
Without P	62.67 a	96.88 b	1.40 a	1.76 a

Note: Values in a column with the same letter(s) are not significantly different according to the Student-Newman-Keuls Test at $P \geq 0.05$.

Nitrogen Derived from Fertilizer (NdFF)

Since oil palm is a major source of oil and fat for human, breeders have been working to shorten the process of breeding selection cycle, and also accelerate the breeding progress (Soh, 2004). However, being a perennial crop, oil palm breeding cycle could easily require 10 to 15 years (Norziha *et al.*, 2008). Additionally, only 148 palms can be planted on 1 hectare, and thus, oil palm progeny trails require much larger areas, cost, and maintenance. This study incorporates the isotope labelling technique in order to provide more genotypic information for plant breeders. In the previous studies, ^{15}N isotopes had been applied as tracers in estimating N_2 fixation (Alves *et al.*, 2000; Chalk, 1996; Hamilton *et al.*, 1991; Danso, 1986; Chalk, 1985), N deposition in field (Böhme & Russow, 2005) and to determine the leaching of nitrogen from fertilizers (Bergstrom & Kirchmann, 2004). Additionally, ^{15}N isotopes were also used in quantifying nitrogen uptake efficiencies of Arabidopsis (Chardon *et al.*, 2010), and nitrogen recovery efficiencies of coated urea in potato fields (Zvomuya *et al.*, 2003).

As shown in Table 5, the results revealed that only the N yield derived from labelled fertilizer (0.31g) of 9-month old oil palm seedlings is significantly higher ($p < 0.01$) as compared to the other genotypes, while the 6-month old oil palm seedlings are not significantly different ($p = 0.90$) across the genotypes. The other parameters, namely total N increase and total %Ndff at 6-month old ($p = 0.72$ and 0.73, respectively) and 9-month old ($p = 0.56$ and 0.22, respectively) seedlings, were also not significantly different between the genotypes. Generally, genotype A demonstrated superior N uptake performance, which could be observed from both the 6-months old and 9-months old oil palm seedlings.

Meanwhile, the ^{15}N labelling technique has provided an insight into the nitrogen use efficiencies (NUE) of the oil palm, whereby genotype A achieved the highest NUE as much as 6.12%, genotype F at 3.27%, while the other genotypes utilized less than 2% of the applied labelled fertilizer. Genotype A contained as much as 10.48% of ^{15}N within the plants as compared to the total N of the plant, followed by genotype F which contained 7.62% of ^{15}N in total N of the plants. The specific Dura from Johore Labis was selected due to its favourable characteristics, such as very big bunch size with low bunch number, thinner shell, and Ulu Remis Dura was chosen because of its ability to reduce sex ratio without reducing the yield. Meanwhile, Banting Dura was used in crossing due to the higher oil content in its fruit bunches, thick mesocarp, and thin shell. Avros Pisifera 2367/17 and 2318/17 were close siblings whose parents were selected due their favourable characteristics, such as high general combining ability and exceptional vigorous vegetative growth.

Genotype A, which was produced from UR X JL X AV, could have higher genetic diversity to inherit more favourable genotypic characteristics as compared to the genotypes which were produced through selfed mother Dura crossed with Avros

TABLE 5
The Effects of Oil Palm Genotype on Total N Increase, Total %Ndff, and N Yield Derived from ^{15}N Fertilizer

Genotypes	Total N Increase (g plant^{-1})				Total %NdFF				N Yield Derived from ^{15}N Fertilizer (g plant^{-1})			
	6 months		9 months		6 months		9 months		6 months		9 months	
A	0.50	a	1.15	a	47.11	a	27.49	a	0.17	a	0.30	a
B	0.37	a	0.73	a	44.57	a	32.43	a	0.15	a	0.09	b
C	0.53	a	0.36	a	48.23	a	32.84	a	0.15	a	0.07	b
D	0.45	a	0.71	a	51.81	a	35.91	a	0.16	a	0.09	b
E	0.42	a	0.69	a	49.25	a	30.43	a	0.16	a	0.09	b
F	0.51	a	0.67	a	45.87	a	27.69	a	0.16	a	0.16	b
G	0.36	a	0.43	a	45.48	a	29.43	a	0.12	a	0.03	b
H	0.54	a	0.59	a	44.45	a	27.79	a	0.17	a	0.09	b
I	0.29	a	0.78	a	44.59	a	28.14	a	0.11	a	0.07	b

Note: Values in a column with the same letter(s) are not significantly different according to the Student-Newman-Keuls Test at $P \geq 0.05$

Pisifera. Genotype E, which was also produced via mother palm produced from Banting Dura and Ulu Remis Dura crossed with AV, produced a similar favourable growth performance compared to the other genotypes, despite the fact that data were not statistically higher. These two genotypes generally showed higher growth performance average as compared to the other genotypes, despite the fact that genotype E was not statically different from the other genotypes. The result could be partially influenced by the short growing periods of the oil palm which had prohibited the oil palm seedlings to express more differences in terms of their growth performance caused by genetic superiority. Meanwhile, the differences between genotypes A and E with the others could be more prominently distinguished if the experiment time period had been extended. On the other hand, genotype F also showed a promising growth performance and a high nitrogen utilization rate as compared to genotype A. As much as 25000 palms were visually selected based on the well-formed bunches and appearances, and Dura was selfed and sib-mated to produce the mother palm for genotype F. Hence, it is favourable to observe the superior growth of this particular genotype as inherits of vigorous parental characteristic. These genotypes of oil palm were newly developed by Sime Darby Research Sendirian Berhad, and hence, the data obtained from this experiment could be used as a reference for plant breeders so as to produce oil palm seedlings with higher nitrogen uptake ability. Nonetheless, due to the variations in the nitrogen uptake of oil palms, more study are still required to fully understand oil palm physiologies which correspond to genotypic characters, and consecutively identify oil palm genotypes with high N uptake.

CONCLUSION

It is important to note that there is no interaction of P fertilizer with oil palm genotypes. Hence, the P fertilizer gives a uniform effect to all the genotype growth performances, and P fertilizer has synergistic effects on the efficiency of oil palms' nitrogen uptake. Oil palm seedlings at 6 months old did not give a clear indication of the difference in the growth performance across all oil palm genotypes. Meanwhile, the dry matter, total N, and even N derived from the ^{15}N labelled fertilizer were not significantly different across the genotypes; hence, it is safe to conclude that 6-month old oil palms are not sufficient enough to reveal the difference in their genotypic characters. However, 9-months old oil palm seedlings showed significant difference in their total dry matter, total N and N yield derived from the labelled fertilizer across the different genotypes. In particular, Genotype A recorded the highest total dry matter (155.82 g/ plant), highest total accumulated N in plants (2.86 g) and N yield derived from the ^{15}N labelled fertilizer in plants (0.30g), highest nitrogen use efficiencies (6.12%), and highest ^{15}N concentration within the plants (10.48%). Meanwhile, the ^{15}N isotope labelling technique could provide a better insight into the oil palm seedlings' nitrogen uptake efficiencies. In order to detect the differences between oil palms genotypic variability, it is advisable to utilize slightly matured oil palm seedlings, in which the stress symptoms could be more profound than that of the young seedlings. It is also wise to extend the period of palm oil growth in order for the oil palms to show greater difference in their genotype superiority.

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Fire Resistance and Reaction-To-Fire of *Shorea macrophylla* and *Acacia mangium* Particleboards Treated with Boron and Phosphorous-based Fire Retardants

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ABSTRACT

Fire resistance and reaction-to-fire of Engkabang (*Shorea macrophylla*) and *Acacia mangium* particleboards, which were treated with zinc borate (ZBr) and monoammonium phosphate (MAP), were investigated in this study. Ten percent of the fire retardants were incorporated into the particleboards in powder form during resin-particle mixing process. The fire resistance of the boards was assessed using insulation and integrity failures. Meanwhile, reaction-to-fire was conducted to examine the effectiveness of the fire retardants to delay ignition and reduce weight loss. The study showed that ZBr was excellent in improving insulation and integrity failures of the boards as compared to MAP. Zinc borate delayed the increase of unexposed face temperature up to 18 min and reduced the weight loss down to 0.57% (ZBr-treated *A. mangium*), but MAP was shown to be better than ZBr in delaying ignition (i.e. up to 41s for *A. mangium* and 20s for *S. macrophylla*). The ineffectiveness of the fire retardants to reduce weight loss of the boards (MAP-treated and ZBr-treated *S. macrophylla* and MAP-treated *A. mangium* might be due to leaching and volatization of phosphoric acid and boric acid in the formulations of the particleboards which would then cause the chemical loading to be lower than the actual chemical loading. It is suggested to extend the research especially in determining the chemical loading of each treated boards during and after they are exposed to fire. This is essential to prove the claim that chemical loading is decreased due to the leaching of phosphoric acid and volatization of boric acid.

Keywords: Fire retardants, insulation failure, integrity failure, weight loss, particleboard

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INTRODUCTION

Particleboard is often used as in ceiling, partitioning and panelling construction. This product can be manufactured from low quality wood, mill residues, lignocellulosic materials from agricultural waste including wheat, rice straw, kenaf, rubberwood and empty oil palm fruit bunches (Basturk, 1993; Cai *et al*., 2004; Izran *et al*., 2010c; Zaidon *et al*., 2007; Izran *et al*., 2010). It is important to note that particleboard has gained its popularity until now as the prices of lumber are unstable in both developed and underdeveloped countries. Consequently, to meet the demands of particleboard production, exploration of more alternative materials is crucial to ensure continuous supply. Engkabang jantung (*Shorea macrophylla*) and *Acacia mangium* possess qualities that are suitable for particleboard production (Izran *et al.,* 2010b). Particleboards from these fast growing species provide acceptable strength properties that surpass the British Standard requirements (Izran *et al*., 2010a). In order to add value to the particleboards made of *S. macrophylla* and *A. mangium*, especially for safety reasons in high rise buildings, their combustibility properties need to be assessed and reduced. In particular, the combustibility needs to be reduced down to meet the requirements set by Uniform Buildings by Law 1984 (UBBL 1984). This can be achieved through incorporation of fire retardants. In fact, the application of fire retardant can be done in two ways: 1) by treating fibres with fire retardants before they are mixed with resin and compressed to particleboard, or 2) by adding fire retardants during resin-fibre blending process. Both the methods were found to be effective in reducing the combustibility of particleboards (Izran *et al.,* 2010). The combustibility of a material for building construction can be assessed through fire resistance test and reaction-to-fire test. Fire resistance exhibits means of quantifying the ability of an element to withstand exposure to high temperatures through insulation and integrity evaluations (BSI, 1987), whereas reaction-to-fire is a test to examine the time taken for ignition to occur as well as the weight loss of the tested samples after the exposure to fire. This paper reports the fire resistance and reaction-to-fire of particleboards made from *S. macrophylla* and *A. mangium* particles mixed with boron-based and phosphorous-based fire retardants.

MATERIAL AND METHODS

The materials used in this study were the particles of *Shorea macrpophylla* and *Acacia mangium*. Meanwhile, monoammonium phosphate (MAP) and zinc borate (ZBr) fire retardants (10% w/w oven-dried dried particles) were used as treating chemicals. Adhesive E2-grade urea formladehyde resin was used as a binder. The woods were flaked, chipped and screened into particles ranging from 1 to 2 mm in size. Then, the particles were dried to 5 ± 2% moisture content (MC) using an industrial oven which was set at the temperature of 105±2°C for 24 h. A single homogenous layered board (340 mm x 340 mm x 12 mm), with a target

density of 700 kgm^{-3}, was fabricated. The final MC of the particleboards was ca. 12%. The fire retardants were incorporated into the particleboards in powder form during blending of furnish. The particles were first blended with UF resin (12% w/w oven-dried dried particles) + wax (1% of solid resin) + hardener (3% of solid resin) in a mixer. The furnish was then incorporated separately with 10% MAP and 10% ZBr. This was followed by forming the furnish into the former and pressed. The furnish was then hot-pressed for 6 min for the particles treated with MAP and 9 min for those treated with ZBr. For the untreated furnish, it was pressed for 7 min. The variation in the time of pressing was due to the influences of the chemicals on delaying and aggravating curing of the UF resin (Izran *et al*., 2010b). A total of twelve boards were fabricated, with 4 boards each for the untreated, MAP-treated and ZBr-treated. One board from each group was used for the fire resistance test and the remaining three boards were utilized for the reaction-to-fire test. The fire resistance required samples with a dimension of 340 mm x 340 mm x 12 mm, whereas, early burning performance needed slightly smaller sample in the size of 225 mm x 225 mm x 12mm.

Fire Resistance Test

This test was conducted in a fire furnace, in accordance with British Standard 476: Part 22 (BSI 1987). The dimension and weight of the treated and untreated boards were measured. After that, the boards were fixed to the furnace using cement. Four thermocouples were attached on the unexposed side of the tested boards. These thermocouples were connected to a recorder which was responsible to record temperature change of the unexposed side. The temperature of the furnace fire was also measured using thermocouples in the furnace which was connected to a computer. The temperature of the furnace was set at 27-30°C before the test. The sample was then heated by fire in the furnace. At the same time, the temperature increment of the unexposed face was recorded at five-minute intervals until the temperature reached 183°C (insulation failure) or until the board collapsed (integrity failure).

Intergrity is the ability of the particleboard to prevent collapse or sustain flaming. Based on the standard, intergrity failure happens when: (1) the tested sample collapses or sustained flaming occurs for more than 10 seconds on the unexposed face, (2) when fire and hot gases cause flaming to the cotton pad, and (3) when cotton pad is not suitable to be included in the test. The failures are: (i) when the occurrence of 60mm diameter gap gauge can penetrate a through gap and its end projects into the furnace and it can be moved in the gap for a distance of at least 150 mm, or (ii) when the occurrence of 25 mm diameter gap gauge can penetrate a through gap and its end projects into the furnace. Insulation is the ability to delay excessive increase in the temperature of the unexposed face. The standard indicates that insulation failure occurs when: (1) the temperature of the unexposed face increases more than 140°C

above its initial mean temperature, or (2) the temperature recorded at any position on the unexposed face using thermocouples is more than 180°C above the intial mean temperature of the unexposed face. The unexposed face is the particleboard surface which is not exposed to fire in the furnace.

Integrity failure influences insulation failure because as the board collapses, evaluation of insulation failure is stopped even though the temperature of the unexposed face has not achieved the standard temperature. The calculation of the time-temperature relationship in the furnace is automatically done by the software installed in the computer. The calculation is according to the formula stated in the standard for fire resistance test (BSI 1987).

Reaction-to-Fire Test

The boards for the test were oven-dried at 103±2°C until the oven-dry weight was achieved (IW). Before the test was conducted, 1 ml of ethanol was dispersed on the surface of the board. This was to encourage combustion on the board when exposed to fire. Each board was placed inclined at 45°, 3 cm above a bunsen burner and the time taken for the board to ignite was recorded. The combustion on the board was left for 2 min. The burned board was re-weighed (WAB). The weight loss of each board was calculated using Equation 1 below:

$$\text{Weight loss (\%)} = \left(1-\frac{WAB}{W}\right)\times 100\% \qquad [1]$$

The data of the weight loss and flaming duration were analyzed using ANOVA to evaluate the efficacy of the fire retardants on the fire performance of the boards.

RESULTS AND DISCUSSIONS

Fire resistance of A. mangium *and* S. macrophylla *particleboards*

The fire resistance of the boards is presented in Table 1. The treated and untreated boards were found to achieve integrity failure before insulation failure, except for the *A. mangium* boards that were treated with ZBr. The boards collapsed before their unexposed surfaces reached the maximum temperature of 183°C above the mean initial temperature and the minimum 140°C above the mean initial temperature. Compared to MAP, however, ZBr was superior as it was able to delay integrity failure by 8 minutes (for *A. mangium* boards) and 6 minutes (for *S. macrophylla* boards). During intergrity failure, the minimum temperature of the unexposed surfaces of the boards (*A. mangium* and *S. macrophylla*) treated with ZBr was 186°C and 57°C, respectively, whereas the maximum temperature were 226°C and 58°C, respectively. Meanwhile, the *A. mangium* boards that were treated with ZBr suffered insulation failure for both the minimum and maximum temperatures at 19th minute. Similar results were also observed for the boards treated with MAP, where the results varied between the two species used. The MAP-treated *A. mangium* boards performed better than MAP-treated *S. macrophylla* boards, where the times

taken for them to achieve integrity failures were 16 and 13 minutes, respectively. The temperatures of the unexposed surfaces above the mean initial temperature during the integrity failure were also found to be different, with the minimum of 88°C and the maximum of 98°C for MAP-treated *A. mangium* boards, and the minimum of 96°C and the maximum of 152°C for *S. macrophylla*, respectively. The MAP-treated *S. macrophylla* board exhibited almost similar results with those of the untreated boards (both *A. mangium* and *S. macrophylla*); however, it failed to reduce the increment in the temperatures of the unexposed surfaces more effectively than the untreated boards (minimum 88°C and maximum 98°C for *A. mangium* and the minimum 96°C and maximum 152°C for *S. macrophylla* above the mean initial temperature). The untreated boards faced integrity failures at 12 minutes after the exposure to fire in the furnace (minimum 80°C and maximum 97°C for *A. mangium* and minimum 71°C and maximum 77°C for *S. macrophylla* above the mean initial temperature).

TABLE 1
Fire resistance of *A. mangium* and *S. macrophylla* particleboards

Samples	TIF (min)	Temp UE (°C) Min (≤140°C)	TempUE (°C) Max (≤183°C)
AM-Cont	12	80	97
AM-ZBr	20	186 (Insulation failure at 19 min)	226 (insulation failure at 19 min)
AM-MAP	16	88	98
SM-Cont	12	71	77
SM-ZBr	18	57	58
SM-MAP	13	96	152

TIF: Time for integrity failure, TempUE Min: Minimum temperature of the unexposed face, TempUE Max: Maximum temperature of the unexposed face. Note: All the samples achieved integrity failure before insulation failure, except for the *A. mangium* boards treated with ZBr

Physical Observations on Fire Resistance Test Samples

Observations were also made to record the physical changes of the boards during the test until they collapsed. In particular, the physical changes of ZBr-treated *S. macrophylla* boards began 2 minutes after the exposure. At this time, smoke was found to be present on the upper **horizontal part** of the board. This happened constantly until the 12th minute. At the 12th minute, the smoke became denser, indicating the exposed face started to burn badly. At the 14th minute, char established on the top left side of the unexposed face and continuous flaming occurred at the 18th minute, and this brought to integrity failure. The same result was observed for the *A. mangium* board treated with ZBr (see Fig.1).

The MAP-treated *A. mangium* board started to show changes after 2.5 minutes, with a leakage of smoke at the top of the unexposed surface of the board. Later, the leakage spread to both the lateral edges of the board. Meanwhile, char started to form at the smoke leakages after 12 minutes. After 15 minutes, cracks began to appear along the upper most surface of the board and ignition occurred after 16 minutes

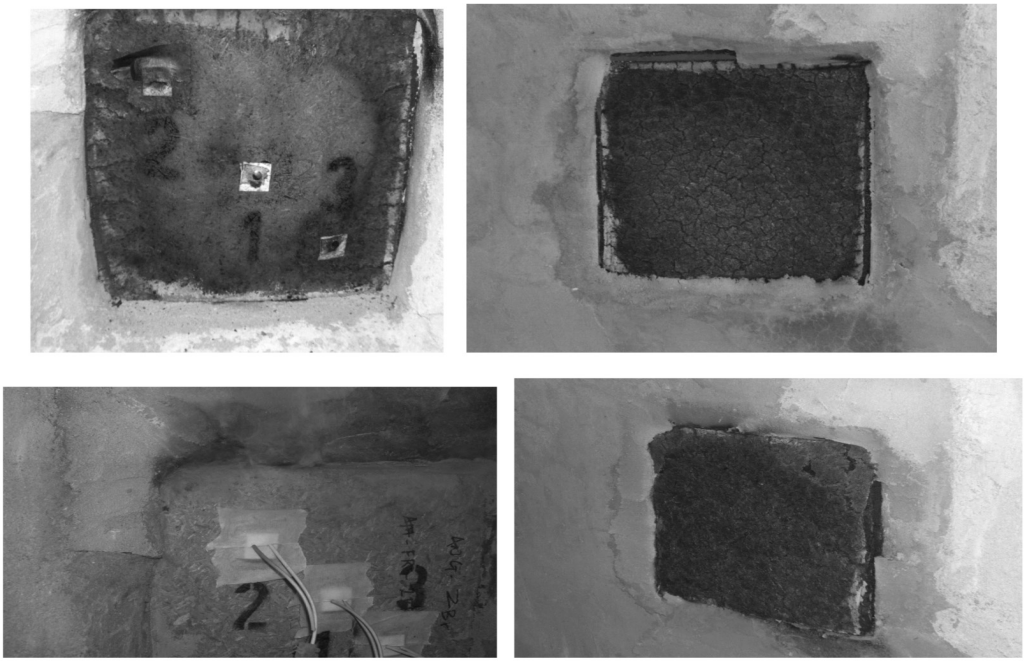

Fig.1: (From left to right) Before and after the test of the ZBr-treated boards (Above: *A. mangium* and below: *S. macrophylla*)

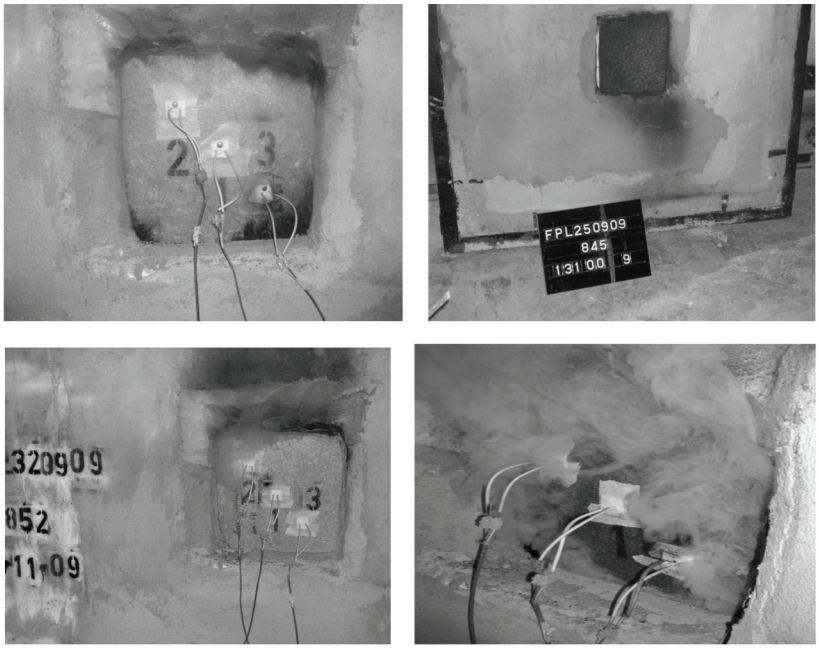

Fig.2: (From left to right) The MAP-treated boards before and after the test
(Above *A. mangium*; Below: *S. macrophylla)*

with a continuous flaming for more than 10s (Fig.3). As for the MAP-treated *S. macrophylla* board, smoke leakage was detected along all sides of the vertical joints between the wall surface and the tested board at 1.22 minutes. There were no significant changes until the 10th minute, where the board started to bend inside the furnace and charring occurred on the four sides of the board. The charring became worse on the right side of the board after 12 minute-exposure and caused a gap of 6mm to occur at the bottom-right side of the board. The gap increased to 25 mm at 13th minute, contributing to integrity failure (Fig. 2).

Char appeared on the untreated boards of *A. mangium* and *S. macrophylla* after 12 minutes. The untreated boards reached integrity failure when cracks in a size of more than 6 mm were formed. The cracks encouraged flaming for more than 10 seconds, and this constituted to the failure (Figure 3). The test revealed that the fire performance of the *S. macrophylla* and *A. mangium* particleboards improved when treated with ZBr and MAP. For fire resistance, ZBr showed a better efficacy than MAP, suggesting that boron-formulated fire retardant performed better than the phosphorous-based fire retardant in term of delaying insulation failure and integrity

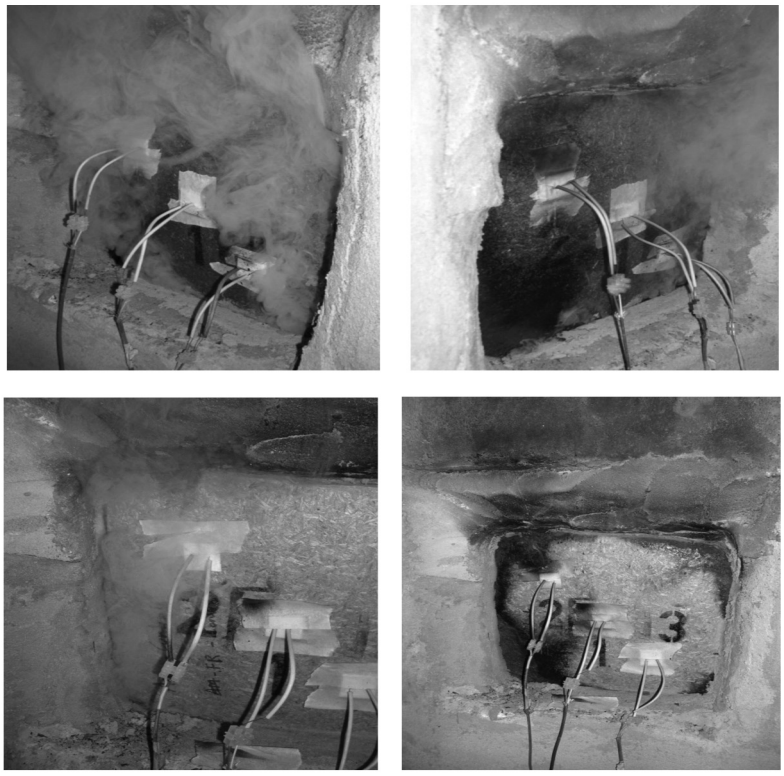

Fig.3: (From left to right) The control samples before and after the test
(Above: *A. mangium*; Below: *S.macrophylla*)

failure. The findings are in agreement with those by Izran *et al.* (2010). Boron increases the production of carbon rather than carbon monoxide or carbon dioxide. The creation of surface layer of char helps to block oxygen from the surface and cause the gases to escape much slowly (Stark *et al*., 2009). Phosphorous compounds in MAP acts similar to boron, but the boron compounds in ZBr can penetrate deep into wood particles covering their outer parts to provide a perfect protection, and thus, prolonging the time taken for the heat to transfer through the cross-section of the board (Kolowski & Wladkya, 2001). These may explain why the ZBr-treated boards have a low rate of unexposed surface temperature increase as compared to the MAP-treated boards.

Reaction-to-Fire

The results of the reaction-to-fire test are summarized in Table 2. The results indicate that the treatments are ineffective in reducing weight loss, but are effective to lengthen flaming duration. The results seem to contradict that of the previous study which revealed that boron-based and phosphorous-based fire retardants should be effective in reducing weight loss (Izran *et al.,* 2010; Abdul Rashid & Chew, 1990). Meanwhile, the treated boards experienced larger weight loss as compared to the untreated ones, except for the *A. mangium* that was treated with ZBr. The weight loss recorded for the untreated *A. mangium* and *S. macrophylla* was 0.87% and 0.41%, respectively. Meanwhile, the *Acacia mangium* and *S. macrophylla* treated with ZBr had weight losses of 0.57% and 1.04%, respectively. In comparison, compared to the untreated boards, the ZBR-treated *S. macrophylla* boards suffered larger weight loss by 157.54%. Different results were obtained for the *A. mangium* boards that were treated with the same fire

TABLE 2
Reaction-to-fire test for *A. mangium* and *S. macrophylla* particleboards

Samples	Weight (g)		Weight loss (g)	Percentage of weight loss (%)	Flaming duration (s)
	Before test	After test			
			S. macrophylla		
Untreated	439.73[7.59]	437.94[7.38]	1.79 [0.83]^{a}	0.41 [0.19]^{a}	10.33 [2.10]^{a}
ZBr-treated	443.19[3.18]	438.58[8.16]	4.61 [5.37]^{b}	1.04 [1.22]^{b}	10.17[10.07]^{b}
MAP-treated	408.88[25.98]	404.36[28.40]	4.52 [3.04]^{c}	1.13 [0.78]^{c}	20.12 [21.55]^{c}
			A. mangium		
Untreated	388.87[48.21]	385.37[46.71]	3.5 [2.01]^{d}	0.87 [0.43]^{d}	8.00 [0.49]^{d}
ZBr-treated	436.02[7.67]	433.54[9.56]	2.48 [1.9]^{e}	0.57 [0.45]^{e}	19.27 [9.5]^{e}
MAP-treated	445.68[17.36]	434.60[21.48]	11.08[7.86]^{f}	2.51 [1.81]^{f}	41.21 [8.51]^{f}

Values are means of 3 samples. The values in parentheses are standard deviation; Means within a column followed by the same alphabets are not significantly different at $p \leq 0.05$ between the species; Means within a column followed by the same numbers are not significantly different at $p \leq 0.05$ between the chemicals.

retardant, where weight loss was observed to be smaller than the untreated ones by 41.12%. This means a larger weight loss was recorded for the boards treated with MAP. The weight loss for *A. mangium* and *S. macrophylla* was larger than the untreated boards by 216.57% and 152.51%, respectively.

However, the fire retardants were found to be effective in lengthening the on-set of flaming for ignition, except for the *S. macrophylla* boards that were treated with ZBr. Meanwhile, monoammonium phosphate (MAP) was more effective than ZBr in delaying ignition. *Shorea macrophylla* and *A. mangium* boards treated with MAP each took 20 seconds and 41 seconds for ignition, whereas those treated with ZBr took 10 seconds and 19 seconds each for ignition, respectively. The comparisons show that the MAP-treated *A. mangium* boards were the most difficult to ignite. As for the control samples, *S. macrophylla* and *A. mangium* boards took 10 and 8 seconds to ignite, respectively.

The percentage loss in weight can be used as a measure of the tendency of the boards to burn once they are ignited (Abdul Rashid, 1982). Thus, it can be concluded that the MAP-treated *S. macrophylla* and *A. mangium* boards were relatively easier to burn even though they were much harder to ignite. The ineffectiveness of the fire retardants to reduce weight loss was expected due to the hygroscopicity of the fire retardants (MAP and ZBr). This is because they absorb moisture from the surrounding. MAP is water soluble and leachable (Izran *et al.,* 2009). As for ZBr, apart from its hygroscpicity, it contains boric acid in its formulation which makes it easily volatized when it is exposed to heat due to low chemical stability. Zaidon *et al.* (1995) discovered that different amounts of boric acid volatize at different temperatures. Thus, it was rather expected that the existence of moisture from the surrounding, due to the hygroscopicity and heat from the Bunsen burner, could speed up the leaching of phosphoric acid through water vapours as well as through volatization of boric acid which caused the amount of the chemicals to become slightly lower than the actual chemical loading incorporated into the particleboards. These could decrease the effectiveness of the fire retardants to protect the boards from thermal degradation caused by the fire. These also explain the reason for the greater weight losses recorded for the treated boards as compared to the untreated ones. However, ZBr is leaching resistant and for this reason, the weight losses of the boards treated with MAP were larger than those treated with ZBr. The failure of ZBr to lengthen the flaming duration of *S. macrophylla* particleboards might also be due to the mechanism explained above.

CONCLUSION

The two fire retardants undertaken in this study were effective in improving fire resistance but not the reaction-to-fire of *S. macrophylla* and *A. mangium* particleboards. In specific, zinc borate performed better than MAP in improving the insulation and integrity of the boards,

except for the *A. mangium* boards. The ZBr-treated boards were also found to be better than MAP in terms of weight loss after burning. The MAP-treated boards ignited less readily compared to those treated with ZBr. The results for the weight loss of the treated *S. Macrophylla* particleboards were inferior to the untreated particleboards. Hence, it is suggested that the research be extended, especially in determining the chemical loading of each of the treated boards, during and after they are exposed to fire. This is essential to prove the claim that the chemical loading decreases due to the leaching of phosphoric acid and the volatization of boric acid.

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PCDDs and PCDFs in Pelagic Fish along the Straits of Malacca

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ABSTRACT

Fish and shellfish are rich sources of long chain fatty acids, especially DHA and EPA. High consumption of fish helps to elevate the level of these compounds in the body. However, fish also are easily exposed to chemical contaminants, such as dioxins (PCDDs) and furans (PCDFs). Exposure to PCDDs and PCDFs may lead to negative health effects, such as cancer, chloracne, hyperpigmentation and others. Level and type of PCDDs and PCDFs were determined in 20 pelagic fish samples of six different species collected from the Straits of Malacca using HRGC/HRMS. The most toxic congener (2,3,7,8-TCDD) was found in all the samples at a very low level of 0.04-0.05 pg/g sample, except in Spanish mackerel (south-T2) and Indian mackerel (middle-T1). Meanwhile, the level of the total PCDDs and PCDFs ranged from 0.13 pg/g to 0.38 pg/g of the wet weight of the samples. The value of the total PCDDs and PCDFs was in a descending order of Hardtail scad, Spanish mackerel, Indian mackerel, fourfinger threadfin, silver pomfret and dorab wolfherring. Generally, the results of this study indicate that fish and shellfish caught along the Straits of Malacca are safe as in terms of PCDDs and PCDFs levels and the data can serve as baseline information for future monitoring of these organochlorine compounds.

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INTRODUCTION

The fisheries along Straits of Malacca contribute approximately 70% of the total marine resources of Peninsular Malaysia (Annual Fisheries Statistics, 2004). These resources are made up of two major groups, which are demersal and pelagic. Pelagic fish live in the water column of coastal, ocean and lake waters, but not at the bottom of sea or lake. They can be compared with demersal fish which live near or at the bottom of the sea (Atmadja, Sadhotomo & Suwarso, 1995). The marine pelagic environment is the largest aquatic habitat on earth and it comprises 11% of the known fish species (Atmadja *et al.,* 1995). The families of *Pampus, Megalapsis, Epinephulus, Eleutheronema, Rasterlliger, Chirocentrus* and *Scomberomorus* are some examples of pelagic fish (Lui, 1992).

The concern of dietary recommendation to achieve an adequate intake of long chain (LC) *n*-3 PUFA is growing as knowledge about its beneficial effects has become a more concerning issue among people (Isabelle *et al*., 2008). A few studies reported that a modest increase in the consumption of LC *n*-3 PUFA would have important and beneficial health outcomes (Gebauer *et al*., 2006; Wang *et al*., 2006). Increased fish and shellfish consumption is suggested as a good approach and possible strategy to increase LC PUFA intakes in order to bridge the gap between the current intakes and dietary recommendations (Isabelle *et al*., 2008). At the same time, however, fish and other shellfish are also sources of persistent chemical contaminants that accumulate in the marine environment by bioaccumulation (Giesy *et al*., 1997). As a result, this strategy comes out with some conflict issues, whereby increasing fish intake to elevate omega-3 PUFA intake will also simultaneously increase the intake of contaminants to a level of toxicological concern (Giesy *et al*., 1997).

The Straits of Malacca, which occupies along the west coastal water of Peninsular Malaysia, is one of the world's busiest oil transport routes (Lui, 1992). The marine environment of the Straits may be affected by any accidental oil spills that has occurred. For example, the incidence of oil and grease content spill found on the Perak coast in 1990 had affected the coastal areas of Negeri Sembilan, Melaka and Selangor (Environmental Quality Report, 1990). Coastal marine environment pollution is also one of the major threats to the Malaysian fisheries industry. Besides marine pollution, land-based pollution, destruction of natural habitats and incomplete combustion from factories could also be the major treats to the marine environment (Lulofa, 1977; Sasekumar, 1980). All these may lead to high abundance of persistent organic pollutants (POPs), such as polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in fish and shellfish caught along the Straits of Malacca, thus increasing exposure to human. Therefore, it is important to investigate the presence of PCDDs and PCDFs in fish and shellfish in a way to minimize any health problem consequences.

PCDDs and PCDFs are commonly known as dioxins and furans, respectively, and they are also considered as persistent, bioaccumulative and toxic environmental contaminants (Geyer *et al*., 2002). PCDDs and PCDFs compounds have been shown to accumulate in fish and wildlife (Kadokami *et al.,* 2002; Brunstrom *et al.,* 2001; Huang *et al.,* 1999; Woodford *et al.,* 1998). Therefore, they are likely to be present in oily fish. Their presence in the marine environment leads to accumulation in the food chain of fish, with levels being highest in large predatory species (Jacobs *et al.,* 1998).

More than 90% of the intakes of PCDDs and PCDFs by general population are derived from meat, dairy products, and fish (Schecter, 1997; Bocio & Domingo, 2005; Charnley & Doull, 2005; Huwe & Larsen, 2005). In Tokyo, Japan, 40% of the daily intakes of PCDDs and PCDFs are derived from fish and shellfish (Sasamoto *et al*., 2006). Meanwhile, a study in Spain showed that around 31% of PCDDs and PCDFs were found in fish and other seafood intakes (Llobet *et al*., 2003).

As PCDDs and PCDFs have now become worldwide concerns, studies on these contaminants are therefore needed to monitor fish and shellfish intakes and achieve the nutritional requirement without exceeding toxicological thresholds. The determination of the type and level of these contaminants in food is very important for dietary exposure assessment, protection of public health, and increasing availability of safe marine fish to consumers (Elliot *et al*., 1996). Therefore, the aim of the study was to determine the levels of PCDDs and PCDFs in fresh pelagic fish species available at fish landing areas along the Straits of Malacca. This included viewing the trend of these contaminants in the fresh marine products. The results obtained are useful as baseline information for the control of PCDDs and PCDFs release into the marine environment.

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade. Meanwhile, the chemicals used are such as hydromatrix (Framton Ave, Harbour City), dichloromethane (DCM) (Fisher Scientific, Fair Lawn, New Jersey) and hexane (Fisher Scientific, Leicestershire, UK). These chemicals were used for extraction, pre-treatment and clean-up of the samples before PCDDs and PCDFs determination using high-resolution gas chromatography/ high-resolution mass spectrometry (HRGC/ HRMS).

Fish Samples and Preparation

The stratified sampling method was used in the collection of the samples. Fresh samples consisted of 20 available fish samples from six different species which had been collected from three regions along the Straits of Malacca: North (Kuala Perlis, Kuala Kedah, Teluk Bahang, Pulau Betong), Middle (Melaka, Port Dickson, Muar) and South (Kuala Selangor, Manjung Utara, Matang). All the samples were collected at two different times in August,

2008 (T1) and November, 2008 (T2). The collection of samples was carried out with the help of some officers from Lembaga Kemajuan Ikan Malaysia (LKIM). The pelagic fish consisted of the following species: *Rastrelliger kanagurta* (Indian mackerel), *Scomberomorus guttatus* (Spanish mackarel), *Pampus argenteus* (silver pomfret), *Megalapsis cordyla* (Hardtail scad), *Eleutheronema tradactylum* (Fourfinger threadfin), and *Chirocentrus dorab* (Dorab wolfherring).

The collected samples were brought to the laboratory on the same day. The samples were delivered to the laboratory in a sealed polystyrene box and stored in a freezer (-20°C) at Dietetic laboratory, UPM. For the analysis, the muscle tissues of the fish were taken and weighed in gram (g). The composite sample of the same species from the same region was prepared after the samples had been gutted, washed and filleted. The prepared samples were transferred into a polyester covered cup and stored in a freezer (-20°C) before further analyses. The samples were sent to Doping Control Centre (DCC), Penang, in a cold box to prevent from any damage. The determination of PCDDs and PCDFs in fish lipid samples was carried out at DCC, USM Penang, which is one of the Accredited Laboratories for doping control analyses and recognized by World Anti-Doping Agency, (WADA).

Analysis of the PCDDs and PCDFs Concentrations in the Samples

The procedure utilized for the determination of dioxins and furans was adopted based on the principle laid by US EPA method 8290 for PCDDs and PCDFs by HRGC/HRMS. The method was slightly modified by DCC which provides procedures for the detection and quantitative measurements of PCDDs and PCDFs in variety matrices at part per trillion (ppt) concentrations. The modified procedure uses matrix specific extraction, analyte, specific clean-up and HRGC/HRMS analysis techniques.

Before extraction, the samples were thawed at room temperature and this was followed by spiking 10 g of wet sample of fish into 50 µl C_{13}-labelled internal standard (8999) (Cambridge Isotope Laboratories Inc., USA). Later, 10 g of hydromatrix was mixed into the sample before homogenization with a mortar. The homogenized sample was then dried in an oven (Petaling Jaya, Selangor, Malaysia) at 50°C for two minutes to hydrate the moisture content until powder was formed. The powder was placed in a cell (size 33) that was closed with Ottawa sand (Fisher Scientific, Leicestershire, UK) before entering into Accelerated Solvent Extraction [ASE 200) (DIONEX Corporation U.S Patents, Sunnyvale, USA)] machine for 20 minutes for fat extraction. The fat was extracted using DCM solvent. The mixture of the extracted fat and DCM was dried using a rotary evaporator (BUCHI Labortechnik, Flawil, Switzerland) for 20 minutes and filtered to get crude fat extract. The fat content was determined gravimetrically, and later, hexane was mixed into the extract to form an aliquot.

The aliquot was placed in a fully automated device, Power-Prep Fluid

Management System (FMS) (Fluid Management System, Inc. Waltham, USA) for a clean-up process that involved three types of column (Fluid Management System, Inc. Waltham, USA), silica (CLDS-ABN-STD), alumina (CLDA-BAS-011), and carbon (CLDC-CCE-034). The clean-up process involved 23 steps to remove the bulk matrix components and enrichment of the target analysis. The involvement of the three column chromatographic clean-up procedure was derived from Smith Stalling method outlined in the US EPA Method 8290. After completing the FMS procedure, the collected PCDDs and PCDFs (mixed with solvent) were dried using a rotary evaporator before spiking them with 50 µl external standard (5999). Later, the solvent was dried using heating block (Dri Block DB-20, Staffordshire, OSA, UK) and nitrogen gas (TESCOM Corporation, ELK River, USA) at 60°C. The collected crude PCDDs and PCDFs mixture was placed in a small covered aluminium foil vial before the analysis.

HRGC/HRMS (GC: Thermo scientific, Trace GC Ultra Rodano, Milan, Italy; MS: Hannah-Kunathstr, Bremen, Germany) were used for the analysis of PCDDs and PCDFs. Each analysis included the determination of seventeen dioxin and furan congeners with 2, 3, 7, 8-chloro-substitution (ten PCDFs and seven PCDDs). Meanwhile, calibration standard was used to construct the calibration curve using the QUAN programme in the XCalibur software (Thermo scientific, Milan, Italy). EDF-4141 window-defining standard (Thermo scientific, Milan, Italy) was used to ensure the first and the last eluting analytes in each sample. Concentrations in the fish samples were calculated on a wet weight (w.w.) basis. Recoveries for internal standards were more than 50% for all the congeners.

Toxicity equivalency (TEQ) was calculated using the procedures developed by World Health Organizational (2005). In this study, PCDDs and PCDFs toxicity were expressed as Toxicity Equivalents (i.e., total toxicity) of the seventeen 2, 3, 7, 8-substituted PCDDs and PCDFs congeners. TEQ was calculated by multiplying the absolute concentration of each congener by a numeric factor that expresses the concentration in terms of the most toxic dioxin molecule, 2, 3, 7, 8-TCDD (tetrachlorodibenzodioxin), which is given a value of one. In cases where congeners were reported as non-detects, limit of quantification (LOQ) would be used as result.

RESULTS AND DISCUSSION

This study was undertaken to investigate the levels of PCDDs and PCDFs in selected pelagic fish. Table 1 shows the fat content (%) and the level of PCDDs/PCDFs (WHO I-TEQ) from the analyses of the fish fillet for each sample. The Indian mackerel species from the middle region the and Spanish mackerel from the south region contained high percentages of fat content, with 5% and 4.35%, respectively. The lowest percentage of fat content was observed in the hardtail scad and Indian mackerel from the north region at 0.8% and 2.05%, respectively.

TABLE 1
WHO I-TEQ (pg/g), fat content (%) and percentage of variation of PCDDs and PCDFs in pelagic fish by region along Straits of Malacca

Region	Species	Common name	Fat content (%)		Mean	Percentage of variation (%)	WHO I-TEQ (pg/g)		Mean	Percentage of variation (%)
			T1	T2			T1	T2		
North	*Pampus argentus*	Silver pomfret	3.6	3.7	3.65	1.94	0.19	0.12	0.16	30.94
	Megalapsis cordyla	Hardtail scad	0.6	1.0	0.80	35.36	0.17	0.16	0.13	5.44
	Eleutheronema tradactylum	Fourfinger threadfin	2.0	2.7	2.35	21.06	0.17	0.13	0.15	18.85
	Rastrelliger kanagurta	Indian mackarel	1.0	3.1	2.05	72.44	0.21	0.73	0.14	262.64
Middle	*Megalapsis cordyla*	Hardtail scad	2.8	3.5	3.15	15.71	0.34	0.37	0.21	10.10
	Eleutheronema tradactylum	Fourfinger threadfin	1.8	3.3	2.55	41.59	0.13	0.12	0.17	4.16
	Rastrelliger kanagurta	Indian mackarel	3.3	6.7	5.00	48.08	0.12	0.14	0.18	7.86
South	*Megalapsis cordyla*	Hardtail scad	0.4	3.1	1.75	109.09	0.90	1.57	0.38	124.67
	Chirocentrus dorab	Dorab wolfhering	3.8	3.5	3.65	5.81	0.12	0.14	0.16	8.84
	Scomberomorus guttatus	Spanish mackarel	3.2	5.5	4.35	37.79	0.25	0.30	0.30	11.79

(T1 = trip 1; T2 = trip 2)

On the contrary, high fat contents were found in the species from the middle and south regions compared to the north region samples of the Straits of Malacca. Based on the results obtained, the percentage of fat by trip was higher in the samples taken from trip 1 (T1) as compared to the sample from trip 2 (T2), except for dorab wolfherring species (T1: 3.8%; T2: 3.5%). The differences in the fat content between T1 and T2 could be related to the differences in the maturity of the fish caught as the analysed samples were those available at the point(s) of collection. Previously, Osman *et al*. (2001) reported lower fat contents of Spanish mackerel (1.46%), fourfinger threadfin (2.24%), hardtail scad (3.08%) and silver pomfret (2.91%) species compared to the data of the present study. The higher level of fat in the samples of this study was found to be related to the different extraction methods used, in which ASE operated at higher temperature (125°C) and pressure (1500 psi), and thus allowed an optimum extraction of fat and provided good recovery and precision for determination of organochlorine compounds, as shown in Table 1 (Ezzell *et al*., 1996).

In this study, the levels of PCDDs and PCDFs were reported as WHO I-TEQ in pg/g wet weight of sample. The hardtail scad and Spanish mackerel species taken from the south region showed the highest values of the total PCDDs/PCDFs at 0.38 pg/g and 0.30 pg/g, respectively. Conversely, both hardtail scad and Indian mackerel species from the north region showed the lowest values of PCDDs/PCDFs at 0.13 pg/g and 0.14 pg/g, respectively. Generally, the levels of PCDDs and PCDFs were higher in the samples from the south region compared to the ones taken from the middle and north regions. Besides, the levels of PCDDs and PCDFs were also higher in T2 as compared to T1, especially for the sample from the middle and south regions. It has been well established that biotic and abiotic factors affect the degree and exposure of chlorinated compounds in fish including the proximity of fish to contaminated sediment, magnitude of contamination in their habitats, fish movement patterns, the tropic status, growth rates, fish age and bioavailability of the contaminants (Stow *et al*., 1994; Bentzen *et al*., 1996). Besides, species' specific metabolism and detoxification of contaminants, reproductive and maturational, as well as the level of body fat can affect the accumulation of contaminant in fish tissues (Bentzen *et al*., 1996; Larsson *et al*. 1996). Therefore, some of the above mentioned factors may explain the differences observed.

The findings of this study showed that the PCDDs and PCDFs levels in pelagic fish were not influenced by the fat content of the samples that much since the fat content in the fish sample was not directly proportional to the levels of PCDDs and PCDFs. The concentration of lipid found in the fish samples ranged from 0.4% to 6.7%. A wide variation (1.94% - 109.09%) in terms of the fat content exist between the samples collected in T1 and T2 was due to the differences in the fish's maturity and sizes as the samples were those available

during the sampling time. According to Jacobs *et al.* (2002), younger fish consume lower levels of feed and thus tend to store lower lipid in their adipose tissue compared to mature fish. This situation may reflect the lipid content of the samples (Jacobs *et al*., 2002). Besides the differences in maturity, the variation in the fat contents could also be due to certain individual fish that utilize high-energy diets but deposit little lipid in their flesh, but tend to have greater adiposity in some others (Bell, 1998).

In an Italian study, the reported mean PCDDs/PCDFs in mackerel species was relatively low at 0.22 pg/g WHO I-TEQ (Taioli *et al*., 2005). Meanwhile, in the samples from Japan, Sasamoto *et al*. (2006) found that the total concentration of PCDD/PCDFs and dioxin-like PCBs in fish and shellfish ranged from 0.98 to 0.91 pg/g WHO I-TEQ between 1999 and 2004, respectively. Moreover, the concentrations of PCDD/PCDFs in 40 species of marine organisms from the Korean coastal waters were shown to vary from 0.02 to 4.39 pg/g WHO I-TEQ (Moon & Ok, 2006). A study done by Li *et al*. (2007) in China found that aquatic foods which had been obtained from a local market contained high concentrations of PCDDs and PCDFs at 0.18 pg/g WHO I-TEQ, compared to the other food groups. The findings from various studies revealed that the levels of PCDDs and PCDFs were relatively low, i.e. below the permitted level (< 4pg/g) underlined by World Health Organization (WHO, 2005). The data obtained in the present study also showed low levels of PCDDs and PCDFs in the pelagic fish samples along the Straits of Malacca, ranging from 0.13 to 0.38 pg/g WHO I-TEQ.

There are many fish landing areas along the Straits of Malacca, and they serve as fish and shellfish collection sites (Annual Fisheries Statistics, 2004). Based on the brief observation during the sample collection, there were quite a number of industrial spots near the collection sites. Human activities, such as land development, agriculture and high population density, have been well established as the main causes of marine water pollution (UNEP/GPA, 2006). Previously, Choo *et al*. (1994) reported that the activities of agro-based and pesticide industries along the West Cost of Peninsular Malaysia might have important contributions to PCDDs/PCDFs in the marine environment. The existence of PCDDs and PCDFs in the fish samples also could be due to the strategic location of the strait as a major international shipping lane (Annual Fisheries Statistic, 2004; Choo *et al*., 1994). In fact, the growth of agriculture and industrial sectors, as well as urbanization which predominates the west coast of Peninsular Malaysia, is among the sources of these persistent organic pollutants (Annual Fisheries Statistic, 2004). Additionally, the north, middle and south regions of the Malacca Straits have different industrialization stages and types (Chua *et al*., 1989), and these may have contributed to the release of PCDDs/PCDFs into the marine environment.

The by-products formation resulted from industrial processes such as

smelting, bleaching and processing of paper pulp (Abbot & Hinton, 1996), manufacturing of some herbicides or pesticides, polychlorinated biphenyls (PCBs), pentachlorophenol, and polyvinyl chlorides (PVCs), burning of plastics and toxic waste at high temperatures with waste incinerators or kilns, as well as motor vehicle exhaust, charcoal grills and cigarette smoke are the major sources of PCDDs and PCDFs (Harte *et al*., 1991; Hoffman *et al.,* 1995; Schettler *et al*., 1999; Im *et al*., 2002). The industry which operates within the nearest coastal vicinity is probably one of the factors contributing to the increasing level of persistent organic pollutants in the marine ecosystem which simultaneously increase the levels of dioxins and furans in pelagic fish.

Fig.1, Fig.2 and Fig.3 depict the congeners of PCDDs/PCDFs in three species, with the highest PCDDs/PCDFs in T1 and T2. According to Gene *et al*. (2008), the congeners that contributed to the highest toxicity were 1,2,3,7,8-PeCDD, 2,3,7,8-TCDD, 1,2,3,4,7,8-HxCDD and 2,3,4,7,8-PeCDF. In all the studied samples, 1,2,3,7,8-PeCDD congener was present at the highest concentration compared to other congeners. Seventy-eight percent of the total toxicity was derived from the four congeners averaged toxicities (Schecter *et al.,* 1994). The most toxic congener of PCDDs and PCDFs was 2,3,7,8-TCDD, and it was classified as a Group 1 carcinogen (i.e. a known human carcinogen) by WHO's International Agency for Research on Cancer in 1997 (WHO, 1999). This particular congener was found in all the samples except in Spanish mackerel (south-T2) and Indian mackerel (middle-T1). The concentration of this congener, however, was very low, i.e. at 0.04 – 0.05 pg/g sample. The highest 2,3,7,8-TCDD congener was found in Indian mackerel (middle-T2) at 0.05 pg/g sample.

A summary of the total PCDDs and PCDFs contents is shown in Fig.4, whereby the different levels of these contaminants are clearly found in the different species. Overall, hardtail scad exhibited the highest levels of the total PCDDs and PCDFs contaminations as compared to the other species. Other species of pelagic fish

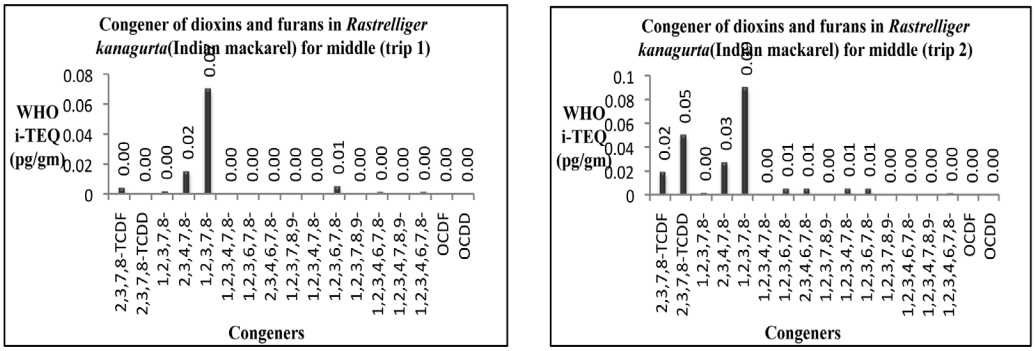

Fig.1: Dioxin and furan congeners concentration in *Rastrelliger kanagurta* (Indian mackerel)

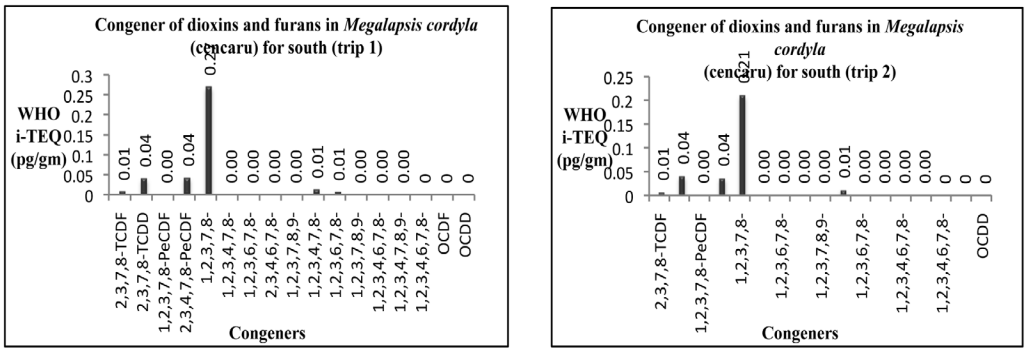

Fig.2: Dioxin and furan congeners concentration in *Megalapsis cordyla* (hardtail scad)

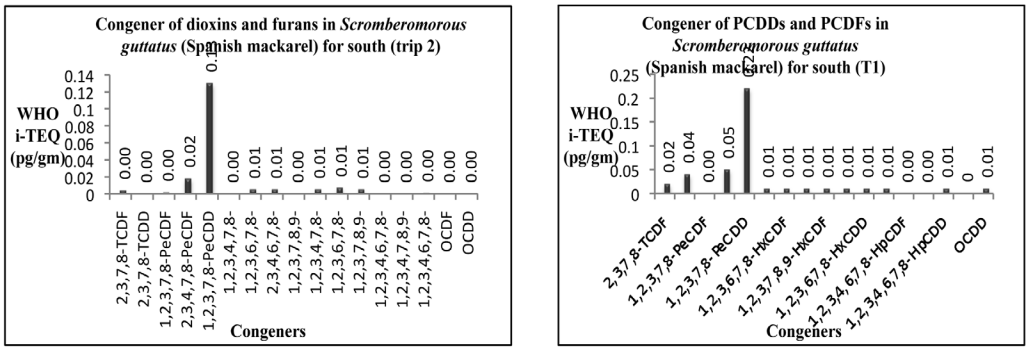

Fig.3: Dioxin and furan congeners concentration in *Scromberomorous guttatus* (Spanish Mackarel)

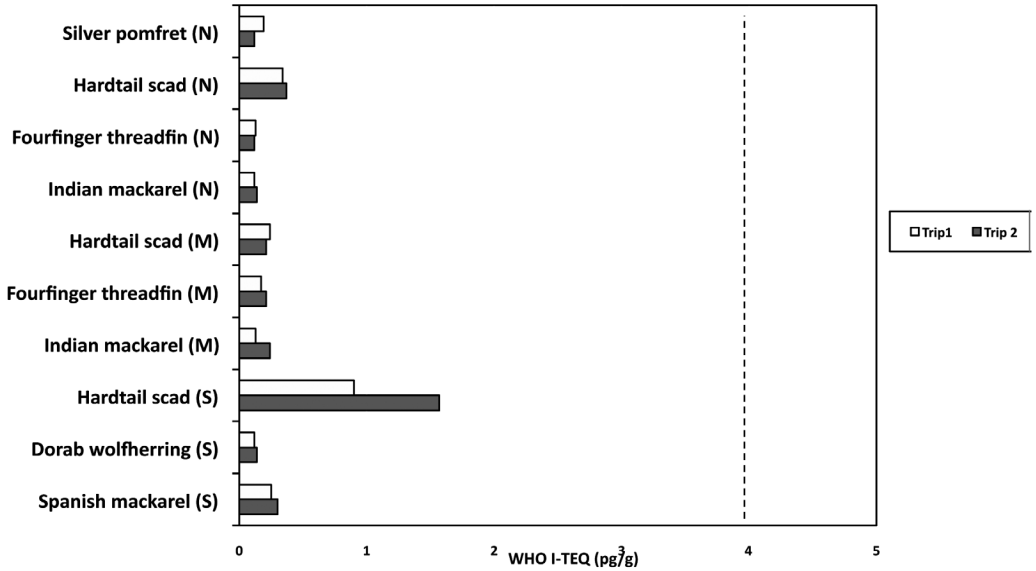

Fig.4: Total WHO i-TEQ of PCDDS and PCDFs distribution in selected pelagic fish by region and trip

had the total PCDDs/PCDFs of less than 0.5 pg/g WHO I-TEQ. Generally, the level of PCDDs/PCDFs in all the samples determined in this study was well below the permitted level of 4 pg/g as prescribed by WHO (2007) for total PCDDs/PCDFs. Although the data are limited, the findings of this study can serve as baseline information to increase public awareness of the PCDDs and PCDFs compounds in the fish samples from the Straits of Malacca.

CONCLUSION

This study has demonstrated the levels of PCDDs and PCDFs in the pelagic fish from Straits of Malacca. In particular, the levels of PCDDs and PCDFs ranged from 0.13 pg/g to 0.38 pg/g of the wet weight of the samples. The values of the total PCDDs and PCDFs were in a descending order of *Megalapsis cordyla* (hardtail scad), *Scomberomorus* (Spanish mackarel), *Rastrelliger kanagurta* (Indian mackarel), *Eleutheronema tradactylum* (fourfinger threadfin), *Pampus argentus* (silver pomfret) and *Chirocentrus dorab* (dorab wolfherring). In general, the detected levels of both PCDDs and PCDF in the studied samples were low compared to the international permitted level, and thus indicated the safety of fish species caught along the Straits of Malacca. These data are important as these serve as baseline information for future studies. However, continuous monitoring of these contaminants is strongly recommended to ensure fish safety.

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Physical and Mechanical Properties of Portland Cement-Bonded Flakeboards Fabricated from *Macaranga gigantea* and *Neolamarckia cadamba*

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ABSTRACT

This study evaluated the properties of cement-bonded flakeboards made from wood of pioneer forest species, namely, Kelempayan (*Neolamarckia cadamba*) and Mahang (*Macaranga gigantea*). The wood species were first evaluated for their compatibilities with cement by looking at their effects on the hydration rate of the cement. The properties of the flakeboards were tested using ASTM D 1037-99. An analysis of variance was carried out to study the effects of accelerator types and concentrations and flake lengths on the boards. There was no significant interaction between the wood species, but there was a significant relationship between the differences of accelerator types and concentrations and flake length at $p<0.05$. Generally, boards treated and fabricated with higher concentration of accelerator and longer flakes had superior performance. The mechanical properties [internal bond (IB), modulus of rupture (MOR), screw withdrawal (SWD) and modulus of elasticity (MOE)] of the boards were significantly influenced by the length of the flake and accelerator concentration — the longer the flake, the higher the accelerator concentration, the better the strength would be. For Kelempayan, the greatest values of MOR, IB, SWD and MOE which were influenced by flake length were 100mm Kelempayan at 1.5% $MgCl_2$ (9.5 MPa), 100 mm Kelempayan at 1.5% $MgCl_2$ (0.37 MPa), 100 mm Kelempayan at 2.5% $MgCl_2$ (519.4 MPa) and 100mm Kelempayan at 1.5% $MgCl_2$ (3329 MPa), respectively. As for the influence of accelerator concentration, the greatest mechanical values were observed from 75 mm Kelempayan at 2.5% $CaCl_2$ (7.47 MPa), 75 mm Mahang at 2.5% $MgCl_2$ (0.38 MPa), 75 mm Mahang at 2.5% $CaCl_2$ (425.51 MPa) and 75 mm Kelempayan

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at 2.5% $CaCl_2$ (3001 MPa), respectively. However, reverse results were recorded for the physical properties of Kelempayan flakeboards added with magnesium chloride ($MgCl_2$).

Keywords: Accelerator type and concentration, pioneer species, flake length, strength

INTRODUCTION

Deforestation has become a big issue for many countries throughout the world. In Malaysia, since the British colonial rule in 1956, the Malaysian economy has involved the expansion of commodity and production. This expansion led to exploitation of natural resources which had divided the economy into two parts, namely, the primary sectors (agriculture, forestry, mining, and fishing) and secondary sectors (manufacturing and construction). The economic growth since that time has caused the demand of the secondary sector higher than the supply of the primary sector (Coutts, 2007). Thus, to meet the demand of the secondary sector, forests were exploited excessively and this led to deforestation. Deforestation rate increases year by year and has never stopped until today, as claimed by Butler (2006). To prevent further deforestation that might have caused many other bigger problems, such as erosion and global warming, government called the wood-based product manufacturers to reduce the usage of wood from the forests and to switch to crops which could be utilized as alternative raw materials in producing wood-based products. It was found that kenaf (Izran *et al.,* 2009), sesenduk (Khairul *et al.,* 2009), engkabang and *Acacia mangium* (Izran *et al.,* 2010a; Mohd Hamami *et al.,* 1998) and coconut (Khairul *et al.,* 2009a) are suitable to be further promoted and industrialized in order to create a sufficient material supply for the wood-based product manufacturers. Pioneer species can also be highlighted to form wider alternative material selection for the manufacturers. Pioneer species is a species that is first to establish itself in an area which was previously devastated by flood, plowing and fire (Anon, 2011). Two promising pioneer species that can be commercialized due to their fast-growing ability are mahang (*Macaranga spp.*) and kelempayan (*Neolamarkia cadamba*). However, information regarding these species is rather limited, and hence, creates obstacles in promoting and utilizing them efficiently.

Cement-bonded boards are readily available and accepted in Europe, United States, Russia and Asia, mainly for roofs, walls and floors. There is a number of researcher which previously carried out on the strength of boards with different types of raw materials and particle geometries (Del Menezzi *et al.,* 2007; Mohamad Hamami *et al.,*1998; Guntekin & Sahin, 2009). These researchers have proven that the boards possess better advantages compared with ones which are produced from organic resins in terms of strength and durability. Therefore, this study attempted to use mahang and kelempayan as raw materials for cement-bonded flakeboards. The effects of several parameters such as accelerators and

flake length on the mechanical and physical properties of the flakeboards were also studied. Accelerators were incorporated into the flakeboards to enhance the compatibility between the flakes and cement.

MATERIALS AND METHODS

Preparations of the Mahang and Kelempayan Flakes

Mahang gajah (*Macaranga gigantea*) and Kelempayan (*Neolamarckia cadamba*), which were extracted from Gua Musang Forest District, Kelantan, were used for this purpose. The binder used was Ordinary Portland Cement (OPC). Through a hydration test, these two species have been found to be better able to trigger the hydration rate of the binder than the other selected pioneer forest species, namely, Memeh, Langian, Terap and Melembu (Noor Azrieda *et al.,* 2010). The hydration rate was evaluated through temperature-time relationship. The most compatible species to be mixed with Ordinary Portland Cement (OPC) should achieve the highest temperature within a short period of time. It is because the higher the temperature, the faster the hardening of the cement is (Barron, 2010).

Firstly, the timbers were cut into billets in a length of 18". Then, they were debarked and shredded to flakes using a shredding machine which is available at Duralite (M) Sdn. Bhd. The initial moisture content of the flakes was 70%. The flakes were soaked in water to remove wood extractives which might affect the cement setting. The description of the boards is listed in Table 1. According to the calculation made, 1.08 kg of flakes, 2.16 g of cement and 0.814 kg of water were required to form a board with a dimension of 450 (l) x 450 (w) x 12 (t), as depicted in Table 1.

TABLE 1
Description of the fabricated cement-bonded flakeboards

Raw material	Mahang gajah and kelempayan
Flake length	75 mm and 100 mm
Targeted board density	750 kgm^{-3}
Board size	(450 × 450 × 12) mm^3
Binder	Ordinary Portland Cement (OPC)
Accelerator types and concentrations:	Calcium chloride ($CaCl_2$) and Magnesium chloride ($MgCl_2$) at 1.5 and 2.5% concentrations besed on cement weight
Material ratio (Cement:Wood:Water	2:1:1

Target density: 800kgm^{-3}

Manufacture of cement-bonded flakeboards

The flakes of those species were oven-dried to reduce the MC to 12 ± 2%, and also hit using a hammermill. After that, the flakes were screened, and only those in the length of 75 mm and 100 mm were selected. They were then blended separately with the cement during board fabrication process. For Mahang, only flakes with 75 mm length were chosen, but for Kelempayan, both the lengths were used. Prior to blending, the flakes were soaked in water for 24 h. Calcium chloride ($CaCl_2$) and magnesium

chloride ($MgCl_2$) were used as accelerators. The binder was mixed separately with two different accelerators at two different concentrations, namely, 1.5 and 2.5% (w/w), to enhance the compatibility between the flakes and the binder. The concentrations were selected based on the hydration test of the OPC mixed with the accelerators and wood flakes (Noor Azrieda *et al.,* 2010). The test revealed that the accelerator concentrations greater than 2.5% gave no effects to the hydration of the OPC. Therefore, to cut cost, the accelerator concentrations from 1.5% to 2.5% were chosen.

The flakes and the binder were mixed in a blender for 20 min and the mixture was put into a former made from metal in a 450 x 450 mm size. The top and the bottom of the mat were covered with caul plates and pressed at 413685.43 Pa pressure to 12 mm thickness. Then, the mat which was placed between the caul plates was clamped and left for 24 h in a conditioning chamber at a temperature of 60±2ºC and a relative humidity of 65±2% to let the flakeboards hardened. The hardened flakeboards were left clamped to cure for 28 days before they were trimmed to standard sizes for the physical and mechanical tests (Table 2) in accordance with ASTM D 1037-99 (ASTM D-1037, 1999). The clamps were removed from the cured flakeboards prior to the trimming. Hence, a total of 72 flakeboards were utilized for the tests. The experimental design is presented in Fig.1.

TABLE 2
Standard sample sizes for the physical and mechanical tests in accordance with ASTM D 1037-99 with 12mm thickness

Type of Testing	Standard size
Bending strength	300 x 75 mm
Internal bond	50 x 50 mm
Screw withdrawal	75 x 75 mm
Thickness swelling	50 x 50 mm
Density	300 x 75 mm

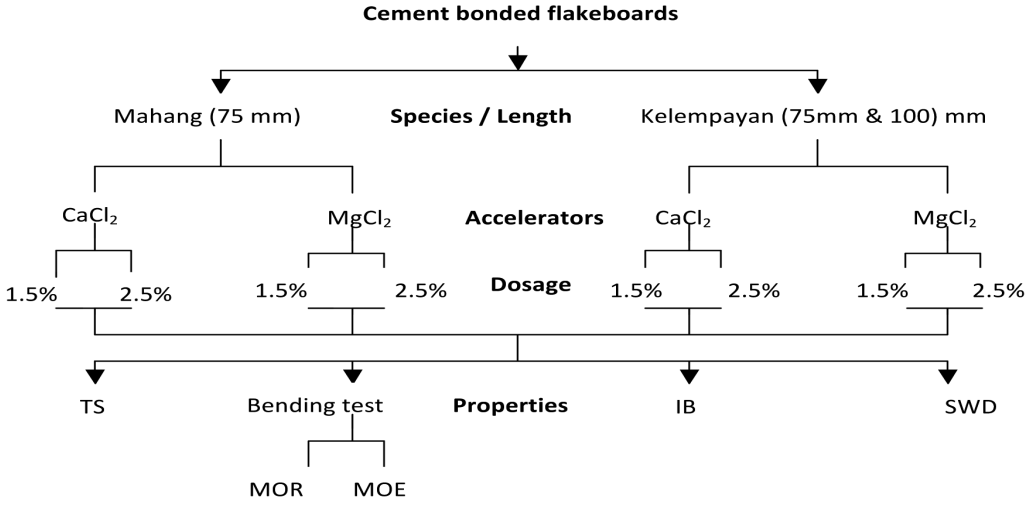

Fig.1: The Experimental Design

Statistical Analysis

An analysis of variance (ANOVA) and mean separation using the least significant difference (LSD) method were carried out to evaluate the effects of flake length, accelerator type and concentration on the strength and dimensional stability of the boards. The ANOVA is available in Statistical Package for the Social Sciences (SPSS).

RESULTS AND DISCUSSION

Performances of the Cement-bonded Flakeboards

The density obtained from the fabricated flakeboards range from 800kg/m^3 to 850kg/m^3. By comparing the data presented in Table 3, it has been determined that accelerator type and concentration are significantly influencing the performances of the flakeboards. Although the influence of flake length was not significant, it was found that the performances increased in line with the increase of flake length. The influence of flake length was only observed for kelempayan. Flakeboards made from 75 mm kelempayan which were added with 1.5% $CaCl_2$ had the mean values for MOR, MOE, IB with 6.9, 2761 and 0.24 MPa, respectively. The SWD mean value was 367.5 N. Higher values were observed for 75 mm kelempayan added with 2.5% $CaCl_2$, whereby the mean values were 7.47, 3001, and 0.33 MPa for MOR, MOE, IB and 367.6 N for SWD. A similar pattern was recorded for 75 mm kelempayan treated with $MgCl_2$. The values increased with the increase of the accelerator concentration from 1.5% to 2.5%, but the values were lower than that of which was added with $CaCl_2$. The values of MOR, MOE and IB for 75 mm kelempayan added with 1.5% $MgCl_2$ were 6.78, 2752 and 0.19 MPa, whereas SWD was 339.1 N. Slightly higher values were found for those added with 2.5% $MgCl_2$. The values were 6.83, 2906, 0.26 MPa and 377.2. For instance, kelempayan flakeboards improved its MOR between 2.86 to 22.03% and MOE between 5.06 to 17.33% when 100 mm flakes were used. On the contrary, the flakeboards added with $CaCl_2$ (100 mm kelempayan) experienced reduction on SWD for both the concentrations (-12.38% for 1.5% $CaCl_2$ and -3.23% for 2.5% $CaCl_2$) as compared with those 75 mm kelempayan flakeboards. However, the values increased with the additions of $MgCl_2$ (18.84% for 1.5% $MgCl_2$ and 27.38% for 2.5% $MgCl_2$).

The use of the accelerators did have significant effects on the boards' MOR, IB and SWD (Table 3), which were made from 75 mm mahang flakes. The accelerators have insignificant effects on MOE, even though there were some increments recorded (2616 MPa for 1.5% $CaCl_2$, 2669 MPa for 2.5% $CaCl_2$) and (2248 MPa for 1.5% $MgCl_2$ and 2684 MPa for 2.5% $MgCl_2$). The results of the mahang flakeboards indicated that higher performances were achieved with higher accelerator concentration. By comparing 75 mm mahang flakeboards and 75 mm kelempayan flakeboards, the 75 mm kelempayan flakeboards were apparently superior to the 75 mm mahang flakeboards. However, SWD of the mahang flakeboards was better than that of kelempayan flakeboards.

TABLE 3
The physical and mechanical values of mahang and kelempayan

	Kelempayan				Mahang			
	$CaCl_2$		$MgCl_2$		$CaCl_2$		$MgCl_2$	
	1.5%	2.5%	1.5%	2.5%	1.5%	2.5%	1.5%	2.5%
	75 mm				75 mm			
	Mean±S.D				Mean ± S.D			
MOR	6.90±0.53[a]	7.47±0.39[b]	6.78±0.35[c]	6.83±0.36[d]	6.31±0.52[a]	6.52±0.24[a]	5.31±0.23[b]	6.84±0.29[b]
MOE	2761±91.16[a]	3001±76.39[b]	2752±60.01[c]	2906±96.49[d]	2616±71.60[a]	2669±61.48[a]	2248±84.34[a]	2684±100.36[a]
IB	0.24±0.02[a]	0.33±0.02[b]	0.19±0.01[c]	0.26±0.01[d]	0.36±0.01[a]	0.37±0.01[a]	0.32±0.01[b]	0.38±0.01[b]
SWD	367.5±17.12[a]	367.6±14.08[b]	339.1±19.01[c]	377.2±28.42[d]	374.73±14.03[a]	425.51±18.51[a]	348.96±16.78[b]	404.62±15.58[b]
TS	1.84±0.16[a]	1.95±0.69[b]	2.14±0.08[c]	2.41±0.09[d]	1.91±0.09[a]	1.79±0.07[b]	2.09±0.11[c]	1.91±0.08[d]
	100 mm							
MOR	7.4±0.59[a]	7.69±0.74[b]	9.5±0.75[c]	8.76±0.44[d]				
MOE	3041±138.1[a]	3161±138[b]	3329±262.6[c]	3168±162[d]				
IB	0.21±0.14 [a]	0.24±0.01[b]	0.37±0.03[c]	0.30±0.02[d]				
SWD	322±22.49 [a]	355.7±30.92[b]	417.8±16.35[c]	519.4±14.37[d]				
TS	2.11±1.96 [a]	1.98±0.15[b]	1.67±0.09[c]	1.81±0.16[d]				

[1]Means within a row followed by the same alphabets under each species are not significantly different at $p \leq 0.05$, [2]MOR=modulus of rupture (MPa), MOE=modulus of elasticity (MPa), IB=internal bond (MPa), SWD=screw withdrawal (N), TS= thickness swelling.

According to MS 934, the standard requirement values for MOR, MOE, and IB are 9MPa, 3000 MPa and 0.5 MPa, respectively. As shown in Table 3, the flakeboards made from 100mm kelempayan that surpassed the standard value of MOR were only those added with 1.5 % $MgCl_2$. Meanwhile, none of the flakeboards made from 75mm flakes for both the species recorded to have higher MOR mean values than the standard. Amazingly for MOE, the mean stiffness values of all the flakeboards made from 100mm kelempayan were found to be greater than the standard value. The results are in line with the findings of a previous study (Badejo, 1988), whereby the longer and the thinner the flakes, the stronger, the stiffer and more dimensional stability the cement-bonded particleboards would become. A similar MOE performance was exhibited by the flakeboards fabricated from 75mm kelempayan mixed with 2.5 $CaCl_2$. Unfortunately, the IB value for the flakeboard of each species, flake length, accelerator type and concentration obviously failed to meet the standard requirement. It was quite surprising as the IB values were even lower than fibre-reinforced particleboards formed with polymer resins (Izran *et al*., 2009b; Paridah *et al.,* 2009). Perhaps, this is due to the properties of OPC, which is high viscosity and less watery compared to polymer resins. This was expected to limit the spread of the OPC on the surfaces of the flakes, and hence affected interfacial bonding between flakes. The relationship of IB with thickness swelling is discussed further in the next section.

Thickness Swelling

Thickness swelling is to measure the dimensional stability of the flakeboards. Lower thickness swelling value indicates a more stable board. As stated in the MS 934 standard, the standard mean TS value should be less than 2%. The Kelempayan flakeboards that had met the standard requirement were those made from 75mm kelempayan added with 1.5% $CaCl_2$ (1.84%) and 2.5 $CaCl_2$ (1.95%), 100mm kelempayan added with 2.5% $CaCl_2$ (1.98%), 1.5% $MgCl_2$ (1.67%) and 2.5% $MgCl_2$ (1.81%). Meanwhile, the Mahang flakeboards showed impressive TS results because none of the flakeboards had TS value more than 2%, except for those added with 1.5% $MgCl_2$.

Previous research has found that thickness swelling has a direct relationship with internal bond. In particular, a panel with higher IB values can resist the stress due to wood expansion and press opening, resulting in lower TS (Del menezzi *et al.,* 2007). The results derived for the flakeboards made from 75 mm and 100 mm flakes of Kelempayan contradict with the findings of Del Menezzi *et al.* (2007). The increase of the IB values for each concentration and type of the accelerator did not reduce the TS for the 75 mm and 100 mm Kelempayan flakeboards. However, the findings are applied for the mahang flakeboards (0.36MPa IB with 1.91% TS for 1.5% $CaCl_2$; 0.37MPa IB with 1.79% TS for 2.5% $CaCl_2$) and (0.32MPa IB with 2.09% TS for 1.5% $MgCl_2$; 0.38MPa IB with 1.91% TS for 2.5% $MgCl_2$). Such improvement was expected due to the enhancement in

the mahang flakes themselves as a result of the cross-linking with Ordinary Portland Cement (OPC). Mahang was found as the most compatible species to be mixed with OPC (Noor Azrieda *et al*., 2010). This was expected to enhance the bond between the mahang flakes and the binder, and thus reduced the effect of moisture to the dimensional stability of flakeboards made from that particular species. In the analysis by Noor Azrieda *et al*. (2010), kelempayan was found to be less compatible with OPC due to its high sugar and starch contents. This may explain the inefficiency of kelempayan flakeboards in reducing thickness swelling.

CONCLUSION

Kelempayan and Mahang were found as suitable materials for producing cement-bonded flakeboards. Accelerator concentration and type were two of the most important parameters for flakeboards fabrication, as they improved the cement setting, and increased the strength of the flakeboards. A higher concentration of accelerators exhibited better strength performance. Conversely, dimensional stability was reduced when the concentrations were increased especially for Kelempayan added with 2.5% $MgCl_2$. The length of flakes apparently improved the strength and dimensional stability of those added with $CaCl_2$, but contradicting results were obtained for the dimensional stability of those treated with $MgCl_2$. Overall, 100 mm long flakes produced stronger flakeboards as compared to those made of 75 mm long flakes. Thus, it is suggested that the compatibility between the wood species and the binder be improved to enhance the cross-linking between both mediums, as poor bonding has been identified as the main factor for the poor performance of the cement-bonded flakeboards. This could possibly be achieved by adding compatibilizers such as maleic anhydride (MAH) and grafted polyethylene (PE-g-MAH) into the wood-accelerator-cement mixture prior to pressing and curing processes. Nonetheless, a further study on the addition of compatibilizers should be conducted to examine whether or not the compatibilizers can solve the problem.

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Antibiogram Pattern among Cultures of *Listeria monocytogenes* Isolated from Frozen Burger Patties in Malaysia

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ABSTRACT

Forty-one isolates of *Listeria monocytogenes*, which were obtained from raw burger patties, were tested for their susceptibility against eleven antibiotics by using standard disc diffusion method. In particular, 31.7% of the isolates were found to be not resistant to any of the antibiotic tested while the rest showed resistance to at least one antibiotic. The result showed that resistance to tetracycline was the most common (46.3%), followed by erythromycin (36.6%), amikacin (31.7%), and sulfamethoxazole-trimethoprim (17.1%). All the isolates of *Listeria monocytogenes* were sensitive towards imipenem and gentamicin. The findings of the present study revealed the presence of multidrug-resistant *Listeria monocytogenes* isolates in the processed meat products and hence suggested the emergence of antibiotic resistance in bacterial strains in the food chain.

Keywords: *Listeria monocytogenes*, antibiotic, susceptibility, resistance, dendrogram pattern, standard disc diffusion method

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INTRODUCTION

Listeria monocytogenes is transmitted to human through contaminated food. Ingestion of this bacterium may cause severe adverse health effects to a group of well-defined high risk people such as pregnant woman, neonates and elderly. Researchers defined those infected individuals with altered or deficient immune system, or

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contracted with non-invasive febrile gastroenteritidis as particularly at higher risk for *listeria* infection (Dalton *et al*., 1997; Heitmann *et al*., 1997; Aureli *et al*., 2000). However, mild to moderate symptoms will also be manifested in healthy adults when the ingested dose is high, i.e. approximately 10-100 million CFU (Farber *et al*., 1996). The non-specific flu-like symptom is always complicated with other illnesses, causing it be probably under-diagnosed and eventually leading to fatality case.

Thus, early diagnosis is important so that appropriate antibiotic treatment can be applied to cure *listeria* infection before the occurrence of more serious consequences. Jones and MacGowan (1995) asserted that *L. monocytogenes* is generally susceptible to all antibiotics. The selection of the antibiotic for listeriosis therapy was further narrowed down by Rota *et al*. (1996) and Teuber (1999), who later specified some antibiotics that are commonly used, including ampicillin, penicillin, trimethoprim, tetracycline, erythromycin, and gentamicin.

Despite the fact that there are many reports published on the susceptibility of *L. monocytogenes* against antibiotics, Charpentier *et al*. (1999) pointed out that the first antibiotic-resistant strains of *L. monocytogenes* were reported as early as 1988. In this particular case, tetracycline resistance was the first encountered antibiotic resistance in *L. monocytogenes* (Poyart-Salmeron *et al*., 1990). Since then, Charpentier and Courvalin (1999) have reported the antibiotic resistance of particular isolates from sporadic clinical cases, food, or environment towards antibiotics. The researchers further added that the emergence of antibiotic resistant bacterial strains had been accelerated by the selective pressure caused by over-prescription of drugs in clinical settings and heavy use as growth promoters in livestock husbandry. Meanwhile, the emergence of antibiotic-resistant *L. monocytogenes* strains implicates the possibility of clinical treatment failure for listeriosis in future.

Antimicrobial susceptibility testing has emerged as one of the effective tools to provide *in vivo* prediction on the success or failure of an antibiotic therapy (Govan, 2006). Besides, antimicrobial susceptibility testing also determines the relatedness of a group of isolated bacterial strains based on their antibiotic resistant pattern. Numerous researchers have used this useful epidemiological marker because it is simple to perform and less time-consuming (Aureli *et al*., 2003; Yucel *et al*., 2005; Arslan & Ozdemir, 2008; Harakeh *et al*., 2009).

The objective of this study was to determine the susceptibility of *L. monocytogenes* isolates against eight groups of antibiotic, and cluster them according to their resistant patterns.

MATERIALS AND METHODS

Isolation of L. monocytogenes

Forty-one isolates of *L. monocytogenes* were obtained from retail burger patties. These isolates were collected from each positive palcam agar plate (MERCK, Germany), in which typical presumptive *L. monocytogenes*

colonies were subcultured onto tryptic soy agar (TSA; MERCK, Germany) and confirmed using PCR assay (Wong *et al*., 2011). The number of isolates collected from each sample was determined by the level of *L. monocytogenes* contamination (Chasseignaux *et al*., 2001). At low level, only one isolate could be obtained, whereas at high levels, up to four isolates were collected. In this study, fifteen strains and eighteen strains of *L. monocytogenes* were obtained from beef and chicken patties purchased from supermarket, respectively, while eight other strains were obtained from vegetarian burger patties purchased from a retail shop.

Antimicrobial Susceptibility Testing (AST)

This was performed using Standard Disc Diffusion method according to NCCLS standard reference procedure (National Committee for Clinical Laboratory Standards, 1993). For this purpose, a total of eleven antibiotics were used: ampicillin (10 µg); penicillin-G (10 U); imipenem (10 µg); vancomycin (30 µg); amikacin (30 µg); gentamicin (10 µg); tetracycline (30 µg); erythromycin (15 µg); chloramphenicol (30 µg); rifampicin (5 µg); sulfamethoxazole-trimethoprim (1.25/23.75 µg). Meanwhile, the antimicrobial impregnated discs were purchased from Oxoid (England).

All the isolates were revived in tryptic soy broth (TSB; Merck, Germany) containing 0.6 % (w/v) yeast extract at 37°C for 24 hours (Yucel *et al*., 2005). The inoculums were swabbed using sterile swabs onto Mueller-Hinton agar (MERCK, Germany) to form a uniform lawn of *L. monocytogenes* on the surface of agar. It is important to note that *Listeria monocytogenes* ATCC 19155 and *Staphylococcus aureus* ATCC 25923 were used as reference strains in this study. The antimicrobial impregnated discs were then dispensed onto the agar using Antibiotic Disc Dispenser. The plates were incubated at 37°C for 48 hours.

To determine the antibiogram pattern of an isolate, the diameter of the inhibition zone was measured to the nearest millimetre. Each isolate was classified as resistant (R), intermediate (I) or susceptible (S), to the antibiotics, using NCCLS guidelines (2004). Intermediate-resistant isolates were classified together with resistant isolates for further interpretation of the data (Wayne, 2006).

Multiple Antibiotic Resistance (MAR) Indexing

Each isolate was assigned an MAR index, as defined by Krumperman (1983):

$$\text{MAR index} = \frac{a}{b}$$

a = Number of antibiotics to which the particular isolate was resistant;

b = Number of antibiotics to which the particular isolate was exposed.

Antibiotic Resistance Clustering Analysis

The associations of the resistant patterns among all the tested isolates were analyzed and computed using the software BioNumerics Version 4.5 (Applied Maths, Belgium). Meanwhile, Pearson correlation coefficient and unweighted average linkage

(UPGMA) were employed to construct a dendrogram for clustering analysis. Susceptible results were coded as '0' and resistant results were coded as '1'.

Statistical Analysis

A statistical analysis was performed using SPSS version 16.0. Friedman test was used to determine whether there is significant difference in the percentages of the antimicrobial resistance levels among the different antibiotics used, followed by a post hoc Nemenyi test.

RESULTS AND DISCUSSION

Results for the susceptibility rates of *L. monocytogenes* isolates against eleven antimicrobial agents are shown in Table 1 and Table 2. All the isolates were found to be sensitive towards both imipenem and gentamicin. More than 50% of the isolates tested showed susceptibility towards all the antibiotics. Meanwhile, the antibiotics other than tetracycline, erythromycin, and amikacin were found to have exhibited effective inhibition on the growth of more than 80% of *L. monocytogenes* isolates.

Resistance pattern of forty-one isolates of *L. monocytogenes* towards eight classes of antibiotic is illustrated in Fig.1. More isolates were observed to be significantly resistant to tetracycline (46.3%) and macrolide (36.6%) than to the rest of the antibiotic classes tested in this study ($P<0.05$). The isolates of *L. monocytogenes* were moderately resistant to both sulfamethoxazole-trimethoprim (SMZ-TMP) and aminoglycoside. Surprisingly, there were small percentages of isolates that were resistant towards ß-lactams (2.4%), glycopeptides (2.4%) and rifamycins (9.8%) which are often used in the treatments of the listeriosis manifestation.

Table 3 shows the antibiotic resistant patterns resulted from all strains, together with the percentage of the isolates within the same pattern. It is worthy to note that 31.7% (13/41) of *L. monocytogenes* isolates were affected by all types of antibiotics

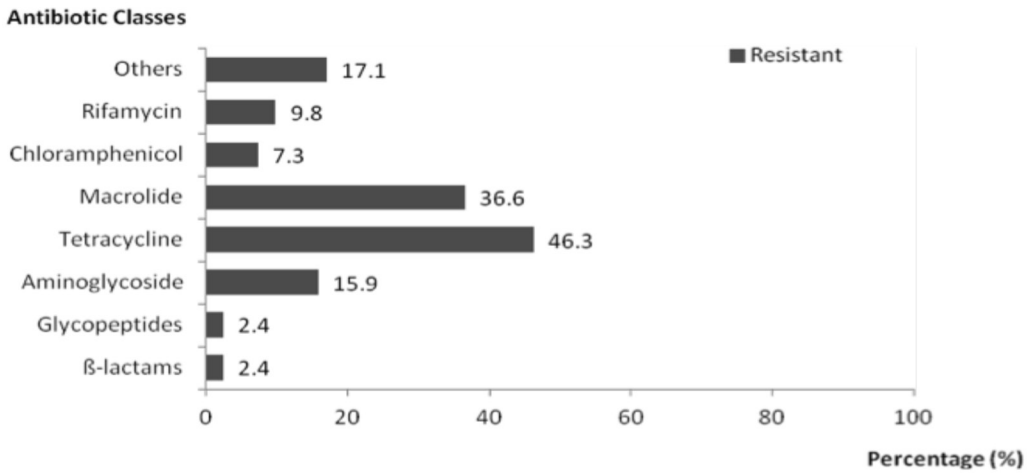

Fig.1: The percentage of *Listeria monocytogenes* isolates resistant to different classes of antibiotic

TABLE 1
Resistant/susceptibility pattern of *Listeria monocytogenes* isolates towards different antimicrobial agents.

Antibiotic classes	Antimicrobial agents (Concentration)	Activity (Prevalence, %)	
		Resistant	Susceptible
ß-lactams	Ampicillin (10 µg)	2 (4.9)	39 (95.1)
ß-lactams	Penicillin (10 U)	1 (2.4)	40 (97.6)
ß-lactams	Imipenem (10 µg)	0 (0)	41 (100)
Glycopeptides	Vancomycin (30 µg)	1 (2.4)	40 (97.6)
Aminoglycoside	Gentamicin (10 µg)	0 (0)	41 (100)
Aminoglycoside	Amikacin (30 µg)	13 (31.7)	28 (68.3)
Tetracycline	Tetracycline (30 µg)	19 (46.3)	22 (53.7)
Macrolides	Erythromycin (15 µg)	15 (36.6)	26 (63.4)
Chloramphenicol	Chloramphenicol (30 µg)	3 (7.3)	38 (92.7)
Rifamycin	Rifampicin (5 µg)	4 (9.8)	37 (90.2)
Others	SMZ-TMP (1.25 + 23.75 µg)	7 (17.1)	34 (82.9)

TABLE 2
The percentage of resistant strains from different sources (%; number of isolate)

Antibiotic	Beef patties	Chicken patties	Vegetarian patties
Ampicillin	50.0 (1)	50.0 (1)	0
Penicillin	100 (1)	0	0
Vancomycin	100 (1)	0	0
Amikacin	61.5 (8)	0	38.5 (5)
Tetracycline	63.1 (12)	5.3 (1)	31.6 (6)
Erythromycin	60.0 (9)	13.3 (2)	26.7 (4)
Chloramphenicol	66.7 (2)	33.3 (1)	0
Rifampicin	100 (4)	0	0
SMZ-TMP	42.8 (3)	42.8 (3)	14.3 (1)

tested in this study. These isolates were obtained from chicken burger patties (12/41) and vegetarian burger patty (1/41). Eight (19.5%) out of 41 *L. monocytogenes* isolates showed resistance to single antibiotic, while 48.7% of the *L. monocytogenes* isolates demonstrated multiple antibiotic resistances (i.e. resistance to at least two antibiotics).

A dendrogram obtained from the clustering analysis is shown in Fig.2, to compare the resistant patterns among *L. monocytogenes* isolated from different sources. From the dendrogram, all the isolates can be clustered with a cut off value at 70% similarity. Meanwhile, thirty-five out of 41 *L. monocytogenes* isolates were differentiated into five discriminatory clusters, namely, C1, C2, C3, C4, and

TABLE 3
Multiple Antibiotic Resistance (MAR) index and antibiotic resistant pattern of *Listeria monocytogenes* isolated from burger patties.

MAR Index	*Antibiotic Resistant Pattern	Isolate number	Percentage of isolate (%)
0.45	AkERdSTe	13	2.4
0.36	AkCES	6	7.3
	AkEPTe	7	
	AkESTe	19	
0.27	AkERd	5	19.5
	AkETe	1, 4, 17	
	AkEVa	2	
	AmpRdTe	11	
	CSTe	9	
	ERdTe	8	
0.18	AkE	22	19.5
	AkTe	12, 16, 18	
	AmpC	25	
	ETe	10, 20, 27	
0.09	Te	3, 14, 15, 21	19.5
	S	33, 35, 41	
	E	38	
0	-	23, 24, 26, 28, 29, 30, 31, 32, 34, 36, 37, 39, 40	31.7

Ak – Amikacin; Amp – Ampicillin; C – Chloramphenicol; E – Erythromycin; P – Penicillin; Rd – Rifampicin; S – Sulfamethoxazole-trimethoprim; Te – Tetracycline; Va – Vancomycin.

C5. The remaining six isolates were left uncategorized into either group. However, these six single isolates were found to possess similarity with clusters C2 - C5 at 71.2%. The isolates from various sources seemed to cluster together into four clusters with only one exception, i.e. cluster C4, which was only occupied by the isolates from the same source (i.e. chicken burger patties). The finding indicated that the *L. monocytogenes* isolates derived from beef, chicken and vegetarian burger patties were highly correlated to each other, as shown in the dendrogram in Fig.2. This may indicate that the *L. monocytogenes* strains isolated from frozen burger patties taken from the supermarkets and retail shop have been exposed to a similar contamination source along the production chain of such product.

Antibiotic or antimicrobial agents are defined as chemical compounds which are either synthesized or derived from natural sources and influence the growth of bacteria. In clinical perspective, it is essential for

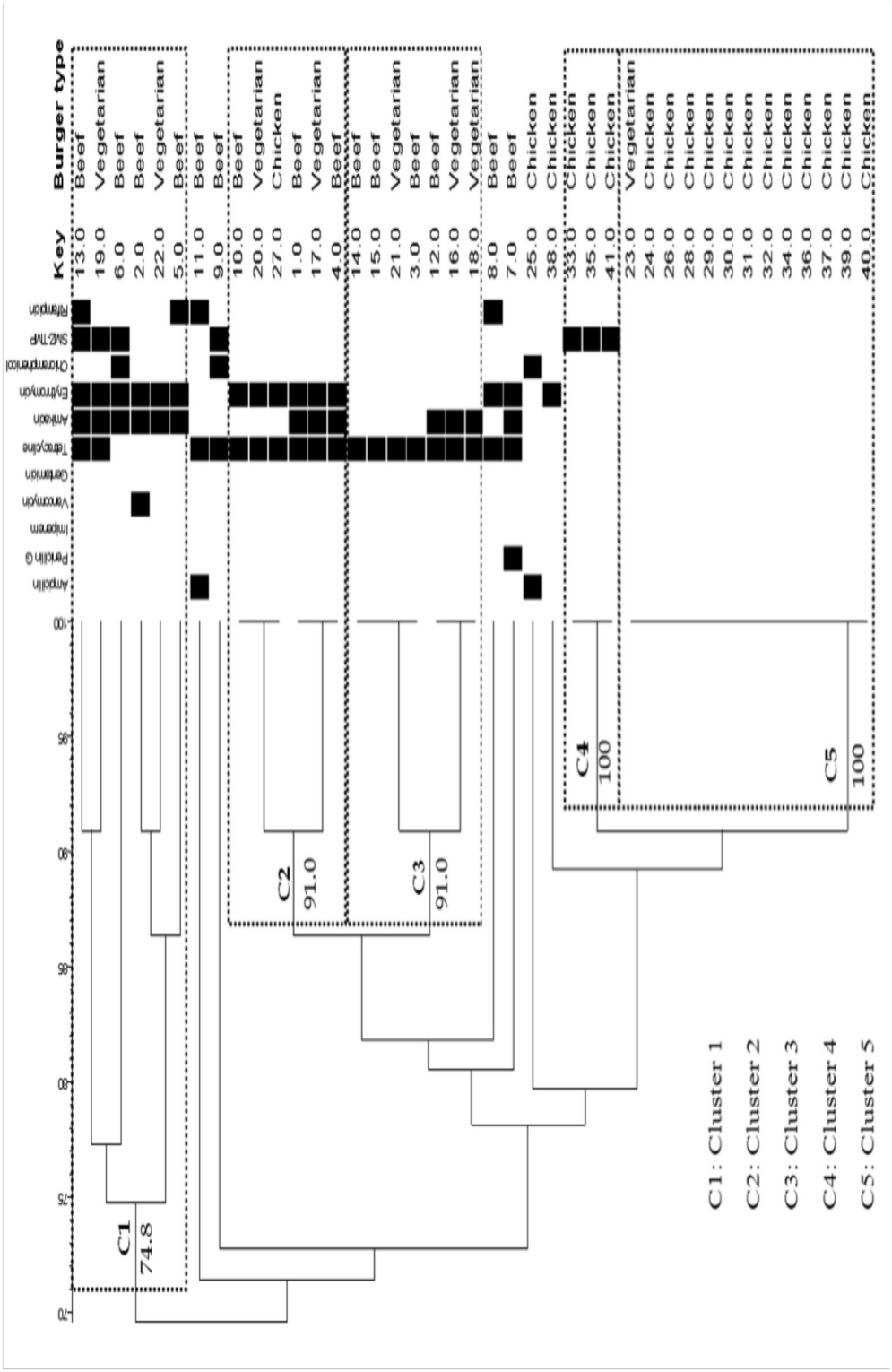

Fig.2: Dendrogram showing the resistance profile using Pearson/UPGMA clustering

treatment of bacteria-causing infectious disease since its discovery. However, due to emergence of bacterial resistant strains, many of the available antibiotics are no longer useful. Antibiotic resistance of bacterial strains is in the realm of public health issues that are of all peoples' concern. All in all, the resulting data from the present study have suggested that the overall incidence of the antibiotic resistance in *L. monocytogenes* is still relatively low. This also reveals that most of the antibiotics used are powerful agents against the tested strains.

From this study, all the isolates were found to be sensitive to at least two antibiotics, namely gentamicin and imipenem. This phenomenon was observed to be uniform among the isolates although they were obtained from different sources and locations, as supported by a study which also reported a similar finding (Conter *et al*., 2009). Besides, *L. monocytogenes* cultures were highly susceptible towards ampicillin and penicillin. In the normal case of listeriosis, the treatment of choice is ampicillin or penicillin G in combination with an aminoglycoside, which is usually gentamicin (Espaze & Reynaud, 1988; Franco Abuin *et al*., 1994; Jones & MacGowan, 1995). High efficacies of these antibiotics against *L. monocytogenes* strains in this *in vivo* result may suggest successful antibiotic treatment for patients. Thus, these antibiotics remain applicable in clinical settings.

SMZ-TMP is the antibiotic used as a second choice therapy for listeriosis, especially for patients who are allergic to ß-lactams (Boisivon *et al*., 1990; MacGowan *et al*., 1990; Lorber, 1997). However, it was found to be ineffective to 17.1% of *L. monocytogenes* isolates in this study. This finding supports other results reported elsewhere (dated back in last five years), whereby the percentages of resistance to SMZ-TMP ranged from 1.6% to 66% (Yucel *et al*., 2005; Arslan & Ozdemir, 2008; Harakeh *et al*., 2009; Conter *et al*., 2009). The emergence of SMZ-TMP resistance strains is of particular importance and it requires more attention since this antimicrobial drug is a successful alternative treatment for listeriosis in human beings.

It is important to note that tetracycline and erythromycin are the most common antibiotics which have shown reduced sensitivity against *L. monocytogenes* isolates, with a prevalence of 46.3% and 36.6%, respectively. This finding is comparatively higher than other published findings. Only 2.4% of *L. monocytogenes* has been reported to exhibit intermediate-resistance towards erythromycin (Conter *et al*., 2009). Similarly, Aureli *et al*. (2003) and Conter *et al*. (2009) reported low prevalence (0–0.8%) of tetracycline resistance of food borne *L. monocytogenes* isolated from food in Italy. Nonetheless, Walsh *et al*. (2001) substantiated that *L. monocytogenes* isolated from various retail foods in Northern Ireland was most commonly resistant to tetracycline. Hence, it is possible to suggest that the isolates from different geographic location

may pose different levels of tetracycline resistance, as noted in the present and other studies. In addition, Chai (2007) speculated that the comparatively lower prevalence of erythromycin and tetracycline resistance in some countries might be due to controlled usage of antimicrobial drugs in the livestock industry and in hospitals. In fact, Denmark, United Kingdom, United States of America and a few other advanced countries have banned the application of tetracycline in agricultural practices (Chai, 2007).

In this study, 48.7% of *L. monocytogenes* isolates demonstrated multiple antibiotic resistances. These isolates were resistant to at least two of the eleven antibiotics tested. Any bacterial strain exhibiting MAR index values lower than 0.2 is deemed to be originated from animals, in which the antibiotics are seldom or never used (lower risk). Meanwhile, the isolates having MAR index values higher than 0.2 are regarded as originating from higher risk sources of contamination such as from animal farms which are greatly exposed to antibiotics. Twelve out of forty-one isolates of *L. monocytogenes* had the MAR index greater than 0.2 (0.27 – 0.45) and therefore, it might be evident that their origin would have been from a high risk source of contamination. Hence, it was not surprising to find 83.3% of these high MAR index isolates originated from the beef patties.

Nowadays, many drugs, antibiotics, and hormones are applied in the livestock industry with the aims to raise cows that can produce more milk and to maximize the return profits from their meat. Based on this observation, it can therefore be assumed that the application of antimicrobial drugs in the poultry farm is much lesser than that in the cow's farm. Recently, Vinesh (2010) reported the usage of herb-based supplement by a local poultry rearing industry to replace antibiotic. The herbs are deemed to boost up chicken's immune system, revitalize their reproductive organs, and improve their digestive system.

Cluster analysis is useful for clustering a set of bacterial isolates corresponding to the dynamic of resistance (Chai, 2007). It is of particular significance in showing epidemiological relatedness and association among isolates with different variables and outputs. In this study, the isolates of *L. monocytogenes* from different sources and locations were classified into five clusters. Nonetheless, there was no significant pattern observed between these clusters. Hence, this might suggest that the isolates could have originated from a similar contamination source. The acquisition of antibiotic resistance genes in *L. monocytogenes* might have occured at the early stage before they were processed into respective products. It would appear that the spread of antibiotic resistance genes might have occurred among *L. monocytogenes* hosted in animals (cow and chicken) in farm and vegetations.

The issue of bacterial resistance to antimicrobial drugs is not only limited to the food industry, animal farms, nor clinical settings, but it also appears to be a global problem. It gives impacts to both industrialized and resource-poor countries since bacterial resistance implicates the

difficulty to treat both community and hospital-acquired infections; this leads to increase the rate of morbidity and mortality, and eventually results in great economic loss to a country. However, there is scarcity of studies which anticipate the cost of antibiotic resistance, particularly in terms of increased morbidity, mortality or cost to hospitals or the society (Liss & Batchelor, 1987; Andersson & Hughes, 2010).

CONCLUSION

The results of this study suggest that the food isolates of *L. monocytogenes* are mostly susceptible to commonly used antibiotics in veterinary and human listeriosis therapy. However, manifestation of resistance towards some antibiotics could be addressed as this might be the sign where *L. monocytogenes* gradually became antibiotic resistant by acquiring potential antibiotic resistant genes from environmental background gram-positive bacteria. To the best of the authors' knowledge, this is the first study carried out on antimicrobial susceptibility testing on *L. monocytogenes* strains isolated from processed meat in Malaysia. A continuous programme focusing on antimicrobial resistance of *L. monocytogenes* is of necessity to assure the effectiveness of listeriosis treatment. Besides, it will also provide insights into the problems of overuse and/or misuse of antimicrobial agents in human and veterinary medicine.

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Relationship between Size of Fish and Parasitic Intensity in Four Freshwater Fish Species from Tasik Merah, Perak, Peninsular Malaysia

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ABSTRACT

A total of 79 fish from Tasik Merah, Perak, Peninsular Malaysia were examined for the presence of fauna. The fish species examined included *Puntius schwanenfeldii*, *Puntius gonionotus*, *Hampala macrolepidota* and *Notopterus notopterus*. Meanwhile, a total of ten species of the parasites were found to be belonging to two major groups of nematode and trematode. The nematodes were *Capillaria* sp., *Spinictus inermis*, *Echinocephalus* sp., *Microtetrameres* sp., and *Cucullanus* sp. The trematodes were *Paradiplozoon malayense*, *Paradiplozoon barbi*, and *Dactylogyrus* sp.

Keywords: Freshwater fish, Tasik Merah, parasite, fish size

INTRODUCTION

The total fish production for Malaysia in 1999 was 1,251,765 metric tons, of which 3,366 metric tons were from freshwater fish (Department of Fisheries, Malaysia Annual Fisheries Statistics, 1995), implying the economic importance of freshwater fish as an important protein source for Malaysians and the world population.

Some observations on the parasites of Malaysian freshwater fish have been reported in the literature, but these are mainly limited to the genus such as *Clarias* sp.*, the* catfish (Fernando & Furtado, 1963; Furtado & Tan, 1973; Leong & Mokhtar, 1981; Lim, 1991; Rahman & Ali, 1991; Rahman *et al.*, 1992; Shaharom *et al.*, 1992; Rahman & Bakri, 2008), or *Channa striatus*, the snakehead (Fernando & Furtado, 1963; Leong & Mokhtar, 1981; Rahman & Ali, 1991; Rahman & Bakri 2008) and *Anabas testudineus*, the climbing perch and *Trichogaster pectoralis*, the snakeskin Gouramy (Fernando & Furtado, 1963; Leong & Mokhtar, 1981; Rahman & Ali, 1991; Rahman & Bakri, 2008). Generally, their findings revealed that

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Clarias sp. are often infected by various species of nematodes and trematodes, while acanthocephalans are also found in *Channa* sp., and *Trichosgaster* sp. usually show low infection rates.

The present paper describes the parasitic fauna of four infrequently reported freshwater fishes from Tasik Merak, Perak, Peninsular Malaysia: *Puntius schwanenfeldii*, *Puntius gonionotus*, *Hampala macrolepidoata* and *Notopterus notopterus*. In this study, the relationship between fish size and parasite intensity was investigated.

MATERIALS AND METHODS

This study was carried out at Tasik Merah, Perak (longitude 100° 32'E, latitude 5° 10'N), Peninsular Malaysia. Only four major species, namely, *Puntius schwanenfeldii (lampam sungai, tinfoil barb) Puntius gonionotus (lampam jawa, java barb), Hampala macrolepidot (sebarau, jungle perch)* and *Notopterus notopterus (belida, grey leatherback)* were included in this research. Twenty fish from each of the species *Puntius schwanenfeldii (lampam sungai or tinfoil barb), Puntius gonionotus, Hampala macrolepidota* and *Notopterus notopterus* were randomly chosen and brought back to the laboratory in an ice box.

Total fish length was measured in cm. The fish were divided into three sub-groups labelled as small, medium and large size-groups because the larger the size, the older the fish were and the longer the exposure of the fish to their environment. For *P. schwanenfieldi*, the fish that measured ≤ 15.5 cm were classified as small, 18.5 – 20.5 cm as medium and ≥ 21.0 cm as large. For *P. gonionotus*, the fish that measured ≤17.5 cm were classified as small, 18.0 – 19.0 cm as medium, and ≥ 19.5 cm as large. As for *H. macrolepidata*, the fish that measured ≤ 21.5 cm were classified as small, 22.0 – 28.0 cm as medium, and ≥ 29.0 cm as large. For N. notopterus, however, the fish that measured < 18.5 cm were classified as small, 19.0 – 20.0 cm as medium, and ≥ 21.0 cm as large.

The external features were visually examined for ectoparasites. The gills of the fish were removed and examined for helminthes under a dissecting microscope. The fish were slit open and the contents were collected on Petri dishes, and examined under a dissecting microscope. All the parasites found were individually picked and kept in small bottles. Trematodes and acanthocephalans were preserved in formaline-alcohol-acetic acid, while nematodes were preserved in 5% glycerine with 70% alcohol. Trematodes were stained with semichon's acetic carmine stain and mounted permanently in Canada balsam. Nematodes and acanthocaphlans were cleared in lactophenol and examined in temporary mounts.

The obtained data were then analyzed by using student t-tests or Wilcoxon-Man-Whitney tests, depending on the number of replicates. H_o was when the mean intensity of parasite in every size-group of fish was similar. H_a was when the mean intensity of parasite in every size-group of fish was different. Nonetheless, the data could not be analyzed by using ANOVA since there were different numbers of replicates in every

subgroup and most of them were less than 6. As a result, comparisons of the mean parasite intensity between the small- and medium-sized groups, small- and large-sized groups, as well as the medium- and large–sized groups for every fish species were carried out.

RESULTS

Eight species of ecto- and endoparasites were found to infect the four fish species (Table 1). The two most abundant parasites were the nematodes *Cucullanus* sp and *Spinitectus inermis*. *Cucullanus* sp was recovered only from *Puntius schwanenfeldii* while *Spinitectus inermis* was found in all the four species of fish. The number of parasites obtained showed no dependence on fish size. The other two nematodes infecting the fishes were *Paradiplozoon* sp. and *Capillaria* sp. The four species of trematodes infecting the fish were *Paradiplozoon barbi*, *P. malayense*. *P. barbi*, *Echinocephalus* sp. and *Dactylogyrus* sp. *Paradiplozoon barbi* infecting *Hampala macrolepidota* were present in a large number in one fish host while only one a single *P. malayense* was found in *P. Schwanenfeldii*. Meanwhile, *Hampala macrolepidota* seemed to be a favourite host for many parasites as compared to the three other species of fish.

For *P. schwanenfeldii* and *P. Gonionotus,* similar results were observed (Tables 2 & 3; Fig.1). The larger the size group, the percentage of the infected fish and the number of parasite would also increase. For *P. schwanenfeldii*, 100% of the fish examined from the medium- and large-sized groups were infected with parasites as compared to 71.4% in the small size-group. Meanwhile for *P. gonionotus*, 14.3% of the fish in the small-sized group were infected and for the medium-sized group, 33.3% were infected. The large-sized group showed a 42.9% infection rate.

In *H. macrolepidota,* the reverse was observed (see Table 4 and Fig.1). The

TABLE 1
The total number of parasites infecting the four fish species in Tasik Merah, Perak

	P. schwanenfeldii	*P. gonionotus*	*H. macrolepidota*	*N. notopterus*	No. of host
Cucullanus sp	79	-	-	-	1
Spinitectus inermis	23	20	1	10	4
Microtetrameres sp	-	-	6	-	1
Capillaria sp	-	-	3	-	1
Paradiplozoon barbi	-	-	10	-	1
P. malayense	1	-	-	-	1
Echinocephalus	-	-	-	1	1
Dactylogyrus sp	-	-	2	-	1
Total no. of parasites	103	20	22	11	
Parasite species	3	1	5	2	

TABLE 2
Various parameters of the various parasite populations in different size groups of *Puntius schwanenfeldii*

	Puntius schwanenfeldii					
	≤15.5 (small)		18.5-20.5 (medium)		≥21.0 (large)	
	No. infected fish (%)	Mean intensity (range)	No. infected fish (%)	Mean intensity (range)	No. infected fish (%)	Mean intensity (range)
Nematode						
Cucullanus sp	2 (28.6)	3.0 (2-4)	5 (83.3)	4.6 (1-10)	7 (100)	7.1 (1-15)
Spinitectus inermis	2 (28.7)	6.5 (5-8)	2 (33.3)	3.5 (3-4)	2 (28.6)	1.5 (1-2)
Trematode						
Paradiplozoon malayense	-	-	-	-	1 (14.3)	1.0 (1)
No. of fish examined	7		6		7	
% of fish infected	71.4		100		100	
No. of parasite species	3		3		3	
Total no. of parasite	20		32		54	
Statistical analysis (1) t-test between ≤15.5 (small) and 18.5-20.5 (medium) showed that the mean intensity of the parasite between them is the same (p≤0.05).						
Statistical analysis (2) t-test between the small and large fish showed that the mean intensity of the parasite between them is the same (p≤0.05).						
Statistical analysis (3) t-test between the medium and large fish showed that the mean intensity of the parasites between them is the same (p≤0.05).						

percentage of the infected fish and the number of parasites decreased with group size. The percentage of the infected fish was the highest in the small-sized group, whereby 62.5% of the in the small-sized group were infected with a total number of 17 parasites, as compared to only 20% of the big sized fish with a total number of 3 parasites.

Notopterus notopterus showed the same result as those of *P. Schwanenfeldii* and *P. gonionotus* (Table 5). However, the total number of the parasites found was only 2 in the large-sized but only 1 in the medium-sized groups. In the small-sized group, on the contrary, no parasite was found.

Based on the obtained data and their analysis, no relationship was found between the mean intensity of the parasites and fish sizes for all the four species of fish. However, if we consider the percentage of infected fish and the total number of parasite obtained from every group, the data were significant.

DISCUSSION

The present study was carried out to show the parasitic infection with the size of

TABLE 3
Various parameters of the parasite populations in different size groups of *Puntius gonionotus*

	Puntius gonionotus					
	≤17.5 (small)		18.0-19.0 (medium)		≥19.5 (large)	
	No. infected fish (%)	Mean intensity (range)	No. infected fish (%)	Mean intensity (range)	No. infected fish (%)	Mean intensity (range)
Nematode						
Spinitectus inermis	1 (14.3)	4.0 (4)	2 (33.3)	4.0 (1-7)	2 (28.6)	4.0 (4)
No. of fish examined	7		6		7	
% of fish infected	14.3		33.3		42.9	
No. of parasite species	1		1		2	
Total no. of parasite	4		8		9	
Statistical analysis (1) t-test between ≤17.5 cm (small) and 18.0-19.0 cm (medium) showed that the mean intensity of the parasite is the same ($p \leq 0.05$).						
Statistical analysis (2) t-test between the small and large fish showed that the mean intensity of parasite is the same ($p \leq 0.05$).						
Statistical analysis (3) t-test between the medium and large fish showed that the mean intensity of the parasites is the same ($p \leq 0.05$).						

the fish rather than species specificity. However, it is interesting to note that most of the parasites found in this study showed specificity toward certain fish hosts. Paul and John (2002) pointed out that it is not uncommon to find a fish harbouring several parasite infections rather than only one single parasite species. In the present study, only a single parasite showed a wide range of host infections, i.e. *S. inermis* was found to infect all the four fish species.

According to Dogiel *et al.* (1970), the diversity of the parasites in a fish host is dependent on the life-span of the host. The longer the life-span, the movement of the host during their life will also increase. This will contribute to the accumulation of the parasites in the host. The age of the fish can be represented by the length, and the longer the length of the fish, the age must be older and vice versa. Thus, a fish of longer length can be assumed to have accumulated more parasites as compared to shorter ones.

The relationship between parasites and the length of host may vary depend on the species of both the hosts and the parasites (Hila Bu & Leong, 1997; 1999). They found that the distribution of twin worm *Paradiplozoon* sp was independent on the size of the infected fish hosts while the number of *Dactylogyrus* sp decreased with the increase of host's size in *Hampala macrolepidota*. The decreasing number of gill parasites in the largest-sized group of fish could be due to the morphological changes of the gill filaments which might

TABLE 4
Various parameter measurements of the parasite population in the different size groups of *Hampala macrolepidota*

	Hampala macrolepidota					
	≤21.5		22.0-28.0		≥29.0	
	No. infected fish (%)	Mean intensity (range)	No. infected fish (%)	Mean intensity (range)	No. infected fish (%)	Mean intensity (range)
Nematode						
Unidentified sp 2	1 (12.5)	2.0 (2)	-	-	-	-
Microtetrameres sp	2 (25.0)	1.0 (1)	1 (16.7)	1.0 (1)	1 (20.0)	3.0 (3)
Capillaria sp	-	-	1 (16.7)	1.0 (1)	-	-
Spinitectus inermis	1 (12.5)	1.0 (1)	-	-	-	-
Trematode						
Paradiplozoon barbi	1 (12.5)	10.0 (10)	-	-	-	-
Dactylogyrus sp	1 (12.5)	2.0 (2)	-	-	-	-
No. of fish examined	8		6		5	
% of fish infected	62.5		16.7		20.0	
No. of parasite species	5		2		1	
Total no. of parasite	17		4		3	
Statistical analysis (1)- t-test between ≤21.5 cm (small) and 22.0-28.0 (medium) fish showed that the mean intensity of the parasite is the same ($p \leq 0.05$).						
Statistical analysis (2) Wilcoxon-Man-Whitney test between the small and large fish showed that the mean intensity of the parasite is the same.						
Statistical analysis (3) Wilcox-Man-Whitney test between the medium and large fish showed that the mean intensity of parasites is the same.						

have affected the attachment of parasites, the acquired immunity of the hosts and other behavioural changes of the host.

According to Poulin and Rohde (1997), however, the host body size is correlated to the number of ectoparasites infecting them. This may be due to the changes in the host's diet with age. The larger the size of a fish, the size of the space and target site for the colonization and infection of parasite will also increase. Meanwhile, the intensity of parasite infection increases with the size of the host due to the longer exposure and larger surface area of the attachment of ectoparasite (Hanek & Fernando, 1978; Liang & Leong, 1991). Furtado and Tan (1973) also have almost the same opinion regarding this matter. They found that larger hosts have greater incidence of being infected by parasite but this is due to the differences in the level of immunity rather than size. However, according to Paul and John (2002), beside the size of host fish, other factors that may influence parasite distribution and variability within infected fish are geographic range and local habitat

TABLE 5
Various parameters of the various parasite populations in the different size groups of *Notopterus notopterus*

	Notopterus notopterus					
	≤18.5 (small)		19.0-20.0 (medium)		≥21.0 (large)	
	No. infected fish (%)	Mean intensity (range)	No. infected fish (%)	Mean intensity (range)	No. infected fish (%)	Mean intensity (range)
Nematode						
Spinitectus inermis	-	-	1 (10.0)	8.0 (8)	1 (20.0)	2.0 (2)
Echinocephalus sp	-	-	-	-	1 (20.0)	1.0 (1)
No. of fish examined	5		10		5	
% of fish infected	-		10.0		40.0	
No. of parasite species	-		1		2	
Statistical analysis (1) Wilcoxon-Man-Whitney test between ≤18.5 cm (small) and 19.0 – 20.0 (medium) showed that the mean intensity of the parasite is the same.						
Statistical analysis (2) Wilcoxon-Man-Whitney test between the small and large fish showed that the mean intensity of parasite is the same.						
Statistical analysis (3) Wilcoxon-Man-Whitney test between the medium and large fish showed that the mean intensity of the parasite is the same.						

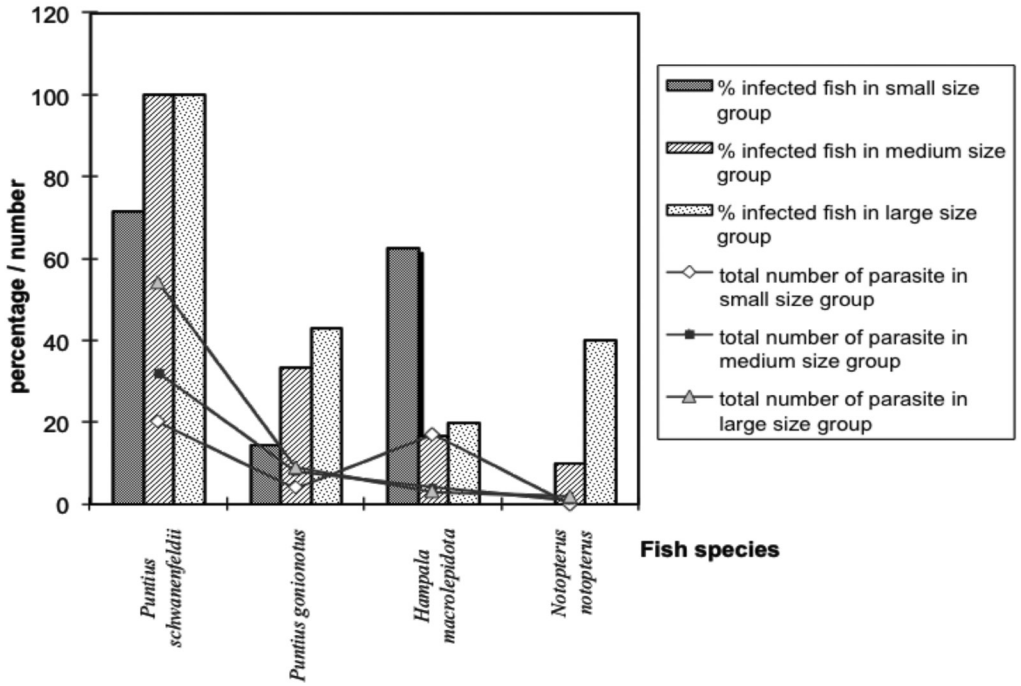

Fig.1: The percentage of the infected fish and the total number of the parasites in the small-, medium- and large-sized groups

used by the fish. The researchers further reiterated that local habitat diversity and species richness will increase the probability of the correct host and habitat requirements for parasites with direct lifecycle or free-living stage will be met.

CONCLUSION

In conclusion, larger fish tend to harbour more parasites as compared to smaller ones. However, this also depends on the species of fish. In this study, only *H. macrolepidota* was found to have shown the reverse. More parasites were found in the smaller size groups as compared to the bigger ones. However, this could be due to the differences in the immune response rather than the size alone.

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Anthropogenic Inputs of Heavy Metals in the East Part of the Johore Straits as Revealed by their Concentrations in the Different Soft Tissues of *Perna viridis* (L.)

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ABSTRACT

Concentrations of Cd, Cu, Ni, Pb and Zn were determined in seven different soft tissues of *Perna viridis* collected in May-November 2006 from the six sites in the Straits of Johore. The metal concentrations in the soft tissues of the samples from the east of the Johore Causeway were generally higher than those of the samples collected from the western partI of it. This indicated that the eastern part of the straits had higher contamination and bioavailabilities of Cd, Cu, Ni, Pb and Zn. Therefore, these results are in agreement with the fact that there are various anthropogenic activities, such as petrochemical plants and port activities, in the eastern part of the Straits of Johore. The findings of this work are useful for future reference, particularly for the semi-enclosed Straits.

Keywords: Heavy metal, green-lipped mussel, *Perna viridis*, Johore Causeway, The Straits of Johore

INTRODUCTION

In 1996, the most interesting issue related to the Johore Causeway was the intention to replace the semi-enclosed Johore Causeway with a bridge. In 2003, the Malaysian government planned to build a "crooked half bridge". On 12 April 2006, however, the Malaysia government scrapped off the plan to build the bridge due to unavoidable circumstances. Among the initial reasons cited for building the bridge were that it would allow free flow of water across both sides of the Straits, whereby the 1909-built causeway artificially divides it into two parts (Yap *et al*., 2006), and that the bridge would help to ease the traffic congestion in Johore Bharu city (Berita Harian, 2006), apart from markedly improving the marine ecology of the Johore Straits, alleviating the stagnation

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and stench, and enhancing the aesthetic value of the marine environment (Berita Harian, 2006). From the ecotoxicological point of view, the demolishment of the Causeway could create substantial impacts on the living organisms on the both parts of the Causeway.

Studies on heavy metals in the green-lipped mussel *Perna viridis* in Malaysia have been widely reported in the literature and its potential use as a biomonitor of heavy metal pollution for the west coast of Peninsular Malaysia has also been well established (Yap *et al*., 2002a, 2002b, 2003, 2004a, 2004b, 2006a). In this study, the Mussel Watch approach was employed for monitoring Cd, Cu, Ni, Pb and Zn. The objective of this study was to compare the heavy metal concentrations in the green-lipped mussel *P. viridis* collected from the west and east parts of the Johore Causeway.

MATERIALS AND METHODS

This study was carried out at the Straits of Johore. The study sites consisted of three sampling sites, namely, each at the western and eastern parts of the Johore Straits which are separated by the Johore Causeway (Table 1 and Figure 1). The samplings were conducted between May - November 2006. After sample collection, the mussels were put into polyethylene bags and kept in a cool box at < 5°C before they were transported to a laboratory at Universiti Putra Malaysia (UPM). Upon arrival at the laboratory, all the samples were placed in a freezer at -10°C until further analysis.

About 30 mussels from each sampling site, with the shell sizes ranging from 60 to 90 mm, were used for metal analyses. The mussels were carefully dissected into seven different soft tissues, namely mantle, muscle, gonad, foot, gills remainder and byssus. Triplicates of each dried category of soft mussel tissues were digested in 5 ml of concentrated HNO_3 (AnalaR grade, BDH 69%). Later, they were placed in a digestion block at 40ºC for 1 hour and then fully digested at 140°C for 3 hours. After cooling, they were diluted to 20 ml with double de-ionized water. The digested samples were then filtered through Whatman No.1 filter paper into acid-washed pill boxes. The samples were determined for their Cd, Cu, Ni, Pb and Zn concentrations by using an air-acetylene flame atomic absorption spectrophotometer (AAS) Perkin–Elmer Model AAnalyst 800. The data are presented in µg/g dry weight basis. To avoid possible contamination, all the glassware and equipment used were acid-washed.

Procedural blanks and quality control samples made from the standard solutions of Cd, Cu, Ni, Pb, and Zn were analyzed after every five samples to check for sample accuracy. The percentages of recoveries for the heavy metal analyses were between 80 and 110%. In addition, the quality of the analytical method was checked using the Certified Reference Material for Dogfish (DOLT-3, National Research Council Canada) and the metal recoveries were shown to be satisfactory (80–100%).

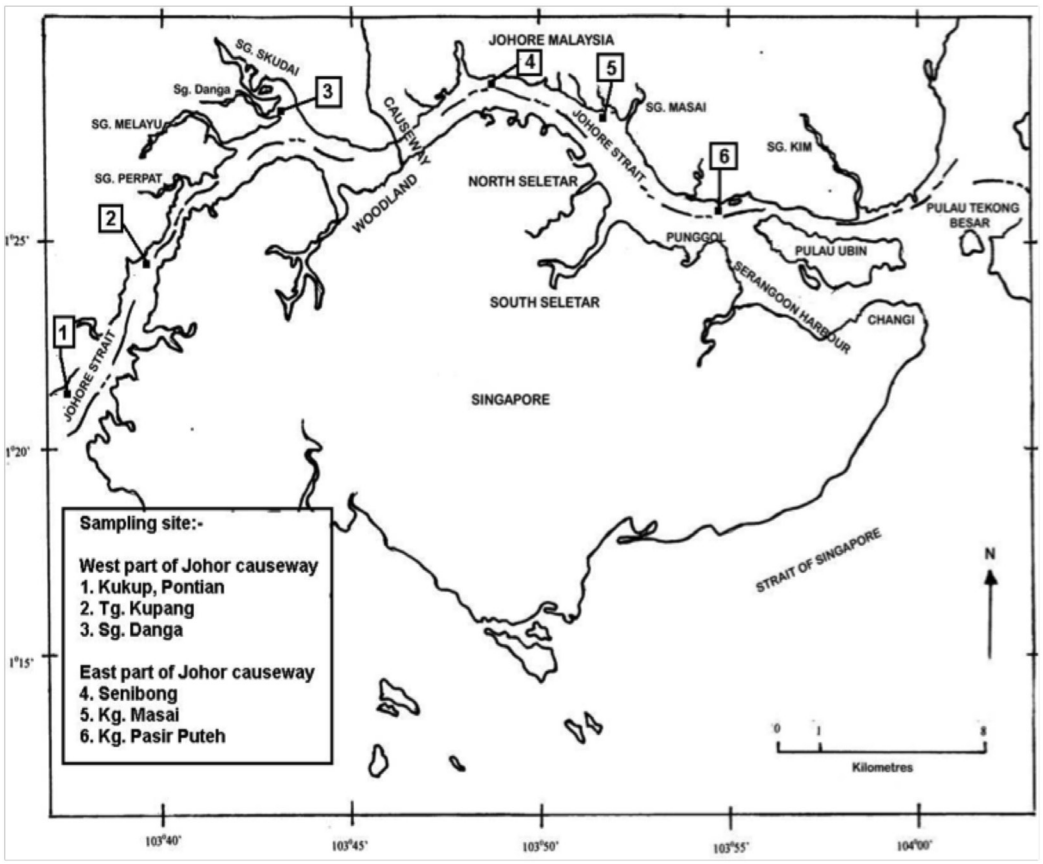

Fig.1: Map showing the sampling sites of *Perna viridis* in the Johore Straits.

For the statistical analyses, independent T-tests were performed to determine the significance levels of any two variables. The statistical analyses were conducted using SPSS Version 13.0 for Windows Release software.

RESULTS AND DISCUSSION

A comparison of the heavy metal concentrations in the different tissues of *P. viridis,* collected from the eastern and western parts of the Straits of Johore which is separated by the Johore Causeway, is given in Table 2. Generally, the eastern part of the Straits recorded higher (although not significantly higher, $p > 0.05$) bioavailabilities and contaminations of Cd, Cu, Ni and Pb and Zn than the western part, as indicated by the metal concentrations in the tissues of the mussels.

The high levels of metal concentrations in the mussels from the eastern part were found in the gill, foot, mantle, gonad, remainder and byssus, indicating the higher metal bioavailabilities and contaminations in this part of the Straits. However, for the concentrations of Cd, Cu and Zn in the gonads, Cd and Pb in the muscles, as well

TABLE 1
Sampling dates, shell lengths (cm) of *Perna viridis* analyzed and descriptions of the sampling sites

No.	Sampling sites	Sampling dates	Shell lengths [mean ± SD]	Description of the sampling sites
1.	Kukup, Pontian	12 May 2006	7.82 - 8.93 [8.25 ± 0.50]	Boat repairing platform, fishing jetty, and an aquacultural area.
2.	Tg. Kupang	24 June 2006	7.5 - 7.89 [7.60 ± 0.20]	Boat repairing platform, fishing jetty, aquacultural area, seaport activities, shipping activities in the surrounding area and coal-powered power plant.
3.	Sg. Danga	23 November 2006	8.53 - 9.00 [8.71 ± 0.20]	Receiving municipal wastes.
4.	Senibong	27 August 2006	9.52 - 10.31 [9.90 ± 0.30]	Port activities and municipal wastes.
5.	Kg. Masai	27 August 2006	6.06 - 7.3 [6.71 ± 0.60]	Port activities and municipal wastes.
6.	Kg. Pasir Puteh	10 June 2006	7.82 - 8.63 [8.19 ± 0.40]	Receiving industrial (petrochemicals) and municipal wastes. Port activities, shipping in the surrounding and a marina site.

as Pb in the byssus, higher concentrations of the metals were found in the samples from the western part compared to those taken from the eastern part. In certain circumstances, the metal concentrations in gonads may be influenced by physiological conditions such as the spawning period of the mussels (Yap *et al*., 2006b). Therefore, the metal concentrations found in the gonads undertaken in this study could not be accurately used as a reflection of the metal contaminations and bioavailabilities of the sites. On the other hand, the big standard deviation (SD) values of the metal concentrations found in every tissue indicated the wide variations in the contents of the Cd, Cu, Ni and Zn. In the mean time, a wider variation of the SD values of metal concentrations observed in the tissues of the mussels from the eastern than the western part could be due to a known metal-contaminated site in Kg. Pasir Puteh (Yap *et al*., 2006b).

The high metal contaminations and bioavailabilities in the eastern part could be due to the discharge of effluents from the domestic and industrial sources nearby. Besides, Kg. Pasir Puteh is also located next to the Pasir Gudang Industrial Estate, where some petrochemical plants and a seaport (Port Pasir Gudang) are located. Moreover, Cu leachate from the antifouling paints of boats and the semi-enclosed topography of the area may further aggravate the pollution problem (Yap *et al*., 2003, 2004, 2006a). The other possible reasons for the high levels of metals found in the mussels from the eastern part could be the riverine inputs from the Tebrau, Skudai, and Segget rivers, which all empty into the waters off Pasir Gudang (Yap *et al*., 2006a). These inputs would carry larger quantities of heavy

TABLE 2
Mean concentrations (mean ± standard error, µg/g dry weight) of the heavy metals in the different soft tissues of *Perna viridis* collected from the western (three sites) and eastern (three sites) parts of the Straits of Johore

Part	Heavy metal	West	East	p
	Cd	0.800 ± 0.200	**1.50 ± 0.700**	0.188
	Cu	11.1 ± 0.900	**12.1 ± 1.90**	0.218
Gill	Zn	95.8 ± 35.8	**123 ± 52.0**	0.377
	Pb	4.50 ± 0.900	**14.2 ± 11.9**	0.452
	Ni	6.80 ± 6.40	**44.1 ± 6.27**	0.485
	Cd	0.300 ± 0.200	**0.800 ± 0.400**	0.395
	Cu	6.20 ± 1.20	**10.6 ± 4.00**	0.349
Foot	Zn	52.5 ± 5.20	**67.1 ± 2.38**	0.349
	Pb	3.80 ± 0.800	**11.4 ± 1.49**	0.390
	Ni	2.30 ± 1.00	**26.8 ± 3.78**	0.427
	Cd	0.600 ± 0.400	**1.50 ± 0.500**	0.282
	Cu	11.0 ± 2.30	**11.9 ± 4.50**	0.080
Mantle	Zn	63.3 ± 8.70	**71.9 ± 3.20**	0.213
	Pb	6.20 ± 1.80	**19.1 ± 2.16**	0.227
	Ni	2.90 ± 1.10	**44.1 ± 6.52**	0.421
	Cd	1.20 ± 0.800	1.10 ± 0.500	0.037
	Cu	10.8 ± 1.20	10.6 ± 1.50	0.049
Gonad	Zn	84.0 ± 5.00	77.3 ± 2.91	0.219
	Pb	7.90 ± 1.90	**13.0 ± 1.12**	0.166
	Ni	3.10 ± 1.60	**31.5 ± 4.33**	0.356
	Cd	1.20 ± 0.800	0.800 ± 0.500	0.050
	Cu	5.20 ± 1.60	**8.40 ± 2.50**	0.245
Muscle	Zn	65.9 ± 15.7	**81.4 ± 16.1**	0.430
	Pb	14.0 ± 4.60	11.0 ± 4.20	0.190
	Ni	2.60 ± 1.20	**31.8 ± 4.36**	0.450
	Cd	1.40 ± 0.600	**2.10 ± 0.600**	0.054
	Cu	22.6 ± 5.50	**26.4 ± 13.8**	0.205
Byssus	Zn	104 ± 16.5	**174 ± 91.3**	0.340
	Pb	16.6 ± 5.70	15.5 ± 5.80	0.102
	Ni	22.8 ± 15.7	**67.2 ± 9.28**	0.427
	Cd	1.20 ± 0.600	**2.20 ± 0.600**	0.096
	Cu	12.0 ± 0.800	**15.6 ± 2.00**	0.180
Remainder	Zn	113 ± 32.9	**101 ± 47.6**	0.163
	Pb	9.60 ± 3.70	**19.3 ± 1.59**	0.274
	Ni	8.80 ± 6.60	**49.7 ± 7.03**	0.427

Note: Values in bold indicate higher concentrations of heavy metals.

metals which could put the local marine ecosystem at risk. Based on the sediment studies by Wood *et al*. (1997), higher metal concentrations were found in the eastern side of the Causeway as compared the western side of the causeway. This reflected the generally greater industrial development on the eastern side of the Causeway which would certainly aggravate the environmental degradation of the fragile semi-enclosed Straits, considering the limited tidal flushing of anthropogenic pollutants. In addition, the neighbouring country, Singapore, also contributed to the high heavy metal contamination of the Johore Straits, as evidenced by the Murray-North Report (The News Straits Times, 2006) which reported that the discharges into the Johore Straits came from the Kranji sewage plant and warm-water discharges from the Senoko plant.

CONCLUSION

Based on the metal concentrations in the different soft tissues of *P. viridis*, the eastern part of the 99-year-old Johore Causeway apparently received environmental impacts from various industrial and shipping activities. Meanwhile, the use of the Mussel watch approach was shown to be useful in identifying the higher metal bioavailability and contamination at the eastern part of the Straits. This finding is very important for future reference, should the Johore Causeway be demolished in the future. In particular, the findings of this study should contribute to the understanding of the present status of pollution in the Straits of Johore. The present data are useful to prompt further ecotoxicological and biochemical studies when the contaminated sites located on the eastern part of the Johore Straits are taken into account.

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The Effects of Crude Oil Boiling Treatment on Physical Properties of *Bambusa vulgaris var. Striata* (Buluh Gading)

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ABSTRACT

Bamboo is a material formed from organic components such as cellulose, hemicellulose and lignin. It is often attacked by biodegradation agents due to the presence of the organic components which affect the physical properties and durability of the bamboo, hence limiting its utilization as an input for production of value-added products. Preservation treatment for lignocellulosic material is not an exception. Boiling treatment was found to be one of the eco-friendly methods to preserve the material. A research was undertaken to study the effects of crude oil boiling treatment on the physical properties and durability of *Bambusa vulgaris var. Striata* (Buluh Gading) against biodegradation agents. Bamboo strips were boil treated in palm oil at 160-200^{0}C for 10 minutes. The untreated and treated strips were tested using physical tests such as relative density, moisture content, swelling and shrinkage. The durability of the strips was also tested using weathering test, where they were exposed to the surrounding for 3 months. The results showed that the boiling treatments improved the dimensional stability of the bamboo and its durability against fungi and boring insects. Meanwhile, the treatments at different temperatures significantly affected the relative density, moisture content, durability, swelling and shrinkage of the bamboo. The moisture content, swelling and shrinkage dropped with regards to the increase in the treatment temperature. The reductions were 18.6% – 2.38%, 16.28% - 7.51%, and 20.72% - 10.94, respectively. The relative density increased to 0.89% when the treatment temperature increased to 160^{0}C, but it decreased from 0.89 to 0.74% as the temperature was increased to 200^{0}C. The durability was assessed in term of percentage of weight loss (%). A smaller

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percentage of the weight loss indicates a better durability. Thus, the treatments were effective to reduce the percentage of weight loss for the above-ground and in-ground graveyard tests. The reductions were found to range from 28.14% - 9.92% and 28.14 – 3.39%, respectively. This study has indicated that bamboo becomes less hygroscopic and more durable when it is exposed to higher temperatures.

Keywords: Bamboo treatment, physical properties, moisture content, swelling and shrinkage, graveyard tests

INTRODUCTION

Bamboo is a type of grass which is commonly grown in Asia. It has a hard, woody and hollow stem. Bamboo is a fast-growing grass even in dense conditions, and matures early (Zhang *et al.,* 2002); thus, the plant is considered as one of the best renewal resources in the world (Anon, 2001). Nowadays, the world is facing decrement of forest resources due to its greater demand compared to its supply; hence, development and exploitation of bamboo are important as well. The properties of bamboo are different that those of timber and for this reason, the readily available processing methods for timber cannot be applied for bamboo. This is why it is essential to create treatment and processing methods that are exclusively for bamboos (Zhang *et al.,* 2002).

Bamboo is a lignocellulosic material. It is susceptible to attacks by decay fungi and boring insects (see Fig.1). In order to reduce the attacks of the biodegradation agents, preservation of bamboo should be conducted. Preservation of bamboo refers to the removal of starch and lignin the plant, as well as to degrade hemicellulose which is the favourite component of fungi and insects to survive (Zhao *et al*., 2010). Even though preservation of bamboos using conventional treatment has been extensively studied, the results are rather discouraging due to the impermeability of the materials (Liese, 1998; Zaidon *et al*., 2007). Therefore, oil treatment was introduced as one of the effective ways to preserve bamboo (Rafidah *et al*., 2010; Razak *et al*., 2004).

Oil treatment can be done using vegetable oils with high boiling point, such as palm oil and hemp oil (Razak *et al*., 2004) because oil can facilitates fast and uniform heat transfer, and hence provide a uniform preservation (Manalo & Acda, 2009). The technique involves immersion of bamboo in an oil bath at different temperatures and durations (Rafidah *et al*., 2010; Razak *et al*., 2004; Manado & Acda, 2009; Zhao *et al*., 2010; Razak *et al*., 2005). Therefore, this paper reports the efficacy of hot oil treatment on physical properties and durability of *Bambusa vulgaris var Striata* when exposed to weathering for 3 months.

MATERIAL AND METHODS

At the age of 3 to 4 years old, *Bambusa vulgaris var Striata* were harvested randomly from their natural stands at the riverside near Kingfisher Residential Area, Kota Kinabalu, Sabah. At this age, bamboo culms were selected for the experiment because they started to mature (Razak *et*

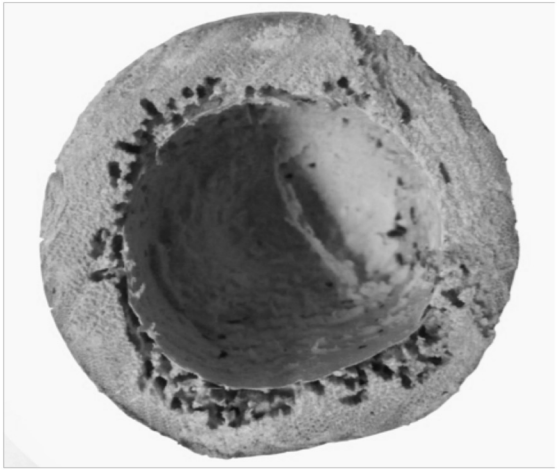

Fig.1: Entrance holes by *Dinoderus minutus* at the cut end of felled culms

al., 2005). A total of 10 culms (from 4th and 6th internodes) were utilised from the fell bamboos. The average moisture content of the green culms was 60%. Meanwhile, crude palm oil was used as treatment media.

Heat Boiling Treatment Processing Using Crude Oil

The treatment temperatures used in this study were 160ºC, 180ºC and 200ºC. The treatments were conducted separately based on the treatment temperature. The fresh bamboo culms were cut into 40 strips with a size of 20 cm long x 5 cm wide (l x w). The strips were divided into 4 groups (30 samples for the treatments and 10 samples as the untreated), as shown in Fig.2. The initial weight of each strip was recorded prior to the treatments. The treatment media were heated in a tank to a temperature of 80ºC. The bamboo strips were then submerged in the heated oil for 10 minutes. It is crucial to make sure that the oil temperature is 80ºC before immersing the bamboo strips to prevent the oil from excessively penetrating into the strips and affecting the evaluations of the physical properties (Razak *et al*., 2005).

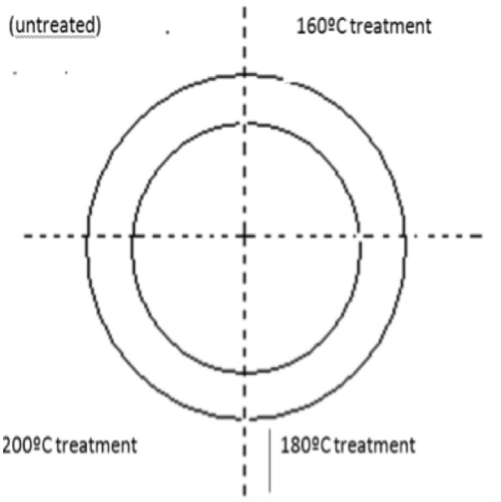

Fig.2: Bamboo culms were divided into four parts (1 part as the untreated, 3 parts for the treatments)

The bamboo strips were further heated until the crude oil temperature achieved the target temperatures (160ºC, 180ºC, or

200ºC). Once the assigned temperatures had attained, the bamboo strips were removed from the heating media, while the surfaces of the strips were blotted using a piece of cloth. Then, the treated strips were dried in an oven which was set with (103) ±2 ºC until a constant weight was attained.

Graveyard Tests

The effects were assessed by weight loss of the bamboo strips in accordance with ISO EN252 (Anon, 1990). The initial weight (W_i) of each strip (untreated or treated) was measured before the tests were conducted. The tests were carried out at a nursery in Universiti Malaysia Sabah. The treated strips were cut into smaller size (1cm x 10 cm) before they were oven-dried. Sixty samples of the same size were reserved for the graveyard tests. The above-ground graveyard test was performed by arranging the treated and untreated strips on the soil at the nursery. As for the in-ground graveyard test, half of the samples length was buried in the soil. Gaps of three centimetre wide should be established between each strip. Thirty strips were utilized for each of the graveyard tests. The bamboo strips were left at the site for 75 days. After 75 days, they were taken to the laboratory, cleaned and subsequently oven dried at 105 ± 2°C until a constant weight was reached before the final weight (W_f) was recorded. The observations of colour change, white rot, brown rot, as well as insect attacks on the samples were also conducted.

Assessments of the Physical Properties

The physical and mechanical properties assessments were done in accordance with ISO 22157-1:2004 (Anon, 2004). The parameters for the physical properties assessed were moisture content, basic density, percentage of swelling after the treatment and percentage of shrinkage at the oven temperature. Ten samples were utilized for each test. The relative density (RD) is the weight of the sample after conditioning at room conditions (Wo) divided by the weight of an equal volume of water (Wwd), as determined using water immersion method in accordance with ASTM D2395-93:1997 (Anon, 1997) (Equation 1):

$$RD\ (gcm^{-3}) = \frac{Wo}{Wwd} \qquad \text{Equation 1}$$

The moisture content (MC) test was conducted in accordance with ASTM D4442-07 (Anon, 2007). The samples were conditioned in a conditioning chamber at a temperature of 25±2 ºC and a relative humidity of 65 ± 2% before their initial weights (W_1), as well as dimensions were assessed. Then, all the samples were oven dried for 24 hours at 103 ± 2ºC. After oven drying, the samples were cooled in desiccators and their final weights (W_2) were measured. The data were used to calculate the moisture content using the following formula (Equation 2):

$$MC = ((W1 - W2) / W2) \times 100\% \qquad \text{Equation 2}$$

Shrinkage and Swelling

The swelling test was performed to study the degree of swelling experienced by the samples after the treatment. The

percentage of swelling (S) was calculated using Equation 3, as follows:

$$\text{Sw}\ (\%) = V_1 - V_2 \ / V_2 \times 100\ \%$$

Equation 3

Where, V_1 is the volume of the samples before the treatment and V_2 is the volume of the samples after the treatment. As for shrinkage (Sh), the test was performed to assess the percentage of the samples' shrinkage after being oven-dried. The formula is as follows:

$$\text{Sh}\ (\%) = Vol_1 - Vol_2 \ / Vol_1 \times 100\ \%$$

Equation 4

Where, Vol_1 is the volume of samples before oven dried and Vol_2 is the volume of samples after oven dried at 103 ºC±2 ºC for 24 h. The statistical analysis was carried out using the analysis of variance (ANOVA) to determine the differences in the properties between the treatment levels. The untreated samples were used for comparison purposes.

RESULTS AND DISCUSSIONS

Table 1 showed that moisture content (MC), relative density (RD), swelling (Sw), shrinkage (Sh), in-ground graveyard (IGGY) and above-ground graveyard (AGGY) tests were significantly affected by treatment temperatures. In particular, the colour of the bamboo was affected by the temperature of the treatment. The treatments changed the colour of the bamboo from light yellow to dark brown with the increase of the treatment temperature from 160°C to 180°C. The bamboo experienced slight burnt when the treatment temperature was increased to 200°C.

Graveyard Tests

The percentages of the weight loss for the samples tested with above and in-ground graveyard tests are summarized in Fig.3. For the above-ground graveyard test, the results revealed that with the increase in the treatment temperature, the weight loss of the samples decreased. Meanwhile, the mean weight loss for the untreated samples, 160°C, 180°C and 200°C-treated samples was 28.14%, 11.03%, 10.29% and 9.92%, respectively. A similar pattern of the results was recorded from the samples of the in-ground graveyard test. The mean weight

TABLE 1
The ANOVA of the heat-treated strips for the effects of different temperatures on the properties of *Bambusa vulgaris var. Striata*

Property	MC	RD	Sw	Sh	IGGY	AGGY
	Temp. (°C)	Temp. (°C)	Temp. (°C)	Temp. (°C)	Temp. (°C)	Temp. (°C)
F-value	80.07	16.31	7.03	6.77	20.25	26.77
P-value	0.0	0.0	0.0008	0.001	0.0	0.0
Significant level	*	*	*	*	*	*

*=significant at p≤0.05; Temp=temperature; MC=moisture content, D=density, Sw=swelling test; Sh=shrinkage test; IGGY=In-ground graveyard test; AGGY=Above-ground graveyard test

loss for the untreated samples was 28.14%, whereas those treated at the temperatures of 60°C, 180°C and 200°C were 10.21%, 7.55% and 3.39%, respectively. Damages by weathering in bamboo were the peeling off of the outermost layer and the vertical checks on the surface.

The decrease of weight loss indicates that the treatment temperature has improved the durability of the samples. This is expected due to the removal of starch, since fungus or pest depends on starch as their food to survive (Rafidah *et al*., 2010). Starch is an important factor for borer infestation and will infest as soon as the culm is felled, which is related to the presence of starch in the parenchyma (Liese, 1998). In addition, the treatment has helped to reduce white rot attack by removal of lignin. White rot consumes cellulose and permits lignin to live. The attack caused the colour of the samples to appear lighter than the original colour (i.e. the colour before they were tested with the in-and above-ground graveyard tests) due to the degradation of lignin by lignin-degrading enzymes produced by white rot (Vaithanomsat *et al*., 2010; Zaidon *et al*.,2000).

Moisture Content

The results for moisture content after treatment are given in Fig.4. The moisture content dropped sharply (more than 100%) when the treatment was applied. The mean moisture content values for the untreated and the samples treated at the temperatures of 160°C to 200°C were 18.6%, 3.55%, 3.43% and 2.38%, respectively.

The results showed that the treatment was effective in eliminating moisture from bamboo. Water elimination is essential to improve durability as decay-causing fungi are generally attracted to lignocellulosic materials that have high moisture content. Decay fungi work best when there are moisture (water), oxygen, and food (lignin, starch and cellulose) to survive (Acaron *et al*., 2010). The attack has been found to be more severe if the moisture content is between 27% to 30%, the condition which allows the spores of the fungi to germinate and develop, and hence causing rotting

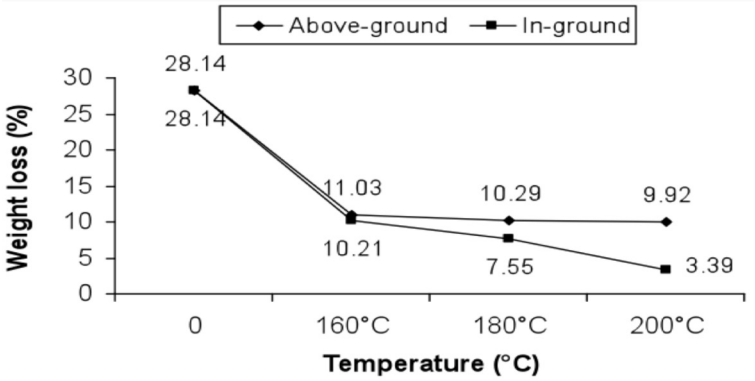

Fig.3: The Graveyard test on the relationship between weight loss and treatment temperature

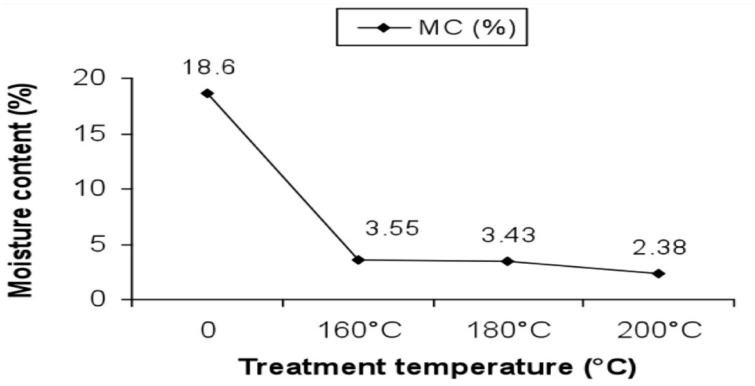

Fig.4: The Graveyard test between treatment temperature and percentage of moisture content

effect (Acaron *et al*., 2010). A high moisture content also attracts insects such as termites (Cowley, 2010; Smith, 2010) and beetles (Koehler & Oi, 2004) which can cause worse destructive effects.

Relative Density

The effects of the treatment temperature on the relative density of *Bambusa vulgaris var. Striata* are shown in Fig.5. It is shown that the relative density values are rather inconsistent between the different treatment temperatures. The control samples exhibited lower relative density value as compared to the ones that were treated at 160°C and 180°C. The samples treated at 200°C presented the lowest mean relative density value. The mean relative density value for the control samples was 0.823gcm^{-3}. Meanwhile, the mean relative density values for the treated samples tested at 160°C, 180°C and 200°C were 0.89 gcm^{-3}, 0.842 gcm^{-3} and 0.74 gcm^{-3}, respectively. The reductions of relative density values are usually related to the degradation of celluloses and hemicelluloses (Rafidah *et al*., 2010). The results showed that the degradation happened when the temperature was increased to 180°C. A further degradation was observed when the temperature was 200°C.

Swelling and Shrinkage

Fig.6 shows the swelling and shrinkage behaviours on the treated and untreated samples. The mean percentage of swelling for the untreated and the samples treated at 160°C 180°C 200°C was 16.28%, 6.29%, 7.64%, and 7.51%, respectively. Higher percentages of shrinkage [after oven dried] were documented, and these were 20.72%, 19.97%, 14.62% and 10.94%, respectively. Greater percentage of shrinkage compared to the percentage of swelling represents the effectiveness of the treatment on moisture elimination.

The results are similar the findings obtained for the moisture content which have revealed higher treatment temperature contributes to lower moisture content. The lower percentage of swelling also indicates that the boiling treatment degrades

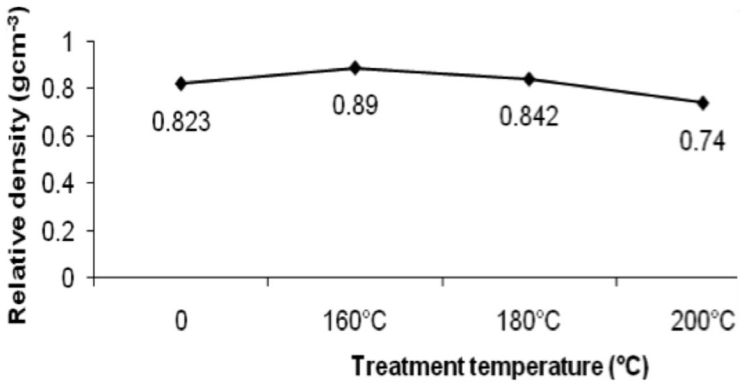

Fig.5: The Graveyard test between treatment temperatures and relative density

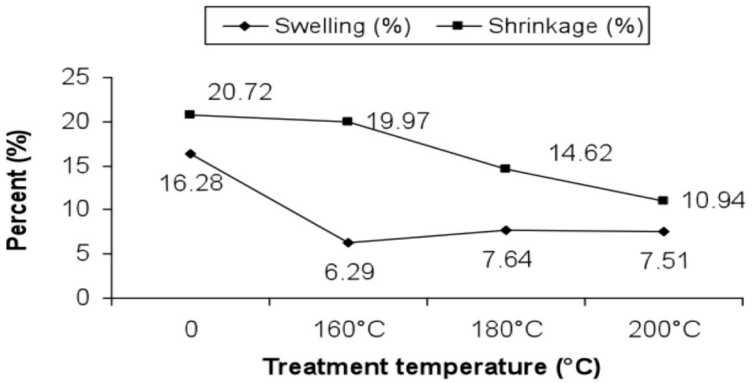

Fig.6: The Graveyard test on the percentages of swelling and shrinkage in relation to treatment temperature

hemicelluloses and modifies cellulose groups, and hence reduces the ability of the bamboo to absorb water (Zhao *et al*., 2010; Rafidah *et al*., 2010; Razak *et al*., 2004) after the treatment. The penetration of oil into the bamboo was also expected to have assisted in minimizing the absorption of water. Razak *et al.* (2005) have proven that palm oil penetration into bamboo cells happened during boiling treatment. It was proven by looking at the presence of palm oil between bamboo cells which was revealed through scanning electron microscopic images obtained from the project (Razak *et al.,* 2005).

CONCLUSIONS

Boiling treatment using crude palm oil was found to be effective in enhancing durability of bamboo against agents of bio-degradation such as insects and fungi. Similarly, treatment temperature has been shown to give significant effects to the increase of dimensional stability of the bamboo by removing moisture and minimizing hygroscopicity. The treatment media

is biodegradable and eco-friendly. The treatment can be used for bamboo and solid wood. Apparently, the advantages that the treatment has can give benefits to managers of wood-based industries who are searching for good preservation treatment. The treatment media is readily available at lower or more affordable price and requires minimum energy contribution, which can be very profitable for the managers. However, continual improvements on the treatment can be made to suit with the production processes which are normally practised in the industry. On-site observations or consultations still need to be done to achieve this.

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Study on *Waxy* Gene Polymorphism and Amylose Content of Breeding Lines Derived from *Oryza rufipogon x Oryza Sativa* CV. MR219

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ABSTRACT

Amylose in rice is controlled by granule-bound starch synthase, a protein encoded by the *Waxy* (*Wx*) gene. This study was conducted to identify the polymorphisms in the partial *Waxy* gene for two BC_2F_7 and nine BC_4F_3, breeding lines and their parents, *Oryza sativa* cv. MR219 and *O. rufipogon*, and also the association between the polymorphism of the partial *Waxy* gene and the amylose content. Sequences of all 13 breeding lines showed that the microsatellite $(CT)_{17}$ located 55bp upstream of the 5'-leader intron splice site and had G at the first nucleotide of the splice donor site of intron 1 of the *Wx* gene. The amylose content analysis revealed that all the samples with similar $(CT)_{17}$ were in the intermediate category (20–25%), except for one BC_2F_7 line (19%). The genotype determined using the microsatelites and SNP markers supports the intermediate category of the amylose content values.

Keywords: Waxy gene, amylose content, *Oryza rufipogon*

INTRODUCTION

Rice is a staple food for the majority of the world's population. As a primary source of carbohydrate, rice is the most important crop in Malaysia. The qualities of rice that appeal to human consumers are flavour, fragrance, and the texture of cooked rice (Henry *et al*., 2006). In Malaysia, intermediate amylose levels in the rice cultivars are considered a good quality (Lim *et al*., 1986). The amylose content in rice has been reported to vary from 15-35% (Ball *et al*., 1998). According to Juliano (1992), the categories

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for amylose content are waxy (0-2% amylose), very low (2-12% amylose), low (12-20% amylose), intermediate (20-25% amylose) and high (25-33% amylose). Rice with low amylose levels are usually tender, cohesive and glossy, while higher amylose cultivars are dry, fluffy and separated when cooked and these percentages are mediated by the proportion of amylose to amylopectin in starch granules. The chemical structure of amylose is linear, as opposed to the highly branched amylopectin that determines the textural properties conferred by starch gelatinization (Juliano, 1971). Unnevehr *et al*. (1992) have shown that the rice in Malaysia contains amylose in the intermediate to high categories. However, local consumers prefer rice with intermediate amylose content. Hence, the high amylose content of the locally produced rice should be improved to better suit the consumers' taste. Pooni *et al*. (1992) suggested that the amylose content might be related to the effects of the maternal plant or cytoplasm, whereas Xu *et al*. (1995) reported that rice amylose content was mainly controlled by the triploid endosperm genotype without any cytoplasmic effect.

In rice, amylose is synthesized by granule-bound starch synthase (GBSS), which is also known as *Waxy* (*Wx*) protein (Jahan *et al*., 2002). This protein is encoded by the *Wx* gene (Tan *et al*., 1999). Non-waxy rice cultivars commonly have two different alleles at the *Wx* locus, namely Wx^a and Wx^b. The alleles encode different levels of GBSS, and is thus involved in controlling the amylose content. The Wx^a allele is predominant in non-waxy *indica* cultivars, while the Wx^b allele is common to the non-waxy *japonica* variety (Yamanaka *et al*., 2004).

The *Waxy* gene has a size of 5499bp and it consists of 13 exons with a 1.1 kb untranslated leader intron (Wang *et al*., 1995; Hirano & Sano, 1991; Umeda *et al*., 1991). A polymorphic microsatellite $(CT)_n$ has been identified in the *Wx* gene, located 55bp upstream of the putative 5'-leader intron splice site (Bligh *et al*., 1995). Tan and Zhang (2001) reported that the cultivars with low $(CT)_n$ repeats ($n \leq 14$) had high amylose content, while those with high $(CT)_n$ repeats ($n \geq 16$) had low and intermediate amylose contents. In addition, a single nucleotide polymorphism (SNP), G or T at the 5' leader intron splice site, influences the gene expression and causes variation in amylose content. In a study by Ayres *et al*. (1997), all of the strains with 18% or less amylose had the sequence AGTTATA, while all the strains with higher proportion of amylose had AGGTATA. These two polymorphisms have been shown to contribute to 81.2-91.2% of the amylose content (Ayres *et al*., 1997; Tan *et al*., 1999; Shu *et al*., 1999) and are regarded as the molecular markers to determine amylose content.

In the present study, the samples were compared based on their partial *Wx* gene sequences, covering both the microsatellite and SNP locations. The specific primer pairs (Waxy-F and Waxy-R) were used for polymorphisms detection in the selected breeding lines from BC_2F_7 and BC_4F_3 generation.

MATERIALS AND METHODS

The Plant Materials

The wild parent *O. rufipogon* (IRGC105491) and the high yielding cultivar *Oryza sativa* cv. MR219 were crossed bred to develop transgressive variants with MR219 being the recurrent parent. The two BC_2F_7 and nine BC_4F_3 variants used in the present study were selected based on the field performance (Bhuiyan, 2010). BC_2F_7 lines G19 and G33 have a mean amylose content of 22.6 and 19.0% (intermediate and low), respectively (unpublished data from Parviz Fasahat). *Wx* gene was only 800Kb away from the grain weight QTL on chromosome-6 (unpublished data from Ngu Mee Siing). Therefore, the nine breeding lines from the population BC_4F_3 (8-1, 10-5, 16-20, 9-12, 11-17, 15-5, 22-9, 29-5 and 29-7) were selected based on their grain weight data and also the level of introgression from *Oryza rufipogon* at the grain weight QTL region. Breeding lines 8-1, 10-5, 9-12, 15-5 and 16-20 have low or similar grain weight as compared to the recurrent parent, MR219. The breeding lines 11-17, 22-9, 29-5 and 29-7 have high grain weights comparable to that of the recurrent parent, MR219. The breeding lines have different levels of introgression from *O. rufipogon* for grain weight QTL on chromosome 6 (Table 1), but only the breeding line 11-17 carried grain weight QTL.

Amylose Content Analysis

The amylose contents of the nine BC_4F_3 samples were determined by the UPM-

TABLE 1
The mMean amylose content (%), number of $(CT)_n$ repeats and single nucleotide polymorphism (SNP) for the 13 samples

Samples	Designation	Level of introgession for grain weight QTL (%)	100 Grain-weight (g)	Amylose content mean (%)	(CT)n repeats	SNP
MR219	MR219	0	2.85	25.3	17	G
Oryza rufipogon	*Oryza rufipogon*	100	n/a	25.1	17	G
G19	R17-1-83-3-B-B	n/a	n/a	22.6	17	G
G33	R14-3-66-4-B	n/a	n/a	19.0	17	G
8-1	UKMRC6-8	50	2.75	20.2	17	G
9-12	UKMRC6-9	25	2.78	22.5	17	G
10-5	UKMRC8-10	0	2.73	21.4	17	G
11-17	UKMRC8-11	100	3.03*	20.6	17	G
15-5	UKMRC11-15	25	2.88	22.8	17	G
16-20	UKMRC13-15	0	2.80	21.4	17	G
22-9	UKMRC14-22	100	2.96	23.4	17	G
29-5	UKMRC19-29	25	2.97	20.9	17	G
29-7	UKMRC19-29	25	2.93	21.3	17	G

* Duncan's Multiple Range Test $P< 0.05$

BERNAS Food Analysis Laboratory, Universiti Putra Malaysia, Serdang, Malaysia. The samples were ground through a sieve and defatted by refluxing with methanol for 6 hours in a soxtec extraction unit (Soxtec™ 2050, FOSS Analytical, Denmark). After defatting, flour was spread and left for two days at room temperature to allow evaporation of residual methanol. Meanwhile, 0.1 gram of the defatted samples was transferred into a 100 ml volumetric flask. Nine ml of 1M sodium hydroxide solution was added to each sample. After adding 1 ml ether, the mixture was heated in a boiling water bath for 10 min. The samples were covered and allowed to stand at room temperature overnight. The volume was adjusted with distilled water and mixed. Then, the amylose content was determined with FIA (flow injection analyser) (FOSS Co., Sweden). The sample was injected into a carrier stream of diluted sodium hydroxide and mixed with the iodine colour reagent and an acetate buffer. Finally, the colour of the iodine-starch complex was measured in a flow cell.

The Wx Microsatellite Allele and Wx Gene Analysis

The leaf samples of the breeding lines were collected at the age of 14 days. DNA was extracted following modified chloroform based on the DNA extraction protocol by Murray and Thompson (1980) and QIAGEN plant minikit.

The PCR amplification of the *Wx* microsatellite was performed using the primer pairs, namely, Waxy-F (5'–ACCATTCCTTCAGTTCTTTGTCT-3') and Waxy-R (5'-TAGCATGTAT GAGACTACTTGTAA-3'). These primer pairs have been reported previously and flanked the beginning of exon 1 and the beginning of intron 1 (Prathepha, 2003). The volume of PCR reaction mixture was 30 µL, containing 25 ng DNA template, 0.2 mM of each primer pair, 1.5 mM $MgCl_2$, 0.1 mM dNTPs and 1.5 U Taq DNA polymerase (Intron). The PCR profile included pre-denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min 30 sec. The final extension was at 72°C for 5 min. Meanwhile, the PCR amplified products were analyzed on 1.5% agarose gel at 90 V for 1 h and visualized under UV light after staining with ethidium bromide (AlphaImager™ 2200). The sizes of the fragments were determined by comparing them with the mobility of 100 bp DNA ladder (Biolabs). The PCR products were then purified using QIAquick Purification Kit (QIAGEN). The purified PCR products were sent to First BASE Laboratories Sdn. Bhd. for sequencing. For each sample, the PCR and forward/reverse sequencing reactions were repeated three times for sequence confirmation. The results were analyzed using Sequence Scanner and ClustalW (2.0.12) multiple alignment tool (http://www.ebi.ac.uk/Tools/clustalw2).

RESULTS

Amylose Content Analysis

The amylose content analysis showed that G33 had the lowest amylose content (19%)

among the 13 samples, while the other 12 samples contained intermediate amylose content ranging from 20.2% to 25.3% (Table1).

The Wx Microsatellite Allele and Wx Gene Analysis

The amplified PCR products of the 13 samples were approximately 250 bp in length, as shown in Fig.1. Based on the DNA sequences, both the microsatellites and SNP were successfully detected (Fig.2). All the 13 samples have the same genotype, $(CT)_{17}$ at the SSR locus, and nucleotide G at the SNP locus.

DISCUSSION

The amylose content of rice is the most important determinant of the cooking quality affecting the textural and organoleptic properties (Juliano, 1971). The textural qualities such as grain cohesiveness, tenderness and glossiness are affected by starch gelatinization properties of rice. The degree of water absorbed by starch granules on heating is affected by the amylose content and its linear structure. Higher amylose content facilitates extensive hydrogen bonding, contributing to the crystallinity of structure which are more resistant to swelling on heating but associated with more water absorption, greater grain expansion during cooking but upon cooling, grains become dry, fluffy and separate. In contrast, starch granules with less amylose gelatinize rapidly and are associated with greater grain gelatinization, stickiness, and glossiness.

The amylose content analysis showed that all the 13 samples could be categorized

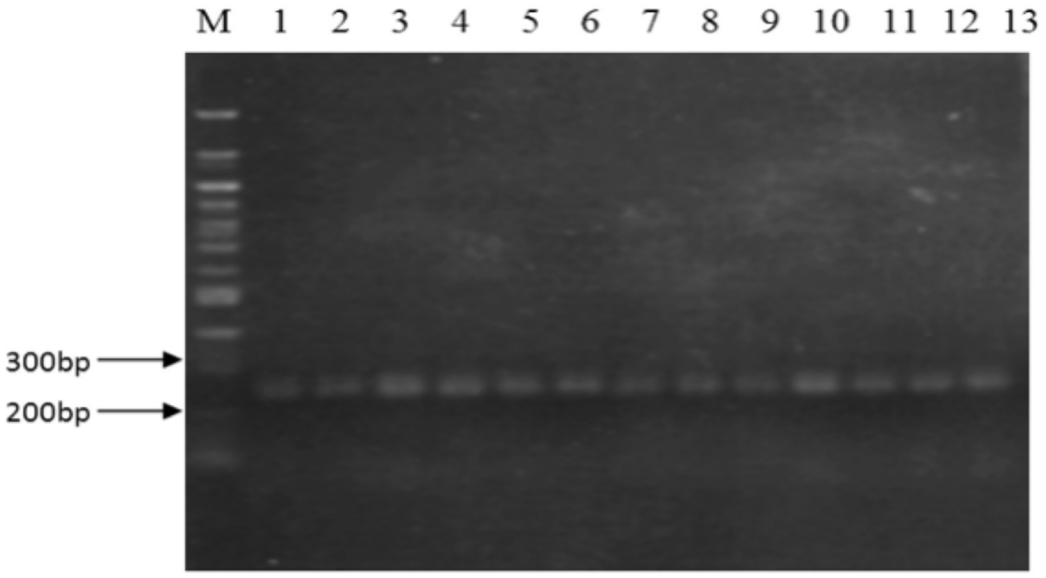

Fig.1: Amplified PCR products of 13 samples. M=100bp ladder. Well 1 to 13 = 8-1, 10-5, 16-20, 9-12, 11-17, 15-5, 29-5, 22-9, 29-7, G33, G19, *Oryza sativa* cv. MR219 and *O. rufipogon* (IRGC105491)

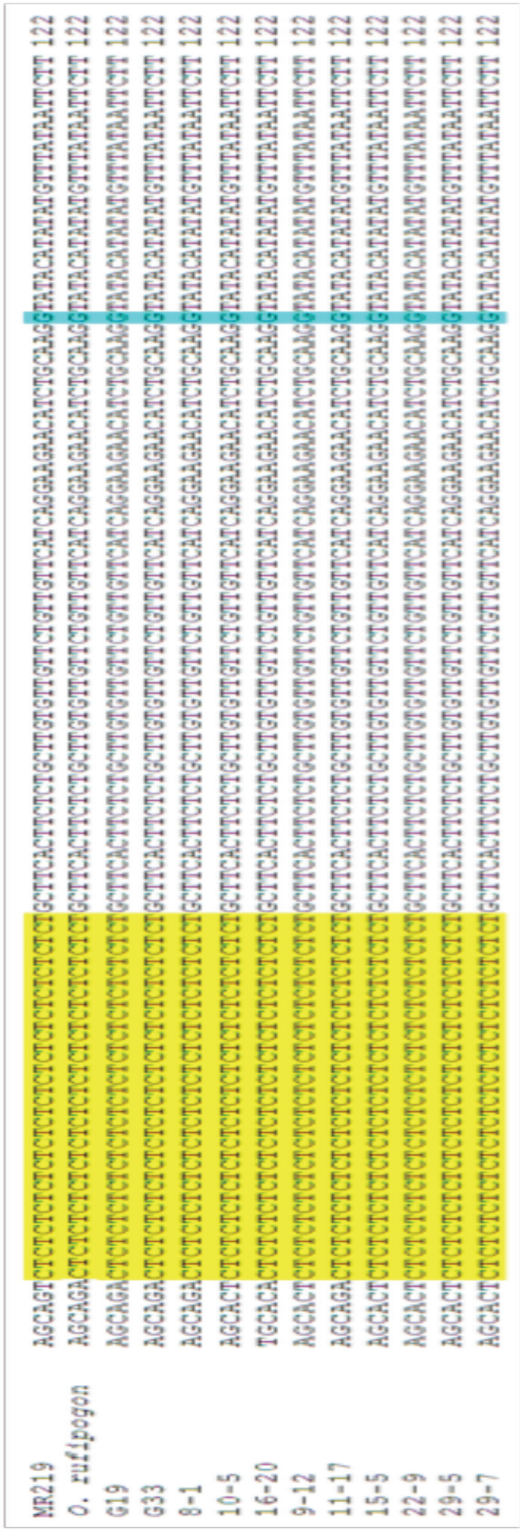

Fig.2: The 122bp fragments, amplified with primer Waxy-F and Waxy-R, were sequenced and compared among 13 samples for the *Waxy* gene. The CT repeats and single nucleotide polymorphism (SNP) are indicated in highlights

as intermediate (20-25%), except for G33. MR219 and *O. rufipogon* have the mean values above the top and bottom range values of the intermediate category. These results correspond to the findings of some previous studies, whereby the rice cultivars with (CT)n repeats, n≥16 would have low to intermediate amylose contents (Tan & Zhang, 2001), and the rice cultivars with base G at the SNP location had more than 18% amylose content (Ayres *et al*., 1997). Jayamani *et al*. (2007) reported that the accessions with $(CT)_{17}$ and $(CT)_{18}$ had both the AGGTATA and AGTTATA sequences. The microsatellite classes $(CT)_{17}$, and $(CT)_{18}$ had similar levels of amylose content (approx. 21%) that were subdivided into 17T/17G and 18T/18G haplotypes, and recorded 20.0/24.3 and 19.7/25.3% amylose content, respectively. Prathepha and Baimai (2004) also reported that non-glutinous rice with intermediate (20-25%) and high amylose content (25%) had the base G at the SNP locus.

Wx gene was very near to a grain weight QTL on chromosome-6 (800kb of physical distance according to the rice genome automated annotation database, http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/) and could increase the amlyose content of the BC_4F_3 breeding lines in the process of population development. However, there is no increase in the amylose content or *Wx* gene polymorphism observed for BC_4F_3 breeding lines with different grain weights. Therefore, these breeding lines, though having introgression from *O. rufipogon* and higher grain weight, have intermediate level of amlyose content which is preferred by local rice consumers. In this study, there was no correlation observed between *Wx* gene and grain weight QTL on chromosome 6.

MR219 and *O. rufipogon* (IRGC105491) had very similar amylose content (25.3 and 25.1%, respectively). As a wild rice species, *O.rufipogon* is expected to have higher amylose content as compared to cultivated rice. Nonetheless, the *O. rufipogon* accession used in this study might have been inter-crossed with *O. sativa* and then gone through many generations of selfing resulting in the lower amylose content. Prathepha (2008) reported that the mean values of amylose content of 212 *O. rufipogon* accessions from Thailand ranged from 12.5-28.1%, and the rice-to-wild gene flow could have a significant impact on the wild populations.

In addition, the amylose content of rice also has a bearing on the consumers' health in relation to the mediating glycemic response after ingestion, which is measured as glycemic index (GI) (Brand-Miller *et al*., 2009; Foster-Powell *et al*., 2002). Rice varieties have been classified as either low-, moderate- or high-GI, depending on the amylose content (Juliano & Goddard, 1986; Behall & Howe, 1995). Some studies indicate that higher amylose contents result in lower glucose and insulin responses (Hallfrisch & Behall, 2000), whereas others suggest that the amylose content alone may not be a good predictor of glycemic response (Panlasigui *et al*., 1991).

Intermediate levels of amylose content in all the breeding lines showed that there is no correlation between the *Wx* gene and grain weight QTL. Thus, determining the amylose content in the breeding lines helps to produce rice which can be favourable to Malaysian consumers as well as worldwide consumers.

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The Effect of Processing Treatments on the Shelf Life and Nutritional Quality of Green Chilli (*Capsicum annuum* L.) Powder

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ABSTRACT

An attempt was undertaken to investigate the effect of various common processing treatments, such as (a) without pedicle and cut longitudinally plus treated with 0.01% potassium metabisulphite (KMS), (b) without pedicle and sliced, (c) without pedicle as a whole, and (d) as a normal whole green chilli with pedicle, on the shelf life during storage in high density polyethylene (HDPE) and low density polyethylene (LDPE) packages at room temperature. The nutritional quality in terms of proximate compositions, Vitamin-C, beta-carotene and mineral contents of green chilli powder were also assessed. The chilli powder from the treatment (a) showed the highest stability up to 195 days in the HDPE pouches. In relation to proximate compositions and mineral contents, the processing treatments had a significant effect on them, except for Vitamin-C content at $P<.0001$. The results showed that the nutritional quality in all the samples of green chilli powder was better than that of the red chilli powder. Vitamin C content was reduced around 50% in all the samples due to the processing, while beta-carotene content was significantly increased as compared to the fresh green chilli. A simple calculation revealed the potential of green chilli powder as a value added and alternative spice.

Keywords: Green chilli powder, spice, shelf life, mineral content, value addition

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INTRODUCTION

Chilli is generally found to be used in three forms, namely, as fresh green chillies, red grind and raw red. Usually, red chillies are dried in the open sun without any pre-treatment in Bangladesh (Elias & Hossain, 1984). It was reported in the FAO Bulletin (FAO, 1995) that as a general rule, chillies are dried as whole pods without cutting or slicing because the whole pods are more attractive to the consumers than the sliced pods. Red chilli drying and its processing in the form of powder are very common all over the world. Many researchers have studied the processing and preservation of red chillies and reported the nutritional compositions in terms of the proximate analysis, Vitamin-C content and mineral contents (Saimbhi *et al.,* 1977; Khadi *et al.,* 1987; Esayas, 2011; Anon., 2002). Notably, there are very limited research reviews on the drying, processing and preservation of green chillies. Mechanical dehydration of green chillies has been performed by Luhadiya and Kulkarni (1978), Hossain and Bala (2000), as well as Srivastava *et al.* (2006). In fact, the processing of green chillies in the form of powder is still a very new technology. Meanwhile, the preservation of green chillies in the form of paste and mixed pickles has been studied by Ahmed *et al.* (2001) and Molla *et al.* (2007), respectively. Recently, the feasibility of green chilli processing and preservation in the form of powder has been reported by Sarker (2008) and Tummala *et al*. (2008).

In Bangladesh, chillies are ranked first in area and second in production among the spices. The cultivated area and production of chillies in 2006-2007 were 68096 hectares and 154000 tons, respectively (BBS, 2007). During the peak to the end of the harvesting season (February-March), the local variety green chillies are found to be wasted at the farm level due to the lack of proper processing and preservation technology in Bangladesh. The price of green chillies at this period falls to Tk5 to 10 (US$0.07 to $0.14) per kg, while the market price rises up to 10 to 20 times higher than this price during the off season. Green chilli growers are deprived from getting the actual price, and thus, its cultivation sometimes becomes non-profitable to them. Therefore, the processing and preservation of green chillies are very important in order to minimize the field losses as well as enhance the value addition, thus, contributing to the national economy of Bangladesh. In economics, the difference between the sale price and the production cost of a product is the value added per unit.

Before the commencement of this research, there was no sufficient information in scientific literature on the chemical compositions including the mineral contents of green chilli powder and its shelf life in poly packages. Since processing treatments influence chemical constituents, therefore, the present study was designed and undertaken to investigate the effect of common processing treatments on the shelf life and nutritional quality of green chilli powder. This study also aimed to identify an easy and low-cost technique for processing and preserving the green chillies. The

findings from this study may also justify the usage of green chilli powder as a potential value added spice as well as nutritional supplement.

MATERIALS AND METHODS

Collection and Preparation of the Samples

Matured but not over-matured green chillies of a local variety named 'DhaniMorich' (Dinajpur, Bangladesh) were collected from the farmer's field for the purpose of this research. The drying shed (bamboo mat), two types of polyethylene pouches [i.e. low-density polyethylene (LDPE) and high-density polyethylene (HDPE)] and KMS (Potassium Meta-bi-sulphite) were obtained from the local market.

The fresh green chillies were cleaned and washed manually in this study. Then four samples as per type of the processing treatments were prepared as follows; Sample-A: without pedicle, cut longitudinally and treated with 0.01% KMS, Sample-B: without pedicle and sliced, Sample-C: without pedicle as a whole, and Sample-D: as a normal whole green chilli with pedicle. A total of 40 kg samples of 10 kg per treatment were simply dried using the sun drying method by scattering them on a bamboo mat. During drying, air temperature and relative humidity were observed in the range from 25 to 32°C and 55 to 65%, respectively. The dried green chillies were ground using a local grinding mill which is normally used for grinding the red chilli powder. The samples were then packed in poly packages and stored at room temperature for the shelf life study and further quality evaluation. All the analyses were done after 180 days of storage of the samples in HDPE.

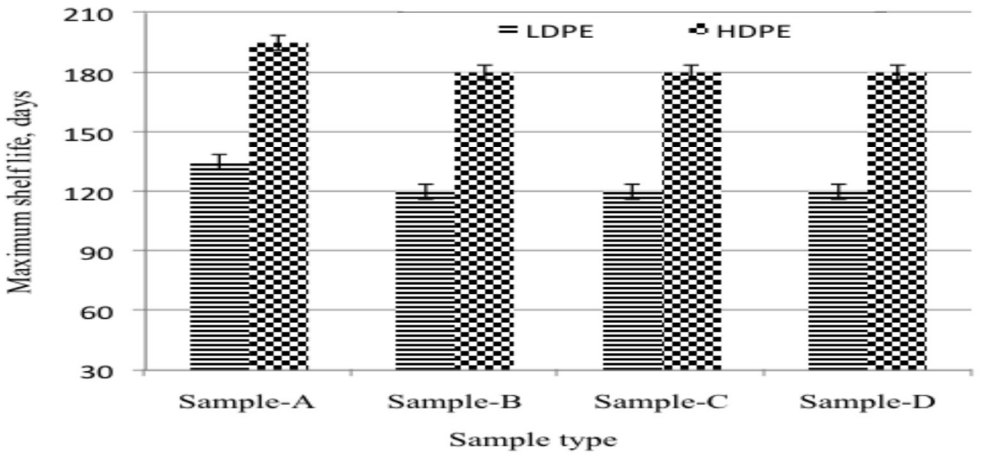
Shelf Life Study of Green Chilli Powder

The samples of chilli powder were manually packed in two types of polyethylene pouches. Chilli powder of 125 g was filled in each package having a dimension of 200 mm x 150 mm. There were 13 packs for each sample, including replications while the total number of pack was 52. All the packages were quickly sealed using an electric-impulsed hand sealer and then stored at room temperature (20 to 30°C) on a shelf at the laboratory. All the pouches were kept under shade to prevent any effect from direct sunlight and other heat sources. Every sample was drawn at each 15-day-interval for monitoring and evaluating of its shelf life simply by the physical observation of its colour, texture, flavour and moisture absorption, as consumers usually test these physical properties before buying any powdery spice. During the observation, the researcher would either tick (√) or cross (×) the evaluation sheet for acceptable and non-acceptable condition of the powder, as presented in Table 1. The maximum storage life of powder was considered as the time of storage having no significant change of its physical properties in terms of colour, texture and moisture absorption.

TABLE 1
Shelf life of green chilli powder in LDPE and HDPE pouches at room temperature

Sample	Storage periods (days)											
	120		135		150		165		180		195	
	LDPE	HDPE	LDPE	HDPE	LDPE	HDPE	LDPE	HDPE	LDPE	HDPE	LDPE	HDPE
Sample-A	√	√	√	√	×	√	×	√	×	√	×	√
Sample-B	√	√	×	√	×	√	×	√	×	√	×	×
Sample-C	√	√	×	√	×	√	×	√	×	√	×	×
Sample-D	√	√	×	√	×	√	×	√	×	√	×	×

'√' and '×' indicate the acceptability and non-acceptability of the sample, respectively in both LDPE and HDPE pouches during the storage period. LDPE=Low density polyethylene and HDPE=High density polyethylene. Sample-A: without pedicle, cut longitudinally and treated with 0.01% KMS, Sample-B: Without pedicle and sliced, Sample-C: Without pedicle as whole and Sample-D: Normal whole green chili with pedicle



(LDPE = Low density polyethylene and HDPE = High density polyethylene.
Sample-A: without pedicle, cut longitudinally and treated with 0.01% KMS,
Sample-B: Without pedicle and sliced,
Sample-C: Without pedicle as whole and Sample-D: Normal whole green chilli with pedicle)

Fig.1: The maximum shelf life of green chilli powder in LDPE and HDPE pouches stored at room temperature

Determination of Compositions of Fresh Green Chilli, Green Chilli Powder (Four Samples) and Red Chilli Powder

The proximate analysis for the fresh green chillies and dried green chilli powder (four samples) and red chilli powder of the same variety was done to determine the moisture, fat, protein and ash contents, as per method recommended by the Association of Official Analytical Chemists (AOAC) and Ranganna (1986). The fat content was determined by the solvent extraction method using the Soxhlet apparatus (Model Acm-54097, India). The percentage of protein (N × 6.25) present in the samples was determined by the Micro-kjeldhalmethod (Esayas, 2011). Meanwhile, the total ash content was determined using the Memmert drying oven (model 40050), whereas carbohydrate was calculated by the difference method. Vitamin C was determined by the titration method while beta-carotenecontent was determined using a spectrophotometer according to the conventional method of Srivastava (1994).

Determination of the Mineral Content of Green Chilli Powder

The most common mineral contents (Ca, Mg, K, P, S, Fe, Mn and Zn) of the different samples packed in HDPE were determined using the Atomic Absorption Spectrophotometer (AAS), VARIAN model AA2407, California, USA.

Quality and Sensory Evaluation of Green Chilli Powder

The colour, taste, flavour and overall acceptability of the green chilli powder were evaluated by a panel of 10 experienced taste panellists (Tummala *et al.,* 2008). The taste panellists gave scores for their preferences of colour, flavour, taste and overall acceptability. The sensory evaluation of the green chilli powder was carried out by preparing these two common items, namely, beef curry and potato mash with other normally used spices. The two items were analyzed for the sensory evaluation by a panel of 10 trained judges, to whom the items were supplied earlier and prepared with the red chilli powder. The panellists evaluated the samples admixed with cooked rice at the beginning and the end after six months of storage of the samples. The scores were noted over a hedonic scale with a maximum score of 9 for "like extremely" and minimum of 1 for "dislike extremely". The hedonic rating test in a scale of 1-9 marking was given as follows: like extremely (9), like very much (8), like moderately (7), like slightly (6), neither like nor dislike (5), dislike slightly (4), dislike moderately (3), dislike very much (2), and dislike extremely (1).

Statistical Analysis

All the means of the triplicate values and standard deviations from the obtained data were calculated and statistically analyzed using SAS version 9.1. Meanwhile, Duncan's multiple range test was employed to determine the differences in the different compositions among the samples.

RESULTS AND DISCUSSION

Shelf Life of Green Chilli Powder

The results of the shelf-life study are presented in Table 1 and Fig.1. All the samples exhibited acceptable shelf life in both types of the packages up to 120 days of storage. Table 1 displays the data startingfrom this period. The powder from Sample-A (i.e. without pedicle, equally cut along the length and potassium meta-bi-sulphite treated) showed the highest stability up to 195 days in the HDPE pouches. This might be due to the packaging material having less permeability and variation of its processing treatment. Besides, potassium meta-bi-sulphite, which is a proven preservative against yeast and moulds in dried products, might enhance the shelf life of the green chilli powder. However, a satisfactory shelf life up to 180 days was observed in HDPE for the other samples. A minimum shelf life up to 120 days was noticed in all the samples when packed in LDPE packages because of the rapid change in physical properties of powder, such as colour, flavour, as well as texture and moisture absorption. An earlier study also reported similar results during the storage of red chilli powder in HDPE (Remya, 2007). Nonetheless, a further study should be carried out to check the shelf life of this powder up to the maximum time by preserving in other packages such as vacuum, metallic and high quality poly packages.

Proximate Analysis, Vitamin-C and Beta-carotene Content ofChilli and Chilli Powder

The fresh green chillies, the green chilli powder of the four samples and the red chilli powder were analyzed in this study. Their chemical compositions are presented in Table 2. Similar proximate results were observed in the fresh green chillies, green chilli powder and red chilli powder of three Ethiopian varieties (Anon, 2002; Esayas *et al*., 2011; Tummala *et al.*, 2008), respectively. However, significant variations in the fat content in comparison with Tummala *et al.*'s (2008) data were noticed, and this was probably due to varietal and methodological differences. However, the results showed that the nutritional quality in an average of all the samples of the green chilli powder was better or similar compared to the red chilli powder. Vitamin-C content was reduced around 50% in all the samples due to drying and processing. On the other hand, beta-carotene content was found to be significantly increased after drying and processing, and this might be due to the increase in its concentration for removing moisture.

Mineral Content of Green Chilli Powder

The common mineral contents in the green chilli powder obtained from the four samples are displayed in Table 3. The results gave interesting information that all the samples of the dry powder contained a very good amount of minerals. It is noted that no published research literature has

TABLE 2
The compositions of fresh green chilli, green chilli powder and red chilli powder

Sample Name and No.		Proximate Analysis					Vitamin-C (mg/100gm)	Beta-Carotene(µgm/gm)
		Moisture Content (%)	Fat (%)	Protein (%)	Ash (%)	Carbohydrate (%)		
Fresh green chilli		85.54± 0.78^{a}	1.05 ± 0.14^{c}	5.42 ± 0.43^{c}	0.582 ± 0.07^{c}	7.41 ± 0.87^{d}	110 ± 2.21^{a}	3.914 ± 0.26^{c}
Green chilli powder	Sample-A	8.47 ± 0.17^{d}	2.67 ± 0.20^{a}	7.93 ± 0.68^{b}	10.64 ± 0.67^{b}	70.29 ± 0.9^{a}	67.6 ± 2.99^{b}	11.89 ± 0.89^{b}
	Sample-B	9.05 ± 0.28^{cd}	2.05 ± 0.15^{b}	8.05 ± 0.45^{b}	9.74 ± 0.73^{b}	71.11± 1.23^{a}	59.08± 0.47^{b}	13.80 ± 1.03^{a}
	Sample-C	8.80 ± 0.30^{cd}	2.58 ± 0.33^{a}	7.87 ± 0.28^{b}	11.06 ± 0.81^{b}	69.68 ± 0.76^{a}	62.48 ± 6.87^{b}	11.90 ± 0.71^{b}
	Sample-D	9.45 ± 0.22^{c}	2.50 ± 0.28^{ab}	9.97 ± 0.46^{a}	13.92 ± 0.93^{a}	64.15 ± 0.70^{c}	66.06 ± 3.68^{b}	14.40 ± 0.65^{a}
Red chilli powder		10.68 ± 0.23^{b}	2.06 ± 0.37^{b}	8.78 ± 0.51^{b}	10.57 ± 0.64^{b}	67.9 ± 0.54^{b}	60.80 ± 6.92^{b}	12.40 ± 0.95^{b}

Values are mean ± SD of three replicates $^{a-d}$The test values along the same column carrying different superscripts foreachcomposition content are significantly different ($p < 0.05$).
Sample-A: without pedicle, cut longitudinally and treated with 0.01% KMS; Sample-B: without pedicle and sliced; Sample-C: without pedicle as a whole; and Sample-D: as normal whole green chilli with pedicle

TABLE 3
Mineral contents in the green chilli powder

Sample No.	Minerals in mg/100gm powder							
	Ca	Mg	K	P	S	Fe	Mn	Zn
Sample-A	500 ± 4.58^{c}	430 ± 5.57^{b}	1810 ± 14^{d}	620 ± 10.0^{c}	450 ± 5.56^{a}	54 ± 4.0^{b}	5.7 ± 0.50^{b}	3.9 ± 0.30^{b}
Sample-B	350 ± 1.0^{d}	320 ± 7.0^{c}	2700 ± 11.13^{a}	680 ± 11.8^{a}	310 ± 5.29^{c}	45 ± 4.58^{c}	3.9 ± 0.40^{c}	4.5 ± 0.26^{ab}
Sample-C	650 ± 2.65^{a}	680 ± 4.36^{a}	2300 ± 13.23^{b}	650 ± 8.89^{b}	350 ± 8^{b}	66 ± 4.0^{a}	8.4 ± 0.50^{a}	5.1 ± 0.56^{a}
Sample-D	560 ± 3.0^{b}	430 ± 2.0^{b}	2100 ± 17.53^{c}	610 ± 11.14^{c}	300 ± 7.8^{c}	54 ± 3.6^{b}	5.7 ± 0.46^{b}	3.9 ± 0.61^{b}

Values are means ± SD of three replicates. $^{a-d}$The test values, along the same column carrying different superscripts foreach mineral content, are significantly different ($p < 0.05$).
Sample-A: without pedicle, cut longitudinally and treated with 0.01% KMS; Sample-B: without pedicle and sliced; Sample-C: without pedicle as a whole; and Sample-D: as normal whole green chilli with pedicle

been found regarding the mineral contents in green chilli powder. It is interesting to note that the mineral contents of the green chilli powder undertaken in this study seemed to be much higher than the results found in the red chilli powder as reported by Saimbhi *et al.* (1977), Khadi *et al.* (1987) and Esayas (2011). These variations may be due to multiple factors, such as the differences in soil conditions where it is grown, variety of chilli, maturity of chilli, growing season, climatic condition, processing treatments and preservation method.

A significant variation in the mineral contents was also observed among the samples. The highest amount of mineral contents in mg/100gm found in samples A, B, C and D were 450 S, 2700 K; 680 P; 4.5 Zn, and 650 Ca; 680 Mg; 5.1 Zn, respectively. In fact, it was difficult to identify which processing treatment was better in terms of mineral contents through the assessment of all these mineral contents. However, a significant effect of the processing treatment on individual mineral content was found at $P<0.0001$.

Sensory Evaluation of Green Chilli Powder

The analysis of variance showed that there was a significance of processing treatment on every sensory attribute at $P<0.0001$. The degree of differences among the samples was evaluated by Duncan's Multiple Range Test (DMRT), as shown in Table 4. From the DMRT result, it was observed that the chilli powder prepared from the chilli pods without pedicle, equally cut along the length and KMS treated revealed the highest scores for colour, flavour, taste and overall acceptability. Due to the longitudinal cut, the surface area for mass transfer during drying had been increased, and hence, Sample-A took a little bit shorter time to dry and it gave better colour and texture after processing. At the same time, KMS might also influence the preservation of colour. Tummala *et al.* (2008) reported similar results when the sample prepared from 1-cm cuts and longitudinal slits was ground without salt and preserved in a metallic polyethylene (MPE) during the storage period of 180 days. However, the powder

TABLE 4
The overall sensory quality of beef curry with green chilli powder at the end of 180 days of storage in HDPE

Sample No.	Sensory Evaluation Test			
	Colour	Flavour	Taste	Overall Acceptability
Sample-A	8.5 ± 0.53^{a}	8.3 ± 0.67^{a}	8.4 ± 0.70^{a}	8.5 ± 0.53^{a}
Sample-B	7.1 ± 0.88^{b}	6.8 ± 0.79^{b}	6.8 ± 0.79^{b}	6.9 ± 0.74^{b}
Sample-C	5.2 ± 0.79^{c}	4.4 ± 0.84^{c}	4.6 ± 0.84^{c}	4.8 ± 0.79^{c}
Sample-D	3.5 ± 0.53^{d}	3.7 ± 0.82^{c}	3.6 ± 0.70^{d}	3.4 ± 0.52^{d}

Values are mean of scores ± SD of 10panellists. [a-d] The test values along the same column carrying different superscripts for each attribute are significantly different ($p < 0.05$).
Sample-A: Without pedicle, cut longitudinally and treated with 0.01% KMS; Sample-B: Without pedicle and sliced; Sample-C: Without pedicle as a whole; and Sample-D: As normal whole green chilli with pedicle.

prepared from the chillies without pedicle slices and without the KMS treatment was found to be moderately favoured by the panellists.

Based on the above results and discussion, it can be summarized that fresh green chillies can easily be dried and processed as powder by slicing into two parts along the length and treated with KMS or without any chemical treatment. This powder can be used as spice and nutritional supplement either in curries for domestic cooking or for processing of commercial products, such as potato wafers, chips, finger fries, extruded products, sandwiches, pizzas, burgers and others. However, it can also be an alternative spice to red chilli powder and fresh green chillies which are more expensive for many practical purposes.

Value Addition by Processing and Preserving of Green Chilli Powder

The total green chilli powder produced from 40kg of fresh green chillies was around 6.25kg at 9% moisture content. The approximate processing cost was US$2.75 per kg of the green chilli powder. Meanwhile, the expected market price of the product was US$4.125/kg, and the cost of red chilli powder was more than US$4.125 per kg. Value addition was 4.125-2.75 = US$1.375/Kg powder. Thus, at least US$208 of value addition was possible by processing and preserving 1 ton of fresh green chillies in the form of powder.

CONCLUSION

Variations in nutritional quality in terms of proximate analysis and mineral contents and shelf life of green chilli powder, due to different processing treatments, were observed in this study. The powder prepared from the chilli pods without pedicle, sliced into two parts along the length and treated with 0.01% KMS was found to be better than that of the other samples in terms of the shelf life and sensory quality with acceptable nutritional values when it was packed in HDPE. Processing of green chillies in the form of powder and preserving it in low-cost poly packages were identified to be potential techniques to minimize field wastage of green chillies, and hence, chilli growers could benefit from these techniques. This powder can be used with many food adjuncts in place of fresh green chillies and red chilli powder. Further research is recommended for standardizing the efficient and sustainable technique for processing and preserving green chilli powder using other mechanical drying methods such as vacuum, oven drying, freeze drying, fluidized bed drying and others, both in domestic and industrial scales.

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Staphylococcus aureus in Food and Nares of Food Handlers in Kuala Pilah, Malaysia

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ABSTRACT

The outbreak of food poisoning due to *Staphylococcus aureus* has been reported with a significant level of morbidity worldwide. In this study, 128 samples taken from nasal swabs of 64 food handlers and 64 food items were collected and analyzed for the presence of *S. aureus*. The antibiotic susceptibility profiles of the isolates were also determined. Cross-sectional was used as a study design in this research. The isolates were identified as *S. aureus* based on colonial morphology, gram stain, mannitol salt agar fermentation, catalase and coagulase test. Fifteen (23.4%) of food handlers were positive for *S. aureus* nasal carriage and 24 (37.5%) of food items were contaminated with an average of 8.4×10^6 CFU/g of *S. aureus*. All isolates were susceptible to oxacillin and mupirocin respectively. However, 74.4% and 5.1% isolates were resistant to penicillin and erythromycin. Our findings suggest that the prevalence of *S. aureus* carriage is high among our food handlers and food items. Thus, we believed that there is a need for proper training on food safety among food handlers and quality improvement in the food premises.

Keywords: Food poisoning, food handlers, nasal carriers, *Staphylococcus aureus*

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INTRODUCTION

Food poisoning is one of the food borne diseases affecting many people worldwide. It occurs through the ingestion of certain microorganisms or their toxins (Campos *et al*, 2009). It is important to note that

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a wide variety of etiological agents have been associated with food poisoning such as viruses, bacteria and parasites (Gunduz *et al*., 2011). In general, *Staphylococcus aureus* has been reported to be a significant source of food borne infection worldwide due to its ability to produce several enterotoxins such as enterotoxin A, B, C1, C2, C3, D and E (Shimizu *et al*., 2000). Since these enterotoxins are heat-resistant (Reiser *et al*., 1984), high temperature can only eliminate *S. aureus* but not its toxins. However, the proliferation of this microorganism can be controlled by keeping the food items hot or cold to ensure its multiplication does not exceed 10^3 cfu/g, which is considered as harmless to humans (Loir *et al*., 2003).

Several control strategies have been implemented to prevent food poisoning, but so far the number of reported cases has not reduced. According to the Ministry of Health Malaysia (2009) there has been an increase of food poisoning cases from 2005 to 2008, and death cases due to food poisoning have also been reported. It is shown that 10% to 20% of food borne diseases is due to contamination by the food handlers (WHO, 1998). Inappropriate ways of handling the food and unhygienic practices by food handlers could potentially enhance the transmission of the disease to susceptible consumers.

S. aureus can colonize human interior nares (Zahoor & Bhatia, 2007), skin (Noble, 1998), oropharynx (Smith *et al*., 2001) and faeces (Arvola *et al*., 2006). This microorganism can also be transferred from food handlers to food items via unhygienic practices such as sneezing or coughing, touching the food without washing hands, neglecting the importance of a clean work area and exposing the food items at room temperature for too long.

Food handlers' knowledge, attitude and practices (KAP) influence the outcome of food preparation (Ansari-Lari *et al*., 2010; Baş *et al*., 2006; Zain & Naing, 2002). Therefore, having a good KAP can reduce the risk of contamination that leads to food poisoning. The aims of this study were to determine the prevalence *S. aureus* nasal carriers among 64 food handlers in the district of Kuala Pilah, Malaysia and the rate of 64 food samples contaminated with *S. aureus*. The antibiotic susceptibility profile of the isolates was also determined.

MATERIALS AND METHODS

The study area was Kuala Pilah, Negeri Sembilan which is located 77 km from Kuala Lumpur, Malaysia. It was selected due to the differences of geographical area, culture, as well as the backgrounds of food handlers.

A total of 64 food handlers were selected through a purposive sampling method with the help of district health officers in charge of inspecting the food premises. Sixty four food samples were also collected during this study.

Data Collection

Sociodemographic characteristics of the respondents, such as gender, age, educational level, work duration and premise grades were collected during the study. The respondents'

age groups were classified as "youth" (17 to 35 years old) and "adult" (36 years old and above), and the educational background was categorized as "low educational level" (received education up to secondary level) and "high educational level" (received education after their secondary level). The working experience was grouped into "experienced" (working for one year and more) and "inexperienced" (working for less than one year), and the cleanliness of working area was chosen as high grade premises (grades A and B) and low grade premises (grade C and no grade).

Isolation and identification of Staphylococcus aureus

The method used for collecting nasal swabs and food samples were modified from Kozioł-Montewka *et al*. (2006) and Huong *et al*. (2010). The nasal swab samples were taken using sterile cotton swabs with Stuart transport medium. The specimens were obtained from approximately 1cm inside nostrils and rotated five times (Askarin *et al*., 2009). The swabs were streaked on Mannitol Salt Agar (MSA) and incubated at 37°C for 24 to 48h.

Ten grams of the food samples were homogenized in 90 ml sterile saline water (0.85%), and inoculated on the MSA plate and incubated at 37°C for 24 to 48h. The identification of *S. aureus* was based on colonial morphology, gram stain, mannitol salt agar fermentation, catalase and coagulase tests (Acco *et al*., 2003; Lues & Tonder, 2007). American type culture collection (ATCC) 25923 *S. aureus* was used as the positive control and *S. aureus* ATCC 12228 was used as the negative control.

Antibiotic Susceptibility Testing (AST)

The sensitivity patterns of the isolates were performed using disk diffusion method according to the Clinical and Laboratory Standards Institutes (CLSI, 2010). Antibiotics disc used in this study were erythromycin 15 µg, oxacilin 1 µg, mupirocin 5 µg, vancomycin 30 µg, and penicillin G 10 units. The turbidity of the tested microorganisms was set according to 0.5 McFarland Turbidity Standard. The entire Mueller-Hinton agar was streaked with overnight culture and antibiotic discs were applied on the agar and incubated at 37°C for 24 h. The zones of inhibition were interpreted according to CLSI (2010).

Statistical Analysis

The data were analyzed using computer software Statistical Packages for Social Sciences (SPSS) version 16.0. The chi-square test was used to determine the significant relationship between the variables and the risk factors. The level of significance was set at $p<0.05$.

Ethical consideration

The Medical Research Ethics Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM) had approved the study. The respondents were informed verbally as well as in written form about the research. Consent from the respondents was taken prior to the collection of nasal swabs and food samples.

RESULTS AND DISCUSSION

Subjects

Sixty four respondents were involved in this study. A total of 75% of the respondents were female and 25% were males. Forty six of the respondents (71.9%) were youth whereas 18 (28.1%) were adult respondents. Majority of the respondents (68.8%) had lower educational level and the remaining (31.2%) had higher educational level. The inexperienced food handlers comprised 36%, while 64% were experienced food handlers. Only 29 (45.3%) respondents can be classified as food handlers working in high grade food premises and 35 (54.7%) of the respondents were food handlers working in low grade food premises (Table 1).

TABLE 1
Socio-demographic characteristics of the respondents (N = 64)

Variables	n (%)
Gender	
Male	16 (25)
Female	48 (75)
Age group	
Youth	46 (71.9)
Adult	18 (28.1)
Educational level	
Lower educational level	44 (68.8)
Higher educational level	20 (31.2)
Experienced level	
Inexperienced	23 (35.9)
Experienced	41 (64.1)
Food premise grade	
High grade food premises	29 (45.3)
Low grade food premises	35 (54.7)

Microorganism

Growth of yellow colonies was observed microscopically. All isolates that grew on the MSA were gram positive micro-organisms and appeared as cocci in clusters with a few of them appeared singly or in pairs. Identity of the *S. aureus* isolates on the MSA was confirmed through positive coagulase and catalase tests (Ahmad, 2010).

Staphylococcus aureus *Nasal Carriers Identified in Food Handlers and Food Contamination*

Based on Table 2, out of 64 food handlers tested, 23.4% were found to be positive *S. aureus* nasal carriers. The prevalence of *S. aureus* nasal carriage among the respondents was almost equal to the prevalence rate as reported by Neela *et al*. (2008), which was 20.9%. The prevalence rate was lower compared to other studies by Souza and Santos, 2009 (29.5%), Acco *et al*., 2003 (30%), and Oteri *et al*. 1989 (24%). However, this study reported a higher prevalence rate than the study by Gündüz *et al*. (2008), which was 0.77%.

Isolates were recovered from 24 (37.5%) of the 64 food samples. The number of *S. aureus* isolated from the samples ranging from less than 1.0×10^1 to 8.7×10^7 CFU/g with an average of 8.4×10^6 CFU/g. Fifty eight percent of the contaminated food samples contain more than 10^4 organisms/gram and this could potentially cause food poisoning (Loir *et al*., 2003). Food samples that are contaminated by this amount of *S. aureus* is considered unsafe to be consumed because the organism can produce sufficient

TABLE 2
Antibiotic susceptibility profiles of *S. aureus* isolated from the nasal swabs of food handlers and food samples

Source	No. of samples	No. of isolates (%)	Mean (range) CFU/g	Resistance to antibiotic (%)			
				OX	P	MUP	E
Food handlers' nasal swab	64	15 (23.4)	ND[a]	0 (0.0)	11 (73.3)	0 (0.0)	1 (6.7)
Food samples	64	24 (37.5)	8.4×10^6 ($< 1.0 \times 10^1$ – 8.7×10^7)	0 (0.0)	8 (33.3)	0 (0.0)	1 (4.2)
Total	128	39 (30.5)	-	0 (0.0)	29 (74.4)	0 (0.0)	2 (5.1)

[a] ND – not determined
OX: oxacillin, P: penicillin G, MUP: mupirocin, E: erythromycin

amount of enterotoxins. A study conducted by Normanno *et al*. (2005), showed a lower prevalence (17.3%) of *S. aureus* in food and food contact surface swab. In addition, Aydin *et al*. (2011) and Huong *et al*. (2010) reported 13.8% and 21.2% of food contamination by *S. aureus*, respectively. The high number of food contamination suggests a need for improving hygiene practices among food handlers to avoid an outbreak of food poisoning.

Antibiotic Susceptibility Test (AST)

S. aureus was isolated from 39 out of 128 of total samples (Table 2) of which 23.4% and 37.5% were isolated from nasal swabs of food handlers' and food samples, respectively. All isolates were sensitive to oxacillin and mupirocin. Most of the isolates were resistant to penicillin (74.4%), whereas only a few of the isolates were resistant to erythromycin (5.1%). Oxacillin was used in this study as a preliminary test to detect methicillin resistant *S. aureus* (MRSA) (Swenson *et al*., 2007). In addition, all isolates were sensitive to mupirocin. These findings are similar to the study done by Askarin *et al*. (2009) whereby *S. aureus* in this study was also sensitive to oxacillin and mupirocin.

The result of this study showed that 29 (74.4%) of the isolates were resistant to penicillin, while Vaez *et al*. (2011) reported 95.6% penicillim resistant in their *S. aureus*. These results supported the fact that 80% to 90% of *S. aureus* isolates are β-lactamase producers (Livermore, 1995; Smith & Jarvis 1999). In addition, only 5.1% of the isolates were resistant to erythromycin, and this result is in concordance with Vaez *et al*. (2011) who have shown only 6.7% of erythromycin resistance.

Relationship between Staphylococcus aureus *Nasal carriers and Sociodemographic Characteristics Food Handlers (Table 3)*

No significant relationship was observed between male and female respondents ($p = 0.505$), youth and adult respondents ($p = 0.336$), respondents from lower and higher

TABLE 3
S. aureus carriage pattern in the relationship with the socio-demographic characteristic of food handlers (N=64)

Variables	Non-carrier [n (%)]	Carrier [n (%)]	n (%)	χ^2	p	Prevalence ratio (C.I)
Gender						
Male	13 (81.2)	3 (18.8)	16 (25)	0.261	0.609	0.692 (0.168-2.850)
Female	36 (75)	12 (25)	48 (75)			
Age group						
Adult	15 (83.3)	3 (16.7)	18 (28.1)	0.640	0.427	0.567 (0.139-2.306)
Youth	34 (73.9)	12 (26.1)	46 (71.9)			
Educational level						
Higher	14 (70.0)	6 (30.0)	20 (31.2)	0.698	0.403	1.667 (0.500-5.559)
Lower	35 (73.9)	9 (20.5)	44 (68.8)			
Experience level						
Experienced	34 (82.9)	7 (17.1)	41 (64.1)	2.575	0.109	0.386 (0.118-1.259)
Inexperienced	15 (65.2)	8 (34.8)	23 (35.9)			
Food premise grade						
High grade	24 (82.8)	5 (17.2)	29 (45.3)	1.135	0.287	0.521 (.155-1.748)
Low grade	25 (71.4)	10 (28.6)	35 (54.7)			

educational levels (p = 0.533), inexperienced and experienced respondents (p = 0.051), as well as respondents from high and low grade food premises (p = 0.469) (Table 5). There was no variable that can predict the carriage pattern among the food handlers because the percentage of the carriers is small compared to the non-carriers. VandenBergh and Verbrugh (1999) stated that the nasal carrier status of *S. aureus* can be determined by genetic make-ups, sex, age, hormonal status in women, and anatomic alterations of the nares. This study found that gender and age were not significantly associated with *S. aureus* nasal carriers (p>0.05). Peacock *et al*. (2001) demonstrated that, the status of nasal carriers can also be determined by the receptors for bacterial adherence found in the nares, an immune response that can either eliminate or tolerate the *S. aureus* and also anti-staphylococcal constituents that are found in the nasal secretions. Hence, the determination of *S. aureus* nasal carriers involves many factors and it is still not well understood. However, food contamination can be prevented by good personal hygiene practices especially during food preparation and handling.

CONCLUSION

This study indicated that the prevalence of *S. aureus* among food handlers was high. Contamination of food was also high indicating lack of hygienic practices

among the food handlers. All the isolates were sensitive to oxacillin and mupirocin. However, none of the variables studied can significantly be associated with *S. aureus* nasal carriers among the respondents.

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The Effects of Culture Systems and Explant Incision on *In vitro* Propagation of *Curcuma zedoaria* Roscoe

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ABSTRACT

Three types of culture systems, solid medium system, liquid shake flask system, and temporary immersion system (TIS) were used for testing their efficiency in propagating *Curcuma zedoaria* plantlets. The proliferation medium used in shoot multiplication was the Murashige and Skoog medium supplemented with 0.5 mg/L 6-benzylaminopurine (BA) and 0.5 mg/L Indole-3-butyric acid (IBA). Among the three systems used, the liquid shake flask system significantly induced more shoot formation and larger shoots from the shoot explants of *C. zedoaria*. Meanwhile, divided shoot explants produced significantly higher number of shoots than the undivided shoot explants. The *in vitro* plantlets derived from the three different culture systems produced healthy and morphologically similar to the mother plants after acclimatization and being transferred to the field.

Keywords: Acclimatization, *Curcuma zedoaria,* liquid shake flask system, temporary immersion system

INTRODUCTION

Curcuma zedoaria Roscoe is one of the important medicinal plants belonging to the family *Zingiberaceae*. It is known as temu kuning in Malaysia and temu puteh in Java, Indonesia. In particular, *C. zedoaria* has anti-tumour (Kim *et al*., 2000), hepatoprotective (Matsuda *et al*., 1998), anti-inflammatory (Jang *et al*., 2001) and analgesic properties (Navarro *et al*., 2002). It is used as a colouring and flavouring agent (Islam, 2004). The rhizomes oil of *C. zedoaria* is used as a tonic and also in perfume. The rhizomes are traditionally used as appetizer, anti-helmenthic, anti-pyretic, for the treatment of leucoderma, piles, tuberculosis and also against enlargement of spleen (Bharalee *et al*., 2005).

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The market demand of *C. zedoaria* is continuously increasing due to its multiple uses. The conventional way of propagation cannot fulfill the increasing market demand. Besides this, the conventional propagation of *C. zedoaria* by rhizome cutting is also season dependent and it requires a long time to be built up for the commercial quantities. However, the *in vitro* culture techniques are used to overcome the present demands of most of the medicinal and aromatic plants (Rahman *et al*., 2004). Meanwhile, the tissue culture technique could be used as an alternative method for the mass propagation of *C. zedoaria* in the production of useful secondary metabolite. The *in vitro* propagation protocols have been successfully developed for many species of the *Zingiberaceae* family, such as *Alpinia galanga* (Borthakur *et al*., 1999), *Kaempferia galanga* (Shirin *et al*., 2000), *Curcuma longa* (Rahman *et al*., 2004), *Zingiber officinale* (Sharma & Singh, 1997; Khatun *et al*., 2003), as well as *Curcuma zedoaria* and *Zingiber zerumbet* (Stanly *et al*., 2010).

The most common method of micropropagation involves proliferation of the shoots via solid medium. However, this conventional way of propagation usually involves a high production cost and is time consuming. To overcome these problems, the use of shake cultures using liquid medium has been promoted. It is reported that liquid medium is ideal for micropropagation because it reduces production cost and is suitable for automation (Aitken–Christie & Davis, 1995). The liquid culture system can provide much more uniform culturing conditions and the culture media can be changed easily. Furthermore, it allows a close contact with the tissue which stimulates and facilitates the uptake of nutrients leading to better shoot and root growth. However, a continuous contact of the plant tissues with the liquid medium will normally cause hyperhydricity (Hussey, 1986) which is responsible for poor growth and substantial losses during and after *in vitro* culture. In contrast, temporary immersion system (TIS) has the advantages over the solid and liquid cultures. TIS generally improves the quality of plant tissues as they can perform better during the acclimatization process compared to the plantlets which are obtained from semi-solid or from liquid medium. Therefore, the main objective of the study was to develop an optimized protocol for *in vitro* propagation of *C. zedoaria*. The specific objectives were to compare shoot proliferation between solid, liquid and temporary immersion system (TIS) and to study the effects of explant type on the shoot proliferation of *C. zedoaria*.

MATERIAL AND METHODS

Plant Material and culture media

The *in vitro* shoot cultures of *C. zedoaria* obtained from the Plant Tissue and Cell Culture Laboratory, School of Biological Science, Universiti Sains Malaysia were used as the study material. The apical shoot explants were inoculated into a 200ml conical flask containing Murashige and Skoog (1962) solid medium supplemented with 0.5 mg/L 6-benzylaminopurine (BA)

and 0.5 mg/L Indole-3-butyric acid (IBA), the optimum shoot proliferation medium formulated by Stanly and Chan (2007). All the cultures were kept at 25± 2°C in a culture room under continuous lighting provided by cool white fluorescent tubes, with intensity of 40-42 mmol m^2 s^1. More shoots were proliferated by subculturing onto the same proliferation medium at every four-week interval.

For the solid medium, 7.5g/L agar (Algas, Chile) was added into the MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA. The same constituent was used for the liquid medium (without gelling agent). The pH of the medium was adjusted to 5.7 – 5.8 for both the liquid and gelled medium prior to autoclaving at 121°C for 11 minutes. A volume of 30 ml liquid medium was dispensed in a conical flask for shake flask system and 100ml of the liquid medium was used for the temporary immersion system.

Description of Temporary Immersion System (TIS)

Reusable Nalgene® polysulfone filtration system (Nalge Nunc International, USA, catalogue number-KH06730-52) was modified and used as the TIS vessel. The modified TIS consisted of two compartments, of which the upper compartment was occupied by the shoot explants and the lower one by the liquid medium. The two compartments of the Nalgene® polysulfone filtration system was modified by connecting each of the compartments with a tube fitted with a filter (0.2 µm) so that when the pressure was applied to the lower compartment, the medium would be pushed to the upper compartment. Thus, the plant materials in the upper compartment were immersed in the liquid medium as long as the pressure was applied. The pressure can escape through the outlet on the top of the vessel. This process helps to aerate the medium and agitate the plants. When the pressure is removed, the medium returns to the lower compartment. The air that enters the vessel is filtered through sterile 25mm nylon non-pyrogenic hydrophilic syringe filters (0.2 µm) (Sartorius) so as to prevent contamination.

The Effects of the Solid and Liquid Culture Systems on the Shoot Proliferation of Curcuma zedoaria

For this purpose, three aseptic shoots (1.5 cm) were cultured into each 200 ml conical flask containing 30ml of MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA, the shoot proliferation medium, and solidified with 7.5 g/L agar (Algas, Chile). For the liquid medium, three aseptic shoots were inoculated in a 200 mL conical flask containing 30 mL of the liquid shoot proliferation medium. The shake flasks were agitated on a rotary shaker (Panasonic G Series, Speed Controller, DUX940W) at 120 rpm. Five experimental units were used for the solid and liquid cultures, respectively. All the cultures were kept at 25± 2°C under a continuous cool white fluorescent light with an intensity of 40-42 mmol m^{-2} s^{-1}. The biomass and the number of shoots produced from each explant were

recorded after three weeks of culturing. The means were compared using Independent T-test at $p \leq 0.05$.

Temporary Immersion System (TIS) and Shake Flask System

The shoot biomass and the multiple shoots formation were compared between the temporary immersion system and the shake flask system. Five shoots were inoculated into each temporary immersion vessel containing 100ml liquid shoot proliferation medium with an immersion period of 15 minutes once a day. For the shake flask system, five aseptic shoots were inoculated into 250 ml conical flask containing 100ml liquid medium of the same constituents. Five units of temporary immersion vessels and shake flasks were used respectively. All the shake flask cultures were agitated on an orbital shaker at 120 rpm. The temporary immersion and shake flask cultures were maintained at 25± 2°C under continuous cool white fluorescent light, with an intensity of 40-42 mmol m^{-2} s^{-1}. After three weeks of culturing, the increases in the biomass and number of shoots produced from each explant were determined and the data were analyzed using the Independent T-test at $p \leq 0.05$.

The Effects of Shoot Incision on Shoot Proliferation

In order to investigate the effect of shoot incision on the shoot multiplication of *C. zedoaria,* the shoot explant used either undivided whole aseptic shoots or the shoot were divided into two halves, with each half shoot used as an explant. The divided and undivided shoots were cultured into a 250 mL conical flask containing liquid proliferation medium. Three shoot explants were used for each flask and six experimental units were used for each explant type. The increments in the biomass and the number of shoots per explant were recorded after three weeks of culturing and the data were analyzed using the Independent T-test at $p \leq 0.05$.

Rooting and Acclimatization

Seventy-five *in vitro* shoots were separated into individual micro-shoot and inoculated into basic MS medium for rooting. The rooted plantlets of *C. zedoaria* were thoroughly washed with tap water to remove traces of the nutrient medium. Old yellowish leaves were removed and the explants were transferred into plastic containers (30 x 40 cm) containing a mixture of organic soil and sand (1:1) with a relative humidity of 80-90% under green house conditions (28 ± 2°C during day time and 24 ± 2°C during night time).

RESULTS AND DISCUSSION

Three types of the culture systems, solid medium system, liquid shake flask system, and temporary immersion system (TIS) were used to evaluate their effects on the *in vitro* propagation of *C. zedoaria*. After three weeks of culturing on the MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA, the number of shoots produced per explant and the biomass of the formed shoots were found to be varied depending

on the culture system used. An average of 2.2 ± 0.3 shoots per explant was obtained from the shake flask liquid medium system (Fig.1A) which was higher than 1.7 ± 0.3 shoots per explant obtained on the solid medium (Fig.1B). The result of this study also showed that the explants cultured on the liquid medium produced bigger shoots with an average of 1.4 ± 0.1 g per shoot biomass as compared to 0.58 ± 0.1 g per shoot biomass cultured on the solid medium (Fig.2). This result indicated that the shoot explants of *C. zedoaria* which was cultured in the liquid medium produced a significantly higher number of shoots and a heavier biomass (bigger shoot) as compared to that cultured on the solid medium (Fig.1c). As a solidifying agent, agar was found to have effects on the growth and development of *in vitro* cultures (Scholten & Pierik, 1998). The main reason was that the gelling agent of the solid medium provided less oxygen to the cultured explants as dissolved oxygen was hindered by the agar (Kohlenbach & Wemicke, 1978). In contrast, the use of agitated shake flask stimulated and facilitated the nutritional and hormonal uptake, leading to better shoot and root growth (Ziv, 1989; Smith & Spomer, 1994; Sandal *et al.,* 2001). Moreover, a continuous

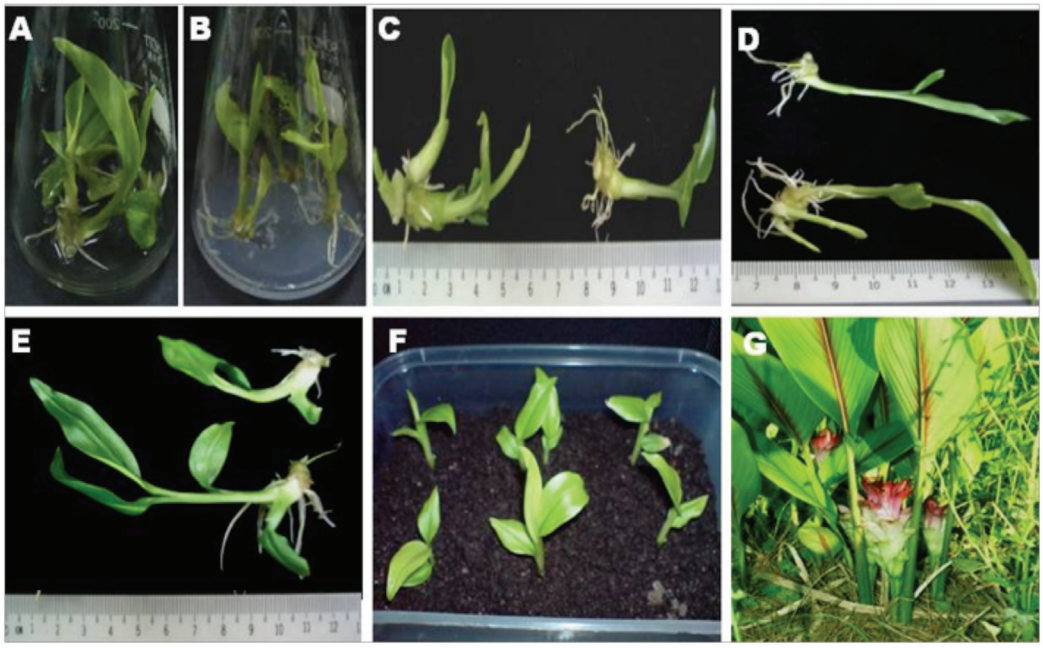
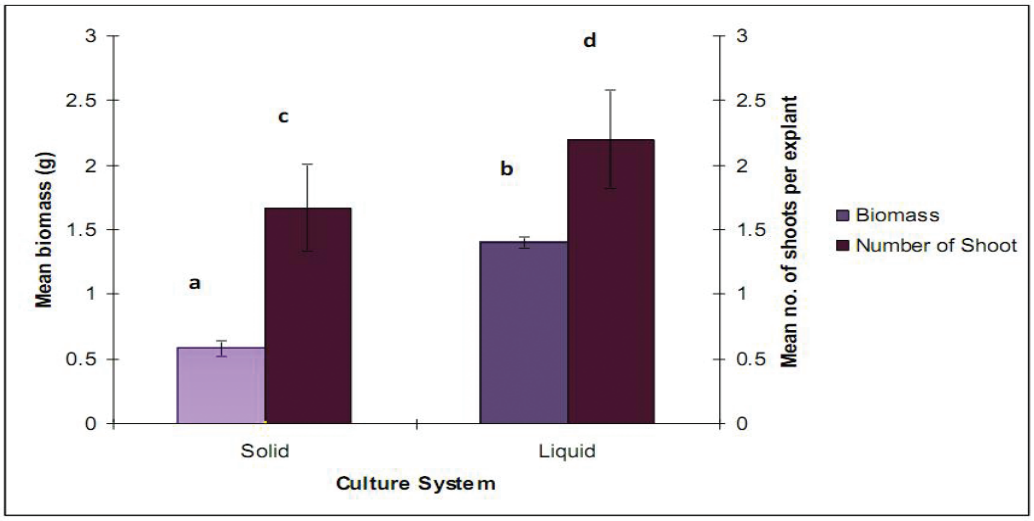


Fig.1: The effects of the culture system and explant types on the *in vitro* micropropagation of *Curcuma zedoaria* after three weeks of culture; A) Multiple shoots formation on liquid medium; B) Multiple shoots formation on solid medium; C) Multiple shoots and shoots size of explants cultured on solid medium (Right) and liquid medium (Left); D) Multiple shoots and shoots size of the explants cultured in TIS (Top) and shake flask (Bottom); E) Shoot size derived from the undivided (Top) and divided (Bottom) shoot explants cultured in shake flask; F) Plantlets acclimatized in the soil mixture containing black soil:sand (1:1) (Day 14); and G) Mature plant established in the soil under field conditions

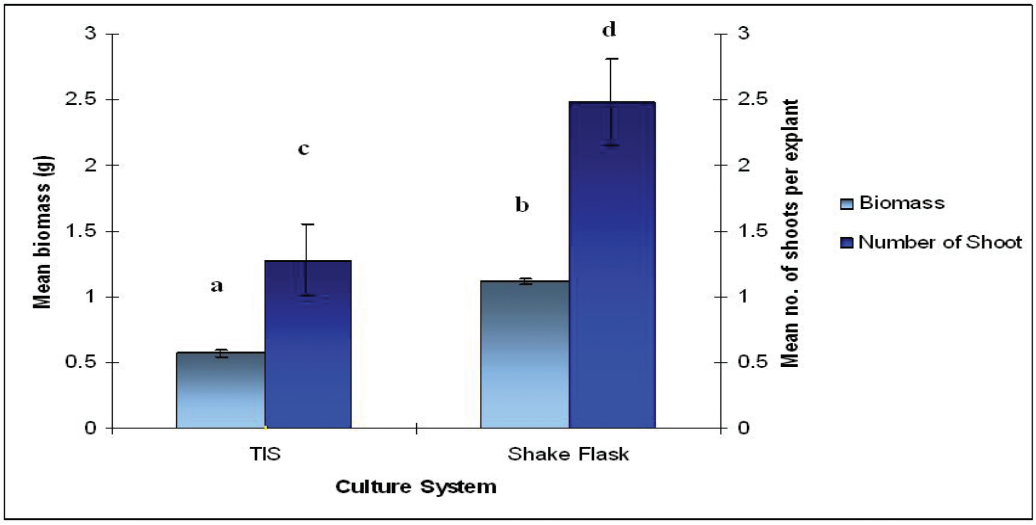


The mean value of each parameter, followed by different alphabet, are significantly different using the Independent T-test, $p \leq 0.05$.

Fig.2: The effects of the solid and liquid media on the shoot proliferation and shoot biomass of *Curcuma zedoaria*

shaking of the medium provided ample oxygen supply to the shoot explants and a better aeration which ultimately enhanced growth and multiplication (Mehrotra *et al*., 2007). Furthermore, the liquid medium allowed a greater uptake of nutrient and plant growth regulators due to the large surface of absorption provided by the partially submerged shoots (Arshad *et al*., 2005). The higher rates of shoot multiplication and improved growth in different plants using the liquid medium have also been reported (Rizvi *et al*., 2007; Douglas, 1984; Nadgauda *et al*., 1990; Liu *et al*., 2004). In a different study by Murch *et al*. (2004) the liquid culture was proven to be more efficient for the production of biomass than the solid medium and there was no hyperhydricity observed in any of the cultures grown in the liquid medium.

Comparative studies between temporary immersion system (TIS) and shake flask system revealed that shoot multiplication and growth were more efficient in shake flask system (Fig.1D). The shake flask system yielded 2.5 ± 0.3 shoots per explant, whereas the temporary immersion system produced 1.3 ± 0.25 shoots per explant (Fig.3). Similarly, Wawrosch *et al*. (2005) observed that the total number of the multiple shoots of *Charybdis numidica* produced by TIS was only half of the total number of the multiple shoots produced by the shake flask system. A higher multiplication rate in the shake flask system has been explained as attributing to the continuous contact between the shoots and the liquid medium, which enables a constant supply of nutrients as well as a continuous aeration to the explants. Here, the result of this study showed that the liquid shake flask system was better than TIS. However, in

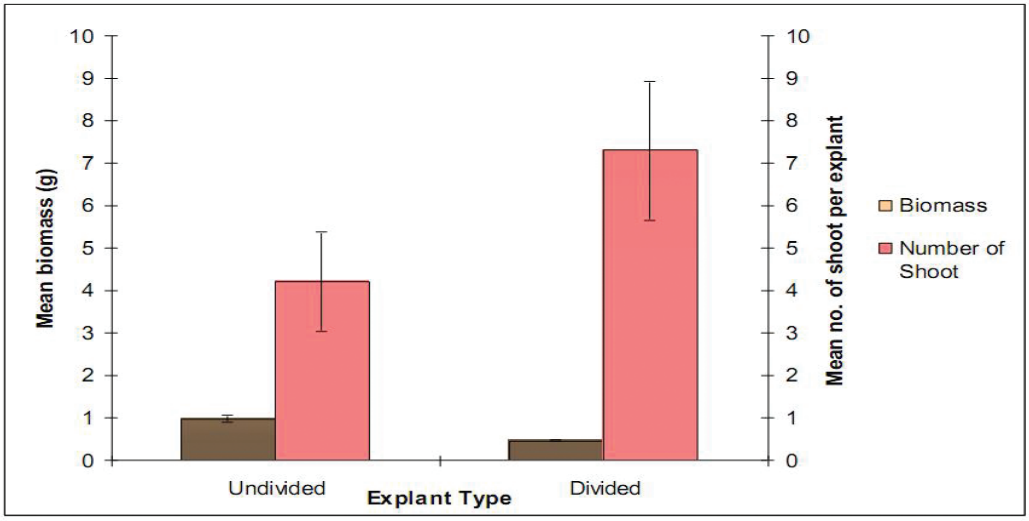


The mean value of each parameter, followed by different alphabet, is significantly different using the Independent T-test, $p \leq 0.05$.

Fig.3: A comparison between TIS and shake flask system on the multiple shoot formation and biomass of *Curcuma zedoaria*

the previous study Stanly *et al*. (2010), TIS was reported as the best choice of the *in vitro* propagation technique for the production of normal *C. zedoaria* and *Z. zerumbet* plantlets. In addition, Murch *et al*. (2004) reported that both the fresh and dry weights of *Crescentia cujete* plantlets grown in TIS were significantly higher than those cultured in the semi-solid medium and the shake flask culture. Moreover, in a different study Grigoriadou *et al*. (2005) reported an equal number of microshoots per explant of *Olea europaea* derived from the TIS and liquid flask cultures. This indicates that the efficiency of a culture system is dependent on many cultural factors and plant species.

The results obtained indicated that dividing the shoot explants longitudinally into halves could enhance the formation of multiple shoots. After 3 weeks of culturing, the divided shoot explants produced an average of 3.6± 1.0 shoot per half explant and hence approximately 7.0± 2.0 shoots could be obtained from a single shoot. On the other hand, the undivided shoot yielded 4.2± 1.1 shoots per explant. Biomass produced by the divided shoot was 0.98± 0.1 g while undivided shoot was only 0.46 ± 0.1 g (Fig.4). The divided shoot explants produced bigger shoots as compared to the ones derived from the whole shoot (Fig.1E). The superiority of the divided shoot over the non-divided shoots was already reported for *Alocasia longiloba* 'Watsoniana' by Chan and Chong (2010) who observed that when the shoot explants were divided longitudinally into halves and cultured into the liquid proliferation medium using the shake flask system, a total of 10 to 12 buds were produced from each whole shoot



The mean value of each parameter, followed by different alphabet, is significantly different using the Independent T-test, p = 0.05.

Fig.4: The effects of shoot incision on the multiple shoots formation and biomass of *Curcuma zedoaria*

within four weeks as compared to the non-divided shoot explants which produced only 3-4 buds within the same duration.

After three week of culture in basal MS medium, all the micro-shoots *C. zedoaria* produced roots. The *in vitro* plantlets of *C. zedoaria* with well-developed roots were removed from the rooting medium and washed under running tap water in order to remove adhered medium. This is important because the agar contains sucrose and other nutrients that can serve as a medium for growth of disease-causing organisms which may eventually cause the rotting of roots. At this stage, the plantlets are normally delicate and extra care has to be taken when they are being transferred from the culture vessels to the external environment. Plantlets grown *in vitro* have been continuously exposed to a unique microenvironment that has been selected to provide minimal stress and optimum conditions for plant multiplication. Plantlets developed within the culture vessels are normally grown under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity. They may readily lose water when exposed to ambient conditions. Hence, these delicate plantlets need to undergo an acclimatization process before they can be transferred to the field. The washed *C. zedoaria* plantlets were successfully acclimatized and all (100%) the plantlets survived when they were transferred to the soil. The well-developed root system may be assumed as major factor to a proper acclimatization of the plantlets from all the three tested systems. Most of the plants showed normal and healthy growths after 14 days of acclimatization (Fig.1F). Lorenzo *et al*.

(1998) also reported that the sugarcane plantlets obtained using the conventional method, solid culture medium and TIS showed a similar growth. The plantlets of *C. zedoaria* derived from the three culture systems were morphologically similar to their respective mother plants (Fig.1G).

CONCLUSION

It can be concluded that the shake flask system using liquid medium proved to be more efficient than the solid medium and the temporary immersion system in promoting the shoot proliferation and shoot biomass of *C. zedoaria*. However, normal and healthy plantlets could be produced from all the three different culture systems. More research is needed to improve the regeneration efficiency that will provide a better regeneration system for the micro-propagation and transformation of this elite medicinal plant.

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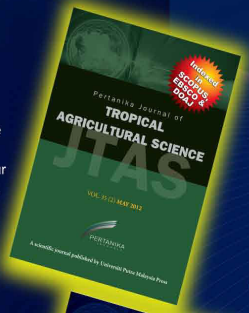

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