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Postharvest Quality of *Carica papaya* var. Eksotika after Foliar Feeding Treatment

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ABSTRACT

A field study was conducted to determine the effects of foliar feeding using of two Frumone® formulations on postharvest quality of Eksotika papaya. The treatments consisted of water (control), Frumone® and Frumone®+neem oil (2 mL.L^{-1} water) applied by foliar spraying to one-year-old plants at 30-d intervals for 4 months. Green with trace of yellow fruits at ripening stage (RS) 2, were harvested and then ripened using calcium carbide (10 g.kg^{-1} fruit) at 27°C for 24 h. Fruit quality at RS 2, 4 and 6 were evaluated. In comparison to the control treatment, the two formulations of Frumone® treatments did not affect peel colour, pulp firmness, soluble solids concentration (SSC), vitamin C content and titratable acidity of Eksotika papaya fruit. However, the pH of the fruit was significantly affected by the treatments. Fruit peel colour, flesh firmness, SSC, vitamin C content, pH and titratable acidity were significantly affected by RS. There were significant interaction effects of foliar feeding x RS on C* values, flesh firmness, SSC and pH. As fruits ripened, h^{o} values correlated positively with pulp firmness and negatively with SSC. This indicated that during fruit ripening, the change in fruit peel colour, from green to yellow, was followed by fruit softening and increase in sweetness. In conclusion, foliar feeding using Frumone® and Frumone®+neem oil did not affect postharvest quality of Eksotika papaya fruit.

Keywords: Foliar feeding, postharvest quality, neem oil, colour, soluble solids concentration

INTRODUCTION

Foliar feeding provides supplemental doses of minor and major nutrients, plant hormones, stimulants and other beneficial substances to plant foliage and stem, via spraying. Plant response is rapid and efficient, with less of its product being needed per feeding (Oosterhuis *et al.*, 2000). Radioisotope studies indicated that foliar feeding was 8-20 times more effective compared to soil fertilizer application for a plant (Anon, 1985). Foliar fertilizer application increased yield and resistance to disease and insect pests, improved drought tolerance and enhanced crop quality. Foliar potassium applications during cantaloupe fruit development and maturation increase fruit firmness, sugar content, ascorbic acid and beta-carotene levels (Lester *et al.*, 2007). However, plant response to foliar feeding depends on species, fertilizer form, concentration and frequency of feeding and stage of plant growth (Kuepper, 2003). Thus, any new foliar fertilizer that is introduced to the agricultural chemical market has to be evaluated before it can be claimed to be beneficial. This is true for the new foliar fertilizer called Frumone® that is claimed to improve fruit set and fruit quality of papaya.

Frumone®, a water-base product, contains a variety of ingredients including phosphorous, potassium, chelated micro nutrients (boron, copper, iron, manganese, molybdenum and zinc), amino acids, synthetic plant hormones, and naturally derived plant growth promoters and adjuvant. Another formulation of Frumone® contains neem oil that has been extracted by cold pressing of the neem (*Azadirachta indica*) seeds. Neem oil has antimicrobial properties and the ability to improve soil fertility

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(Gajalakshimi and Abbasi, 2004). The objective of this study was to determine the effects of the two foliar fertilizer formulations, Frumone® and Frumone®+neem oil, on the postharvest quality of 'Eksotika' papaya fruit.

MATERIALS AND METHODS

The study was carried out from April to July 2005, at the papaya farm of the Department of Agriculture, Serdang, Selangor, Malaysia. Treatments, consisting of Frumone® and Frumone®+neem oil at the rate of 2 $mL.L^{-1}$ water, were applied by foliar spraying on 36 one-year-old papaya plants (selected randomly) at 30 days intervals. The control plants were only sprayed with water. Fruits at ripening stage (RS) 2 that were green with traces of yellow were harvested and then ripened using CaC_2 (10 $g.kg^{-1}$ fruit) at 27°C for 24 h. Fruit qualities at RS 2, 4 and 6 were evaluated.

Determination of Peel Colour

Peel colour was determined using a Minolta CR-300 Chroma Meter (Minolta Corp., Osaka, Japan) using the Illuminate C (CIE, 1976) and results were expressed as lightness (L*), chroma (C*) and hue (h°). The L* value ranges from 0 = black to 100 = white. The h° is an angle in a colour wheel of 360°, with 0°, 90°, 180° and 270° representing the hues red, yellow, green and blue, respectively, while C* is the intensity or purity of the hue. Measurements were carried out at three locations of every fruit of the papaya.

Determination of Flesh Firmness

Flesh firmness was evaluated using the Bishop Penetrometer FT 327 (Alfonsine, Italy). The force required for an 11-mm probe to penetrate the cut surface in two opposite locations to a depth of 5 mm was recorded. The penetration force was expressed in newton (N).

Determination of Soluble Solids Concentration (SSC)

Ten grams of fruit was macerated and the tissue was homogenised with 40 mL of distilled water using a kitchen blender. The mixture was filtered with cotton wool. A drop of the filtrate was then placed on the prism glass of a refractometer (Model N1, Atago Co., Ltd., Tokyo, Japan) to obtain the %SSC. The readings were corrected to a standard temperature of 20°C by adding 0.28% to obtain %SSC at 27°C.

Determination of pH

The remainder of the juice from the SSC determination was used to measure juice pH by using a glass electrode pH meter (model Micro pH 2000, Crison Instruments, Spain).

Determination of Vitamin C Content

Vitamin C content of flesh was determined according to the method of Ranggana (1977) and reading was expressed as $mg.100\ g^{-1}$.

Determination of Titratable Acidity

The remainder of the juice from the SSC determination was used to measure titratable acidity by titrating with 0.1 $mol.L^{-1}$ NaOH and using 1% phenolphtalein as an indicator. The results were calculated as percentage citric acid [(ml NaOH x 0.1 $mol.L^{-1}$/weight of sample titrated) x 0.064 x 100].

Statistical Analysis

The experimental design was a completely randomized design with three replications. Each replicate consisted of four plants with five fruits per plant. Data were analyzed using analysis of variance (SAS, 1998) and means were separated by LSD. Correlation analysis by means of Pearson's correlation matrix was performed to establish any association between peel colour, flesh firmness, SSC, vitamin C content, pH and titratable acidity.

RESULTS AND DISCUSSION

Foliar feeding using Frumone® and Frumone®+neem oil did not affect peel colour of the papaya fruit (Table 1). The main effect of RS indicated that L* and C* values increased while h^o values decreased significantly as papaya fruit ripened from RS 2 to 6. The h^o values of the peel decreased from green to yellow as the fruit attained ripeness. There was a significant interaction effect of foliar feed x RS on the C* values.

Flesh firmness of the papaya was not affected by foliar feeding of Frumone® and Frumone® +neem oil (Table 2). This is similar to studies on the effects of copper and calcium sprays on cherry and apple fruit quality whereby the foliar sprays had no effect on the flesh firmness of the fruits (Brown *et al.*, 1996). As expected, flesh firmness decreased significantly as fruit ripened from RS 2 to 6 (Table 2). The decrease could be due to polyuronide

TABLE 1
Main and interaction effects of foliar feeding and ripening stage on the peel colour (L*, C* and h°) of Eksotika papaya fruit

Factor	Peel Colour		
	L*	C*	h°
Foliar Feeding (FF)			
Control	54.48 a[z]	32.64 a	103.69 a
Frumone®	53.22 a	31.34 a	104.97 a
Frumone®+neem oil	53.72 a	31.31 a	102.61 a
Ripening Stage (RS)			
2	46.20 c	21.39 c	125.62 a
4	53.09 b	31.18 b	102.12 b
6	62.12 a	42.73 a	85.53 c
Interaction			
FF x RS	NS	*	NS

[z] Mean separation within columns and factors by LSD at P ≤ 0.05.
[NS,*]Non significant or significant at P ≤ 0.05.

solubilization in the fruit during ripening (Selamat, 1993). The flesh firmness correlated positively with h° values (Table 3) indicating that as the green peel turned yellow, the flesh became soft.

Foliar feeding did not affect the SSC of papaya fruits (Table 2). Similar findings were reported in cherries and apples (Brown *et al.*, 1996), and tomatoes (Chapagain and Wiseman, 2004). As fruits ripened, the SSC of papaya fruit increased significantly with the maximum value occurring at RS 4. This was followed by a significant decrease of 9.88% in SSC as fruit ripened from RS 4 to 6. This has been reported in papaya whereby the SSC stagnates or decrease once fruits attain a certain degree of ripeness (Sim, 1988). The SSC of the fruits correlated negatively with h° values and firmness (Table 3), indicating that as fruits ripened, with the peel colour changing from green to yellow, the fruits became sweeter and softer.

The fruit pH was significantly affected by foliar feeding (Table 2). The pH decreased at RS 4 and then increased significantly at RS 6 as

TABLE 2
Main and interaction effects of foliar feeding and ripening stage on flesh firmness, SSC, pH, vitamin C content and titratable acidity of Eksotika papaya fruit

Factor	Firmness (N)	SSC (%)	pH	Vitamin C (mg.100 g^{-1})	Titratable acidity (%citric acid)
Foliar Feeding (FF)					
Control	1.28 a[z]	12.06 a	5.45 b	84.19 a	13.65 a
Frumone®	1.14 a	11.53 a	5.52 a	77.00 a	12.55 a
Frumone®+neem oil	1.21 a	12.53 a	5.42 b	86.38 a	12.12 a
Ripening Stage (RS)					
2	2.40 a	9.39 c	5.56 b	75.07 b	7.36 b
4	0.74 b	14.06 a	4.99 c	95.95 a	10.10 b
6	0.49 c	12.67 b	5.83 a	76.56 b	20.87 a
Interaction					
FF x RS	*	*	*	NS	NS

[z] Mean separation within columns and factors by LSD at P ≤ 0.05.
[NS,*] Non significant or significant at P ≤ 0.05.

fruit ripened. For fruit pH, there was a significant interaction effect of foliar feeding x RS. The vitamin C content of papaya fruits was not affected by foliar feeding treatment but increased significantly as fruits ripened from RS 2 to 4, then decreased significantly at RS 6 (Table 2).

The titratable acidity of the fruits was not affected by foliar feeding (Table 2). This was in agreement with the findings of foliar feeding in cherries and apples (Brown *et al.*, 1996), and tomatoes (Chapagain and Wiseman, 2004). The titratable acidity increased significantly as fruits ripened from RS 2 to 6 (Table 2). Similar findings were reported in acerola (Vendramini and Trugo, 2000) and passion fruit (Shiomi *et al.*, 1996). This could be due to an increase in the succinic acid as well as the presence of citric, oxalic, fumaric and malic acids in the papaya fruit during ripening (Ali *et al.*, 1994).

CONCLUSIONS

In comparison to the control, the two formulations of Frumone® treatments did not affect peel colour, pulp firmness, SSC, vitamin C content and titratable acidity of Eksotika papaya fruits. However, the fruit pH was significantly affected by the treatments. Fruit peel colour, flesh firmness, SSC, vitamin C content, pH and titratable acidity were significantly affected by RS. There were significant interaction effects of Frumone® x RS on C* values, flesh firmness, SSC and pH. During fruit ripening, the change in fruit peel colour, from green to yellow, was followed by fruit softening and the increase of fruit sweetness. In conclusion, foliar feeding using Frumone® and Frumone®+neem oil did not affect the postharvest quality of Eksotika papaya fruits.

TABLE 3
Correlation coefficients for peel colour (h^o), flesh firmness (Firmness), soluble solids concentration (SSC), pH, vitamin C and titratable acidity (TA) of Eksotika papaya fruit in response to foliar feeding

	h^o	Firmness	SSC	pH	Vitamin C	TA
h^o	—					
Firmness	0.87**	—				
SSC	-0.60**	-0.73**	—			
pH	-0.22	0.07	-0.40**	—		
Vitamin C	-0.22	-0.25	0.52**	-0.49**	—	
TA	-0.77	-0.61**	0.32*	0.48**	0.04	—

n = 54.
*,** Significant or highly significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

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Apicultural Site Zonation Using GIS and Multi-Criteria Decision Analysis

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ABSTRACT

This paper discusses the application of Geographical Information System (GIS) and Multi-Criteria Decision Analysis (MCDA) technology as a tool to aid decision-making in a case study to locate beekeeping zones in the state of Selangor. The combination of GIS capabilities with MCDM technique provides greater effectiveness and efficiency of decision making while solving spatial decision problems. In this research, land suitability analysis and zoning was carried out with respect to the bee's biotic needs and some other important factors in apiary management. Suitability weighting was determined using the pairwise comparison matrix of the Analytical Hierarchy Process (AHP) and suitability score using Weighted Overlay function in ArcGIS9. The overall consistency ratio value of AHP pairwise comparison was 0.01 which indicates a reasonable level of consistency in the deployment of the pairwise comparisons. The results of the analysis are presented and verified with actual data of the existing apiaries in Selangor. The integration of AHP model with GIS resulted in Non-Suitable, Most Suitable, Moderately Suitable and Suitable beezones. The total Non Suitable Areas (NS) was 34.73%, leaving the remainder as potential areas (65.27%). The remaining are the Most Suitable Areas (S1) 13.72 %, Suitable Areas (S2) of 27.24% and Moderately Suitable Areas of 24.32 %.

Keywords: Geographical Information Systems, Multi-Criteria Decision Analysis (MCDA), Analytical Hierarchy Process (AHP)

INTRODUCTION

Apiculture or beekeeping is a large area of study and application by itself. It is a huge field of agriculture and has been practised by man since the primitive age. The term apiculture as defined by Food and Agriculture Organization of the United Nations (FAO, 2003) is "the science and art of bees and beekeeping", which uses bees as micro-manipulators to harvest plant foods from environmental resources that would otherwise be wasted (FAO, 1986).

Beekeeping is an important component of agriculture and rural development programmes in many Asian countries. Honeybees are natives to the IndoMalaya region where diverse floral sources are available throughout the year. The role of beekeeping in providing nutritional, economic and ecological security to rural communities in Asia cannot be overlooked as it has always been linked with their cultural and natural heritage (Matsuka, 1998).

Bees play a key role in the functioning of agricultural ecosystems as pollinators of crops and flowers. Malaysian Ministry of Agriculture & Agro-Based Industry have started the *'Honeybee Project'* to encourage the honeybee industry in farm families as a main/side income, exploiting the existing resources of plantation. This honeybee industry is expanding and a profitable commercial industry and side income for farmers. The prospect to expand this industry is bright in Malaysia considering that the demand for bee products in Malaysia and worldwide has increased.

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This report demonstrates how GIS can play a role to aid decision-making in locating suitable zones for beekeeping. Land suitability analysis and zonation involve a multiple criteria analysis technique. In this research, land suitability analysis was carried out in respect of the bee's environment and modelled into GIS systems incorporated with Analytical Hierarchy Process (AHP) model for the analysis of the criteria weightage.

Historical Overview

The history of commercial beekeeping in Malaysia started since the establishment of Malaysian Beekeeping and Research and Development Team (MBRDT) in 1981 (IDRC, 1987). The team consists of several institutions namely Universiti Putra Malaysia (UPM), Universiti Malaya (UM), Malaysian Agricultural Research Development Institute (MARDI), Malaysian Industry Smallholders Development Authority (RISDA), Rubber Research Institute Malaysia (RRIM) and Department of Agriculture (DOA).

MBDRT was funded by IDRC (International Dutch Research Council) and the objective of MBRDT is to undertake research and extension activities in promoting modern beekeeping in Malaysia. Although it has been more than 20 years since the establishment of MBDRT, modern beekeeping in movable hives is still not prominent in Malaysia.

Types of bee plants and pollen which are favourable to honeybees have been identified in previous MBDRT research, but the location of the source has not been identified and there is no map for suitable beekeeping locations or zones created using Multi Criteria Decision Analysis (MCDA) and GIS (Geographical Information System) technology. Existing flowering calendars only provides time (month) of blooms but does not contain information such as location and specific time of blooms. Thus, time of the most nectar and pollen production is not known for commercial beekeeping.

The integration of beekeeping with other crop production has been practised in other countries and shown to yield high revenue. According to Akranatul (1987), productive beekeeping depends on good colony management and good beekeeping areas. In order to promote it as a profitable agricultural occupation, areas with a good potential for beekeeping must be located and evaluated.

GIS and MCDA for Land Suitability Analysis

GIS has long been used as a tool for developing alternative uses of agricultural land, precision farming, crop yield or land suitability mapping in determining the best alternatives for agricultural production. It is the capability of GIS for supporting decision making that is of particular importance for the landuse suitability mapping and modelling (Malczewski, 2004). The ability of GIS to integrate, display, and query many types of information at the same time makes it an important tool for decision support in agriculture. Perhaps the most useful tool of all in GIS is its ability to form overlay operations between layers especially in selecting or locating suitable area for agricultural purposes.

The terms Multi-Criteria Decision Making (MCDM) and Multi Criteria Decision Analysis (MCDA) are used interchangeably in referring to the multi-criteria evaluation (MCE) technique, usually carried out for land suitability analysis or in determination of site fitness for any specific application (Malczewski, 2004). The critical aspect of spatial multi-criteria analysis is that it involves evaluation of geographical events based on the criterion values and preferences set with respect to a set of evaluation criteria. The combination of GIS capabilities with the MCDM technique provides greater effectiveness and efficiency of decision making while solving spatial decision problems.

According to FAO (1976), suitability is a measure of how well the qualities of a land unit match the requirements of a particular form of land use. The process of land suitability classification is the evaluation and grouping of specific areas of land in terms of their suitability for a defined use. De la Rosa (2000) stated that land suitability is a component of sustainable evaluation of land use. Suitability together with vulnerability defines the suitability of a land use. The sustainable land use should have maximum suitability and minimum vulnerability, as shown in *Fig. 1.*

Fig. 1: Land use sustainability (after de la Rosa 2000)

Land suitability analysis deals with information, which is measured in different scales like ordinal, nominal, ratio scale etc. Based on the scope of suitability there are two types of classifications in FAO (1976) framework.

- *Current suitability:* refers to the suitability for a defined use of land in its present condition, without any major improvements in it.
- *Potential suitability:* for a defined use, of land units in their condition at some future date, after specified major improvements have been completed where necessary.

Agricultural land suitability is an interdisciplinary approach thus; determination of optimum land use type for an area involves integration of data from various domains and sources like soil science to social science, meteorology to management science. All these major streams can be considered as separate groups; further each group can have various parameters (criteria) pertaining to that group. However all the criteria are not equally important, every criterion will contribute towards the suitability at different degrees (Prakash, 2003).

There are several decision making approaches for analysing land suitability for land-use or land suitability purposes. Today, the widely used methods for land suitability analysis include ranking and ratings, weighted summation (AHP), Simple Additive Weighting Method (SAW), Boolean overlays, Fuzzy techniques and GAM ratings.

Analytical Hierarchy Process

The Analytical Hierarchy Process (AHP) is a comprehensive, logical and structural framework which enhances the understanding to decompose a complex decision making into a more feasible one to resolve hierarchical structure. AHP was developed by Professor Thomas L. Saaty of the University of Pittsburgh in 1980. AHP is based on three basic principles which are decomposition, comparative judgment and synthesis of priorities as shown in *Fig. 2.*

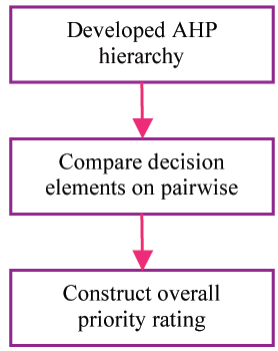

Fig. 2: Three major steps of Saaty's AHP

Comparative judgment by a decision maker requires pairwise comparison assessment between two criteria, including the sub-criteria. The fundamental concept of AHP lies in proceeding from a pairwise comparison of criteria to evaluate the weights that assign relative importance to these criteria. The Pairwise Comparison method was developed in the context of the AHP to create a ratio matrix. The procedure involves input of the pairwise comparison matrix and produces the relative weight as output. Identification of each criterion weightage can be interpreted easily through Pairwise Comparison Matrix; it requires rating scale preferences between two different criteria with values from 1 - 9 as shown in Table 1.

TABLE 1
Scale for AHP comparisons (Saaty, 1980)

Intensity of Importance	Description
1	Equal importance of both elements
3	Weak importance of one element over another
5	Essential or strong importance of one element over another
7	Demonstrated importance of one element over another
9	Absolute importance of one elements over another
2,4,6,8	Intermediate values between two adjacent judgements

The synthesis principle uses the derived ratio scale of the local priorities in the various levels of the hierarchy and constructs a composite set of alternatives (Malczewski, 2004). The procedures involve the utilisation of geographical data, the decision preferences and the manipulation of the data and preferences according to specific decision rules. The critical aspect of spatial multi-criteria analysis involves evaluation of geographical data based on the criteria values and preferences set with respect to a set of evaluation criteria.

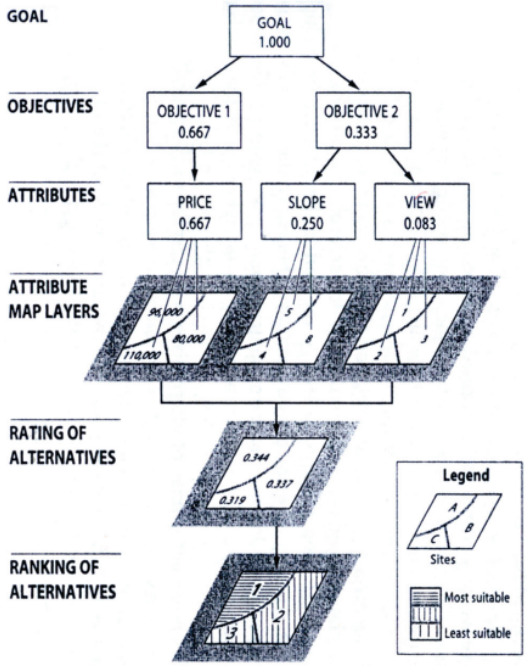

Fig. 3: Analytical Hierarchy Process (modified from Malczewski, 2004)

METHODOLOGY

There are many research methodologies that have been used by various researchers for land suitability analysis, which are all based on the use of GIS and several MCDM techniques as a tool and require certain evaluation criteria.

The methodology framework focuses on decision making as a process which involves a sequence of activities. It starts with problem recognition, criteria and constraints evaluation, data acquisition, AHP weightage analysis, manipulation of the AHP results using GIS and generation of suitability zones, ground truthing and verification, and finally evaluation and recommendations.

Criterion Factor

In multi-criteria analysis using AHP, criteria identification and determination are the most important elements in achieving any set aim or goal. In this research, a suitable bee zone was identified and weighted using AHP mathematical model. Several criterion factors were identified for locating the bee zone. The criterion factor took into account every single factor for locating an apiary for beekeeping as well as other factors such as ensuring migratory beekeeping is successful.

Site assessment of suitable bee settlement areas is important to ensure bees are placed in suitable and favourable areas regardless of abundant sources of nectar and pollen. The criteria used to determine suitable areas for beekeeping was identified based on numerous literature reviews including the FAO (1987b) guidelines on apiculture (beekeeping) and discussions with expert in apiculture. Based on the FAO guidelines, an apiary site should ideally be:

- away from playgrounds and noisy commercial or industrial areas
- near a fresh water supply: the banks of a river, lake or fish-pond, or even a dripping faucet
- near food sources
- fairly dry, away from swampy or flooding valley or any bottom land with stagnant water
- accessible to good roads
- on the leeward side of a hill
- with annual rainfall between 1275 mm and 1875 mm
- away from smoke and fire,
- away from danger of vandalism and unfriendly neighbours

Consequently, information about the relative importance of the criteria is required. This is achieved by assigning a weight to each criterion. After the weightage are derived, these evaluation criteria have to be integrated using multi-criteria decision rules. The decision rules provide the basis for ordering the decision alternatives and for choosing the most preferred alternative.

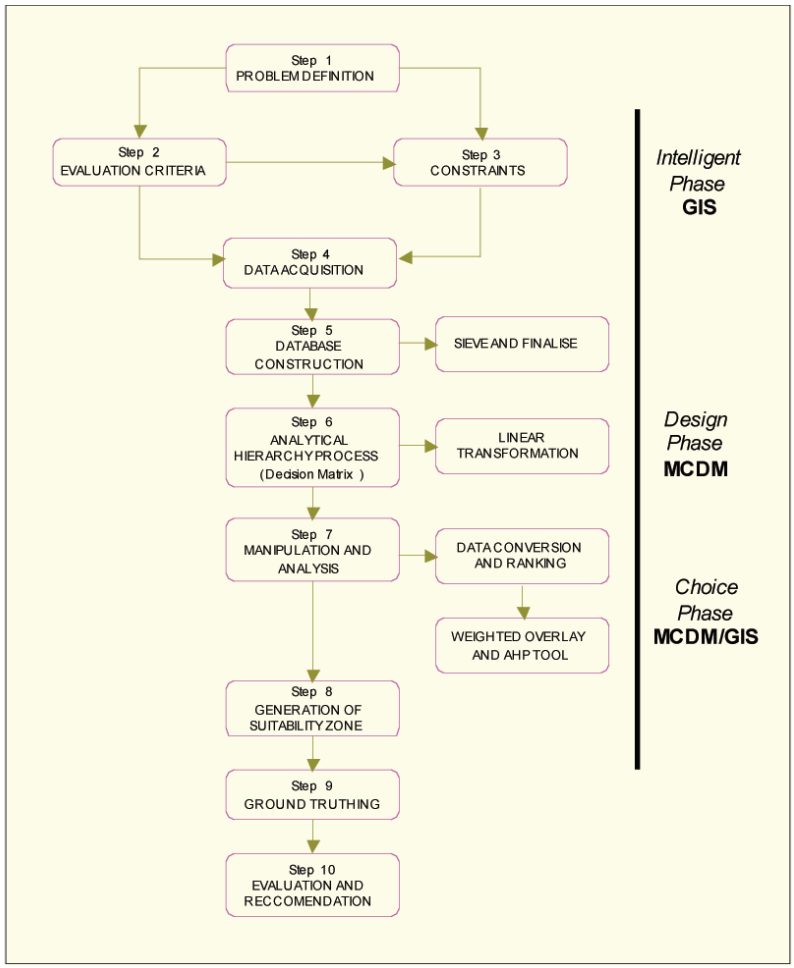

Fig. 4: Research methodology

AHP Weight Analysis Using Expert Choice® Software

Land suitability in this research consisted of generating pairwise comparison matrix for each criterion and sub-criterion of the beekeeping factor using AHP technique. The generation of weight and rank for the suitable area is carried outside the GIS environment or termed as 'loose coupling' using Expert Choice software. Several apiculture experts from the Department of Agriculture Malaysia (DOA) and Universiti Putra Malaysia (UPM) were consulted to determine the preferences and the ratings of AHP. Explanations were given to the experts on the basis of AHP implementation. Several criteria rankings were given as ratios and percentages by the experts and then synthesised to reflect the AHP ratings.

The information on AHP weightage was used to produce a suitability map. Pairwise comparision matrix for each sub-criteria is shown in Table 2 and summary results of pairwise comparison of sub-criteria and criteria are shown in *Fig. 5*.

The estimation of the consistency ratio is one of the important steps in determining the levels of inconsistency in the pairwise comparison. According to Malczewski (2004), the consistency ratio (CR) is designed in such a way that if CR < 0.10, the ratio indicates a reasonable level of consistency in the pairwise comparisons. However, if CR ≥ 0.1, the values of the ratio indicate inconsistent judgement (Saaty, 1980, 1982; Malczewski, 1999), for a complete technical description of AHP technique). The overall weighting values and its consistency of the AHP analysis are shown in *Fig. 6*.

TABLE 2
Pairwise comparison matrix

Comparing the relative importance with respect to : HYDROLOGY FEATURES

	0 - 200m	200 - 500m	500 - 700m	> 700m
0 - 200m from source		2.0	3.0	9.0
200 - 500m from source			2.0	7.0
500 - 700m from source				4.0
>700m from source				

Incon = 0.01

Comparing the relative importance with respect to : ROAD NETWORKS

	< 5 km	5 - 10 km	10 - 15 km	>15 km
< 5 km		2.0	4.0	9.0
5 - 10 km			2.0	7.0
10 - 15 km				4.0
>15 km				

Incon = 0.01

Comparing the relative importance with respect to : TOPOGRAPHY FEATURES

	< 150m	150 - 300m	> 300m
< 150m		2.0	7.0
150 - 300m			4.0
> 300m			

Incon = 0.00

Comparing the relative importance with respect to : NECTAR CLASS

	< 150m	150 - 300m	> 300m
< 150m		2.0	4.0
150 - 300m			2.0
> 300m			

Incon = 0.00

Comparing the relative importance with respect to : POLLEN CLASS

	< 150m	150 - 300m	> 300m
< 150m		2.0	4.0
150 - 300m			2.0
> 300m			

Incon = 0.00

Comparing the relative importance with respect to : GOAL- SUITABLE ZONE

	NECTAR	POLLEN	HYDROLOGY	ROAD	TERRAIN
NECTAR CLASS		1.0	2.0	5.0	8.0
POLLEN CLASS			2.0	3.0	6.0
HYDROLOGY FEATURES				2.0	3.0
ROAD NETWORKS					2.0
TERRAIN					

Incon = 0.01

Linear Transformation

The weight values produced by AHP in Expert Choice software are as pointers. The values need to be transformed as commensurate criterion maps so that they could be further analysed in a GIS environment. Maximum score linear transformation is used to generate the proportional magnitude to the original weight. Linear scale transformation is a frequently used deterministic method for transforming input data into measurable criterion maps. The linear scale transformation method converts the raw data into standardised criterion scores. The two most often used procedures for linear transformation are maximum score and score range procedures. Linear scale transformation formula for maximum score is

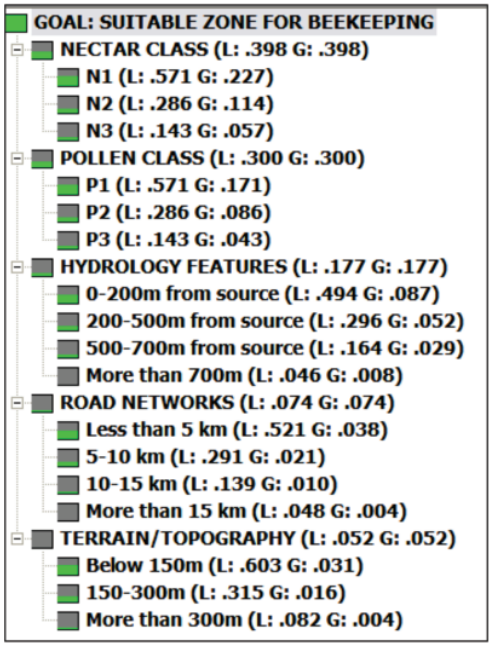

Fig. 5: Pairwise comparison in Expert Choice Software

$$x'_{ij} = \frac{x_{ij}}{x_{ij}^{max}}$$

where, x'_{ij} is the standardised score for the ith object (alternative) and the *j*th attribute, x_{ij} is the raw score and x_{ij}^{max} *is* the maximum score for the jth attribute. The value of the standardized scores would then range from 0 to 1 and according to (Malczewski, 1999) the best standardized score is always equal to 1. The advantage of this method is that it performs proportional (linear) transformation of the raw data. In order to capture the magnitude of the standardised method in a GIS system, it has to be multiplied with an arbitrary multiplier and the formula is

$$x'_{ij} = \frac{x_{ij}}{x_{ij}^{max}} * m$$

The multiplier value in this instance is nine (9) to reflect the maximum values as applied in Saaty's (1980, 1982) AHP. The final value generated was then rounded to the nearest integer value to allow the weight to be input into a GIS environment. Linear transformation results of the final value corresponding to the criterion are shown in Table 3.

Data Preparation for Available Area

In order to determine available areas for beekeeping zoning several steps have to be accomplished, which include sieving of unsuitable areas according to the guidelines provided by FAO (1987b), expert's opinion and availability of bee plants listed by Atim (1981). Only areas that have major bee plants as listed by Atim

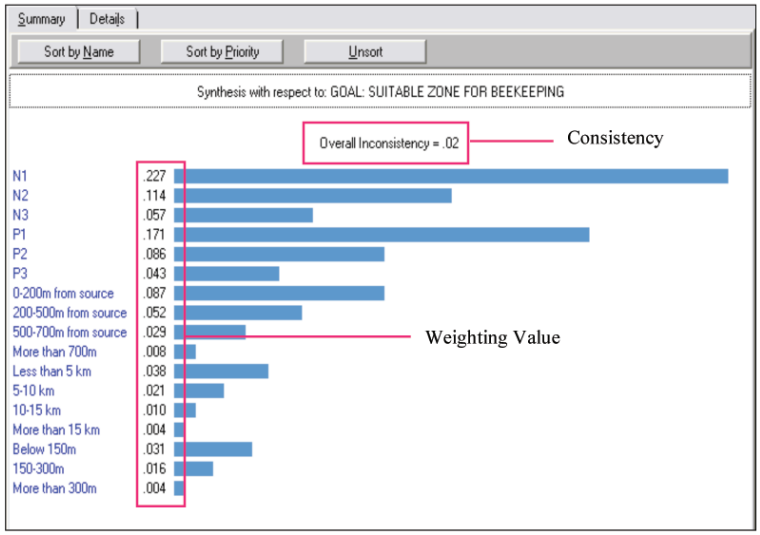

Fig. 6: Overall consistency ratio of AHP

TABLE 3
Criteria and commensurate weight

CRITERION	SUB-CRITERIA	DESCRIPTION	WEIGHT
CLIMATIC	Rainfall	Dry Season	9
		Wet Season	3
PHYSICAL	Topography/ Elevation	Low Land : Below 150m	9
		Hill Land : 150 -300m	5
		High Land: Above 300m	1
	Hydrology Bodies (Water Network)	Best Suited: Below 200m	9
		Moderately Suited : 200-500m	5
		Slightly Suitable : 500-700m	2
		Modifiable : More than 700m	1
EXISTING INFRA-STRUCTURE	Road Networ	Best Suited: Below 5 km	9
		Moderately Suited : 5-10 km	5
		Suitable : 10- 15 km	2
		Unsuitable : More than 15 km	1
FOOD SOURCE/ BEE PLANT	Nectar	High nectar source : N1	9
		Medium nectar source : N2	5
		Low nectar source : N3	3
	Pollen	High nectar source : N1	9
		Medium nectar source : N2	5
		Low nectar source : N3	3
OVERALL CRITERION WEIGHT		Terrain/Topography	1
		Road Networks	2
		Hydrology Networks	4
		Pollen Class	7
		Nectar Class	9

(1981) are selected as available areas. All other landuse occupancies such as built-up areas, mining areas, urban, towns and associated areas and other agricultural areas that are not classified as having bee plants are sieved. The selected agricultural areas which consist of several major bee plants are grouped into classes to determining the volume of nectar or pollen. N1/P1 indicates High Nectar/Pollen Source, N2/P2 indicates Medium Nectar/Pollen Source while N3/P3 indicates Low Nectar/Pollen Source as shown in Table 4.

Data Conversion and Ranking

Data conversion consists of converting map layers, which are in vector format to raster layer. The layers are then ranked accordingly as previously identified through AHP analysis. This is important because the AHP extension tool in ArcGIS9 only allows raster datasets for analysis.

TABLE 4
Classification of bee plants according to nectar and pollen class

CATEGORY	LANDUSE	Nectar Class	Pollen Class
Agriculture	Grassland	N2	P2
Agriculture	Coconut	N1	P1
Agriculture	Coconut/Cocoa	N1	P1
Agriculture	Coffee	N1	P2
Forest	Forest	N1	P1
Agriculture	Orchards	N1	P1
Agriculture	Rubber	N1	P3
Agriculture	Mixed Horticulture	N2	P2
Agriculture	Oil Palm	N3	P2

Spatial analysis for land evaluation comprises of overlaying several thematic layers to find locations that encompass all desired criteria. The relative weights of factors for beekeeping suitability were used as multi-factors to rank and classify the GIS database map layers of the study area in order to generate the suitability map of beekeeping zones.

Criterion 1: Topography

Naturally, bees inhabit lowlands or highlands. In this analysis, topography is divided into three classes; the highest rank is for lowlands which are easier for apiary management in comparison to highlands. The elevation map of the study area is divided into three regions. The topographic features are divided into three classes' lowland (0-150 m above sea level), hilly land (150 - 300 m above sea level) and highland (more than 300 m above sea level) (refer to *Fig. 7*).

Criterion 2: Hydrology

A suitable beekeeping zone must be located near to water resources. Therefore, 200 m, 500 m and 700 m buffers were input into the GIS software to generate surrounding water surfaces. In this research, the main contributing factor was the hydrologic features because in tropical climate bees need water to cool their hives and the water resources must be at least 500 m from their hives. Areas nearest to water resources are of the highest rank. Values for 700 m or more are still acceptable if a proper apiary management could be established by placing dripping faucets near hives in the apiary (refer to *Fig. 8*).

Criterion 3: Road Network

In terms of logistics, a good road network is very important to the location of an apiary or beezone. The highest ranked placed areas are nearest to road networks. Buffering of road networks is executed using Spatial Analyst Tool in which the processes include reclassification of required buffer zone followed by another reclassification according to the AHP weight. (refer to *Fig. 9*).

Criterion 4: Nectar Class

The nectar class map of the study area is divided into three ranks. Each bee plant is classified accordingly to its corresponding nectar class; the highest rank has the most nectar production. The ranking for High Nectar Source (N1) is 9, Medium Nectar Source (N2) is 5 and for Low Nectar Source (N3) is 3 (refer to *Fig. 10*).

Criterion 5: Pollen Class

The pollen class map of the study area is divided into three ranks. Each bee plant is classified accordingly to its corresponding pollen class; the highest rank has the most pollen production.

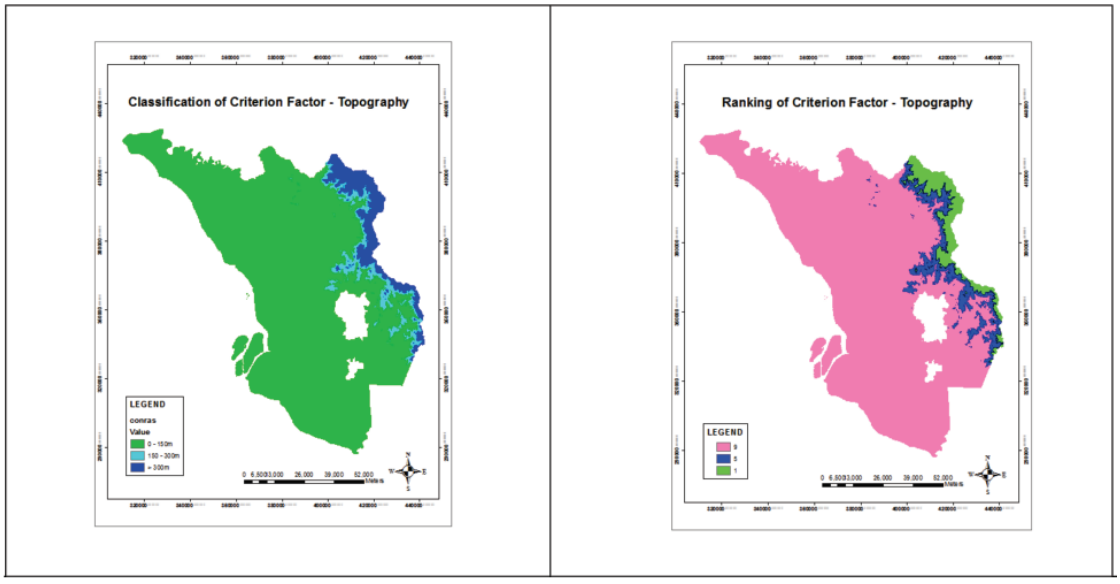

Fig. 7: Topography - Classification and ranking

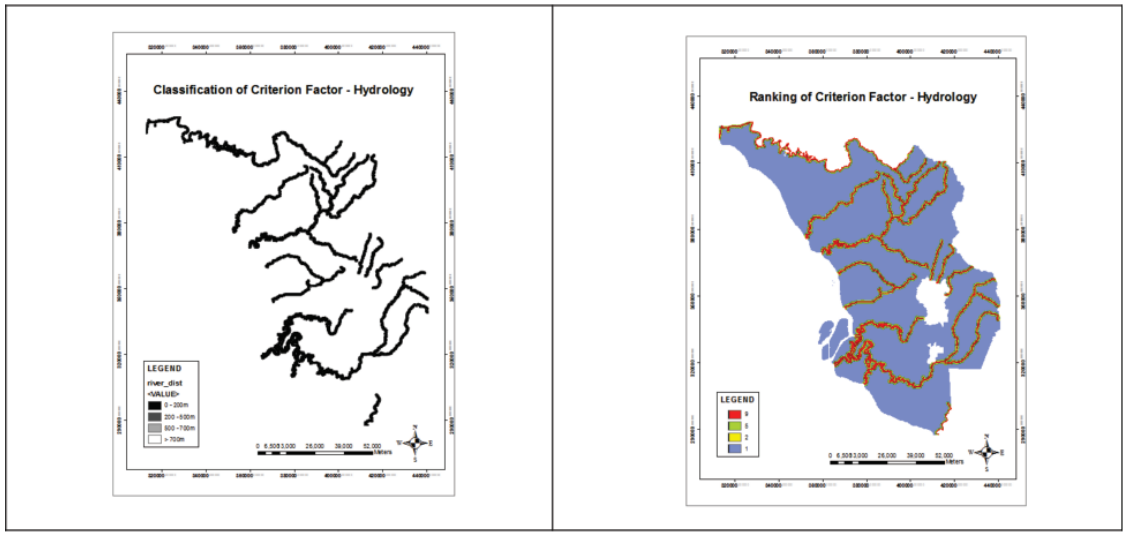

Fig. 8: Hydrology - Classification and ranking

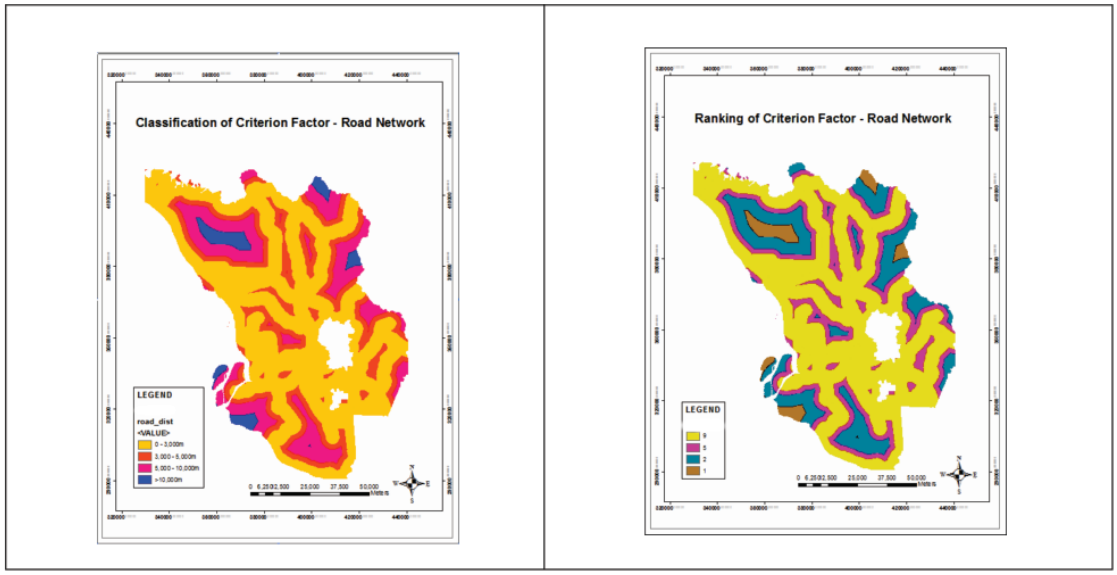

Fig. 9: Road network - Classification and ranking

The ranking for High Pollen Source (N1) is 9, Medium Pollen Source (N2) is 5 and for Low Pollen Source (N3) is 3 (refer to *Fig. 11*).

RESULTS

The ranks for each criterion maps were produced using Linear Transformation according to AHP value; and used for further suitability analysis. The are several methods in performing suitability analysis in ArcGIS9, for instance using map calculator, Weighted Overlay function or using tools that have been developed for AHP analysis.

Suitability Analysis - Weighted Overlay

Weighted Overlay is a technique for applying a common measurement scale of values to diverse and dissimilar inputs in order to create an integrated analysis. Geographic problems often

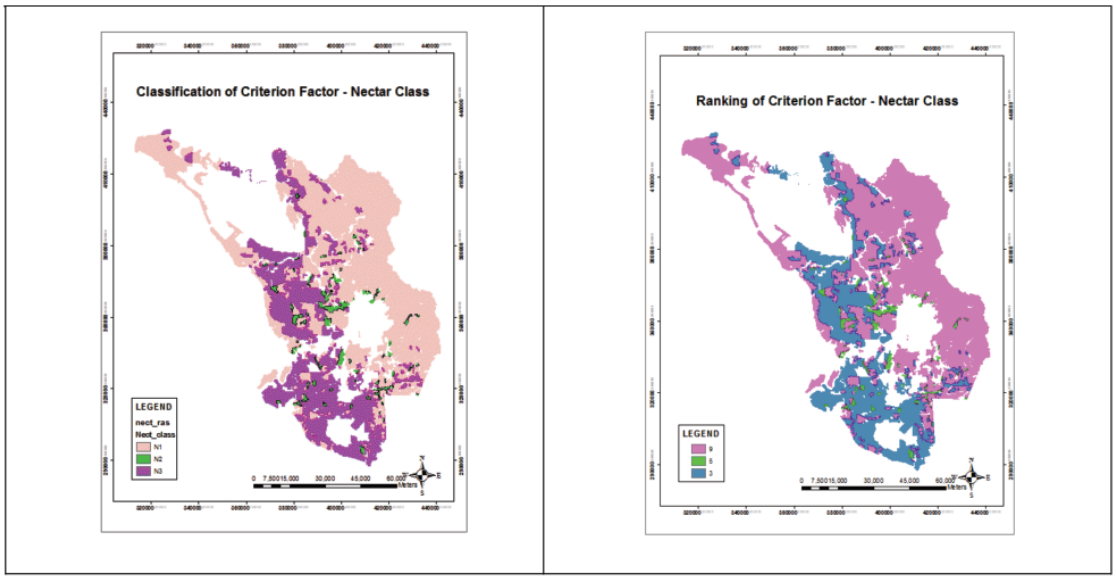

Fig. 10: Nectar class - Classification and ranking

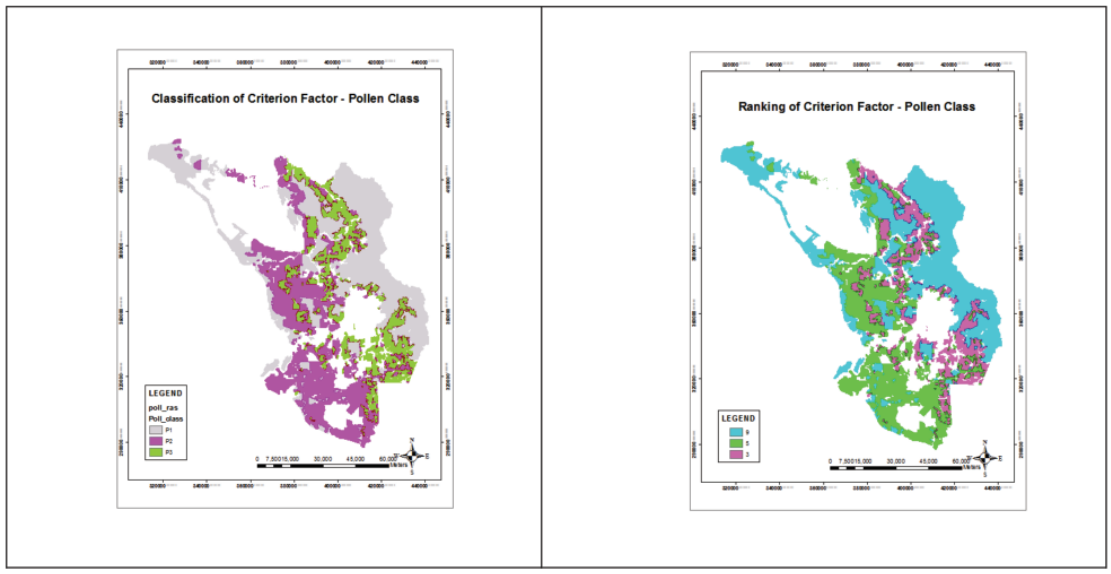

Fig. 11: Pollen class - Classification and ranking

require the analysis of many different factors, for instance, choosing the sites in the beekeeping zones. Every single layer of data must be prioritized accordingly.

Continuous (floating-point) rasters must be reclassified to integer before they can be utilised in ArcGIS9 which has been done during the linear transformation process. Each range must be assigned a single value before it can be used in the Weighted Overlay tool. The assigned weight need to be inserted in each input raster. The weight of each criterion is stated in Table 5.

For suitability analysis, five factors were considered: nectar class, pollen class, hydrology, road network and topography. The goal was to find suitable zones for beekeeping activity. The

TABLE 5
Percentage of weight

Criteria Factor	Weight % (Expert Choice)	Weight (%) Weighted Overlay
Nectar Class	37.4	37
Pollen Class	31.8	32
Hydrology Feature	16.8	17
Road Network	9	9
Topography	5	5

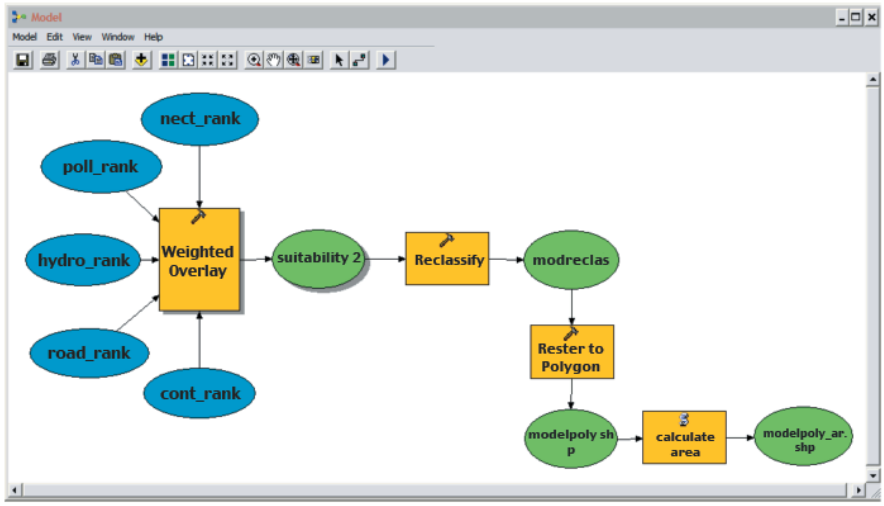

Fig. 12: Model for Generating Beekeeping Suitability Zone

weighted overlay model is displayed in *Fig. 12* as a process generated using Model Builder.

The weighted overlay function dialogue box is shown in *Fig. 13*, the integer values of each criterion was inserted in the percentage of influence column, while the rank was automatically inserted according to existing rank of each layer.

The results of weighted overlay analysis are the creation of a suitability map as shown in *Fig. 14*.

The most suitable areas are ranked as 9 followed by rank 3 for the least suitable area. The classification is accomplished using a Reclassification tool, Equal Interval and determines 3 classes. This analysis produces results in raster format and the suitability map was reclassified to S1 - Most Suitable, S2 - Suitable, S3 - Moderately Suitable as shown in *Fig. 15*.

A calculation of the total area was done to determine the effective suitable area for apiculture in Selangor as shown in Table 6.

DISCUSSION

The site verification was carried out at apiaries in Kuala Selangor and Mardi, Serdang to verify the results of the model. Several areas were visited and visually captured. The coordinate of each beekeeping site was recorded using a Silva Multi-Navigator GPS System. Information about the sites are shown in Table 7. The coordinates were then transferred into GIS system and evaluation of the area assessed in terms of its suitability according to the model developed.

The result is comparatively acceptable whereby the site verification data of the apiaries corresponded to S1 which is the Most Suitable area, as verified with the existing apiaries location as shown in *Fig. 16*.

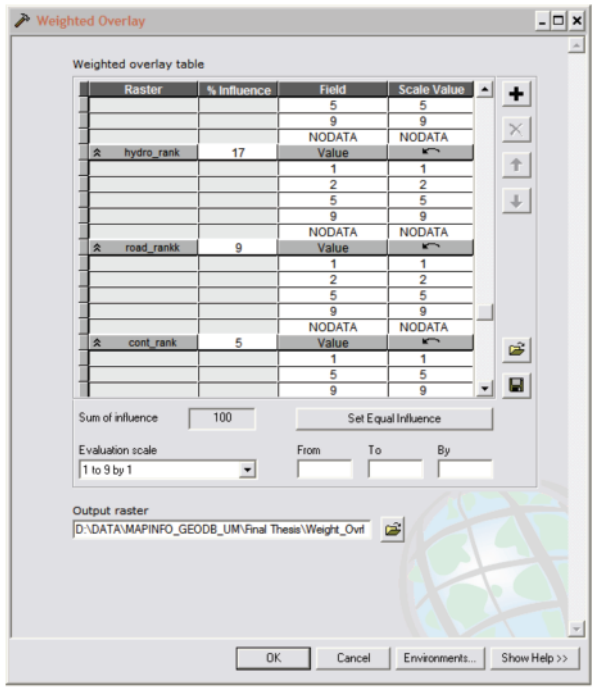

Fig. 13: Weighted overlay function

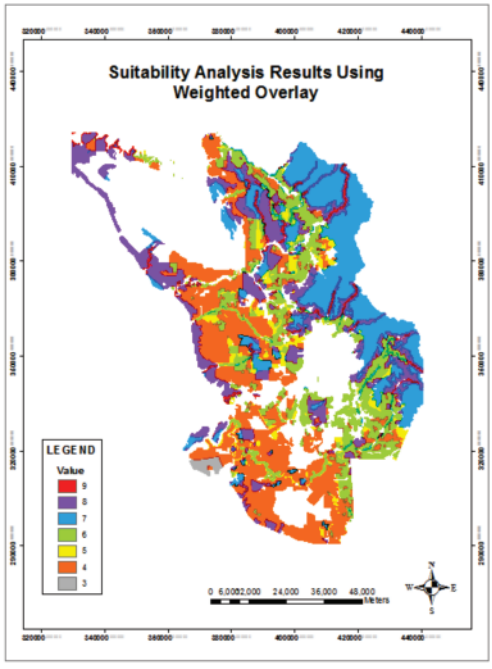

Fig. 14: Suitability map using weighted overlay

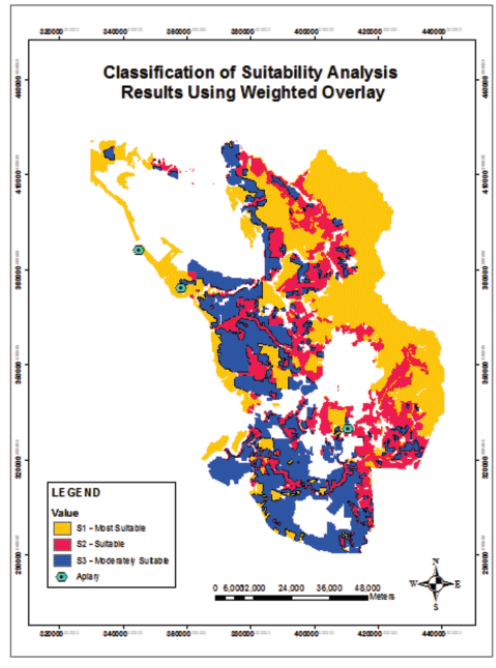

Fig. 15: Beekeeping uitability zone classification using weighted overlay

TABLE 6
Suitability hectarage

Suitability Analysis	Weighted Overlay	
	Hectares	Percentage
S1 - Most Suitable	109, 166.67	13.72%
S2 - Suitable	216, 757.24	27.24%
S3 - Moderately Suitable	193, 498.15	24.32%
Total Suitable area	519, 422.0589	65.27%
Not Suitable	276, 358.66	34.73%

TABLE 7
Information on Apiaries

GPS ID	X	Y	Owner	Address/Area	# Hives	Species
1.	3.38	101.20	Misbah b. Yusof	Kampung Sungai Gulang-Gulang, Kuala Selangor	9	Cerana, Trigona
2.	3.48	101.08	Lee Man Fay	Kampung Sekinchan, Kuala Selangor	100	Cerana
3.	2.98	101.68	Haji Hamzah	Mardi, Serdang	20	Cerana, Trigona, Florea

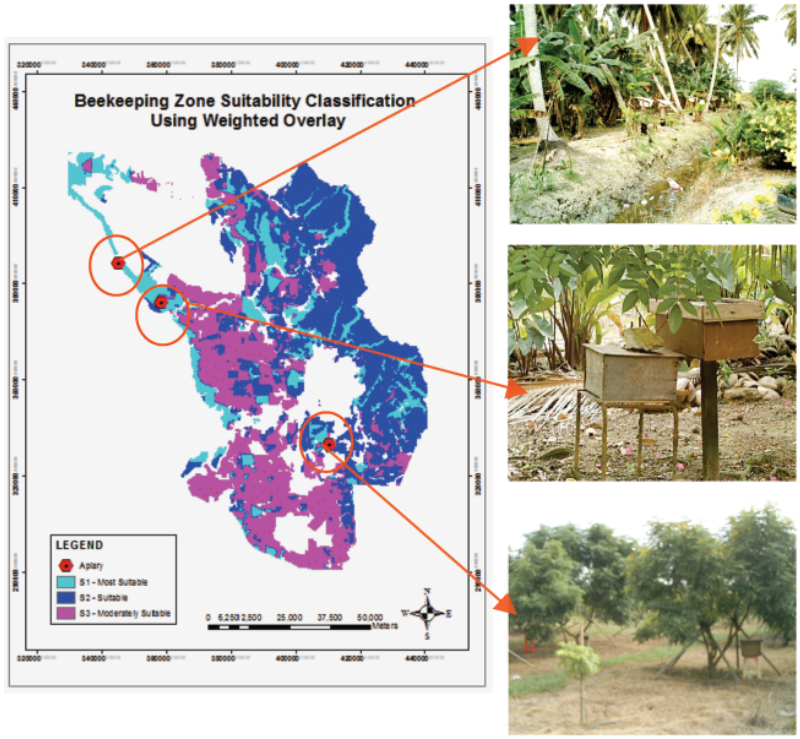

Fig. 16: Verification of suitability maps with existing apiaries

CONCLUSIONS

Integrating multi-criteria decision-making and GIS technology in locating beekeeping areas is one of the ways to determine or evaluate potential zones for beekeeping, since there are several factors that contribute to potential zones and each factor has a different weight. This research achieved its objective of using GIS and MCDA as tools to locate suitable zones for beekeeping. Verification of existing apiaries with the model using AHP techniques provides satisfactory results of weightage of each criterion for beekeeping zones suitability. By using AHP, a mathematical model of criteria that contributes to suitability of beekeeping zones could be established. The analysis is a guideline of suitable factors of beekeeping and the model could be modified to suit certain needs depending on the area of interest. The research outcome could be expanded in further research to forecast the flowering time for migratory beekeeping. Evaluation and zoning of suitable beekeeping areas can contribute to the implementation of beekeeping activity on a large or small scale.

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Studies on Variability of Indigenous Mulberry Germplasm on Growth and Leaf Yield

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ABSTRACT

Twenty-five indigenous mulberry germplasm were evaluated for growth and yield traits in a partial balanced lattice design at Central Sericultural Germplasm Resources Centre, Hosur, Tamil Nadu, India. The leaf yield performance showed high variation among the mulberry accessions. Analysis of variance on growth and yield showed significant variation among the accessions for all the traits. Seasonal variation was also observed in respect of leaf yield. The interaction between accession and season was highly significant for all traits. The coefficient of variation was maximum for single leaf weight (36.32%) and minimum for leaf moisture content (2.08%). The correlation coefficient analysis indicated significant association of leaf yield and other growth traits. The number of branches per plant, length of the longest shoot, total shoots length, leaf moisture content and leaf moisture retention capacity showed associations at different levels of significance with leaf yield. Other traits showed complex relationships with each other. Divergence analysis grouped 25 indigenous mulberry accessions into 7 clusters. Maximum accessions were grouped in cluster I (6 acc.) followed by cluster II (5 acc.) V (5 acc.), VII (4 acc.), IV (3 acc.) and minimum one each in cluster III and VI. The diversity among the accessions measured by inter-cluster distance (D2) showed variation. The cluster group indicates that mulberry accessions were distributed in different clusters irrespective of geographical distribution. The CIMMYT selection index shows that mulberry accession MI-0416 performed better among the accessions studied followed by MI-0308, MI-0376, MI-0437 and MI-0310. Other accessions showed higher index value that is less suitable for selection.

Keywords: Mulberry germplasm, variability, indigenous, growth, leaf yield

INTRODUCTION

Mulberry, a heterozygous perennial plant is the principal food of silkworm (*Bombyx mori* L.). The mulberry germplasm provides suitable material for silkworm feeding and hence the importance lies in maintaining a large number of germplasm to develop improved elite genotypes. Due to the needs of the sericulture industry, improved mulberry varieties are being developed and maintained. The improvement in quantity and quality of mulberry genotypes over the existing ones is a continuous process and breeders want suitable parental material for developing superior genotypes. Moreover, at present, there is increasing demand to develop region and season specific mulberry varieties to feed silkworm races. The central Sericultural Germplasm Centre is maintaining 1100 mulberry germplasm collected from 26 different countries. The mulberry accessions are continuously being characterized and documented. Many of the mulberry accessions have been characterized for morphological as well as important economic traits (Thangavelu *et al.*, 2000). Knowledge of genetic variability is important for breeding (Frankel and Brown 1983; Frey *et al.*, 1983) and useful to improve the specific set of characters in low yielding mulberry germplasm. The diversity, variability and related factors have been highlighted by different authors on growth and yield traits (Fotedar and Dandin, 1998; Tikader *et al.*, 1999; Tikader and Rao, 2002, Tikader *et al.*, 2003; Vijayan *et al.*, 1999). Various authors reported the association of leaf yield with other

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traits in mulberry (Vijayan *et al.*, 1997). Thereafter the selected elite germplasm is needed for testing using appropriate statistical designs to know the actual performance for further assessment. At present, information on germplasm evaluation under field conditions is scanty.

The present study was initiated to identify suitable mulberry accessions based on variability, association and performance over seasons to select suitable genetic materials for further utilization.

MATERIALS AND METHODS

Plant Materials

Twenty-five indigenous mulberry accessions were used in this study. The collections of mulberry accessions were from different states in India as follows: Uttarakhand (8), Karnataka (6), Kerala (5), West Bengal (2) and 1 each from Uttar Pradesh, Assam, Meghalaya and Tamil Nadu (Table I).

Experimental Layout

The experiment was set up in a Partial lattice design with three replications at Central Sericultural Germplasm Resources Center, (CSGRC), Hosur, Tamil Nadu, India. The centre is situated at 12.45° N, 77.51° E and 942 m altitude with tropical dry climate. The average rainfall ranges from 500 – 1000 mm per annum. The soil is red loamy with pH 6.5 – 7.5. The plantation was maintained as low bush with 90 x 90 cm spacing with 9 plants per replication with standard cultural practices (Thangavelu *et al.*, 2000; Tikader and Rao, 2002). The first pruning was done after one year of establishment of the plantation, with four harvests per year after pruning at 90 days interval.

Data Recording

After 90 days of pruning seven plants were randomly sampled from each replication for evaluating eight growth and yield traits. The traits were number of branches per plant (NB), length of the longest shoot (LLS), total shoot length (TSL), internodal distance (ID), leaf moisture content (MC), leaf moisture retention capacity (MRC), leaf yield per plant (LYD) and single leaf weight (SLW). The data on agronomical traits were collected 4 times per

TABLE 1
Details of indigenous mulberry accessions used for the study

Sl No.	Accession	Accession name	Collection/origin (State)
1	MI-0029	Kollegal	Karnataka
2	MI-0080	BC259	West Bengal
3	MI-0154	UP-14	Uttar Pradesh
4	MI-0252	Kalimpong local	West Bengal
5	MI-0290	*Morus alba*	Karnataka
6	MI-0296	Acc.16	Karnataka
7	MI-0301	Acc.1	Karnataka
8	MI-0308	V1	Karnataka
9	MI-0310	Chak Majra	Uttarakhand
10	MI-0312	Gulikadava	Kerala
11	MI-0313	Seekupari	Tamil Nadu
12	MI-0324	ERRC-101	Kerala
13	MI-0326	ERRC-71	Kerala
14	MI-0346	Tingari local	Assam
15	MI-0349	Garobodha	Meghalaya
16	MI-0369	Resham Majri - 6	Uttarakhand
17	MI-0370	Resham Majri - 7	Uttarakhand
18	MI-0376	Kunjagao - 2	Uttarakhand
19	MI-0388	Herbertpur	Uttarakhand
20	MI-0400	Krishnaswami - 2	Karnataka
21	MI-0415	Guhanathapuram	Kerala
22	MI-0416	Keeraithodu	Kerala
23	MI-0431	Saharanpur Road	Uttarakhand
24	MI-0437	Baragarh - 2	Uttarakhand
25	MI-0439	RSRS, Sahaspur	Uttarakhand

year for 3 years resulting in 13 harvests from 2002 – 2006. Standard procedures were followed as described by various authors (Jolly and Dandin, 1986; Machii *et al.*, 1997, 2001; Tikader and Rao, 2002; Thangavelu *et al.*, 2000). The leaf moisture content and leaf moisture retention capacity was calculated as described by Tikader and Roy (1999) and Vijayan *et al.* (1996, 1997).

$$\text{Moisture content (\%)} = \frac{\text{Leaf weigh fresh} - \text{Oven dry leaf weight}}{\text{Leaf weight fresh}} \times 100$$

$$\text{Moisture retention capacity (\%)} = \frac{\text{Leaf weighafter 6 hours dry} - \text{Oven dry leaf weight}}{\text{Leaf weightfresh} - \text{Oven dry leaf weight}} \times 100$$

Data Analysis

Data was y analysed using the SPSS statistical package. Analysis of variance of the eight growth and yield traits were carried out using with the adjusted values. The mean values for eight growth and yield traits were used for correlation matrix and cluster analysis. Pair wise distances between the accessions based on Mahalanobis distances were recorded (Mahalanobis, 1936). Ward's minimum variance cluster analysis was used to group the tested mulberry germplasm accessions (Ward, 1963). The selection index was based on the method described by The International Maize and Wheat Improvement Centre (CIMMYT).

RESULTS AND DISCUSSION

The mulberry germplasm accessions tested in this experiment are presented in Table 1. The accessions were collected from different sources through surveys and exploration and collections from institutes.

Variability Among Mulberry Accessions

The agronomic variation among the accessions is presented in Table 2. A high variation was observed in different growth and yield related traits. The ranges for the different traits are as follows: number of branches per plant (9.30 – 14.39); length of the longest shoot (122.43 – 176.38 cm); total shoot length (891.26 – 1718.38 cm); internodal distance (4.19 – 6.96 cm); leaf moisture content (70.22 – 75.79%); leaf moisture retention capacity (61.00 – 72.75%); leaf yield per plant (496.68 – 1071.08 g) and single leaf weight (1.82 – 6.80 g). The coefficient of variation was maximum in single leaf weight (36.32%) followed by leaf yield per plant (17.83%); total shoot length (16.48%); number of branches per plant (12.03%) and minimum for leaf moisture content (2.08%). The F-ratio indicated that all the accessions were highly significant for all the traits. The seasonal variation was also highly significant (1% probability) except for the number of branches per plant. The interaction between accession x season was also significant at 5% probability level. These results agree with the observations made by Tikader and Roy (2001) and Tikader and Dandin (2005).

Simple Correlation Matrix

Simple correlation coefficient was carried out among the accessions on different growth and yield traits (Table 3). The leaf yield was expressed in combination with other traits such as length of the longest shoot, total shoot length, leaf moisture content and leaf moisture retention capacity. Other traits showed a complex association with each other. Individual traits showed positive association such as number of branches per plant with total shoot length (0.90**) and negative association with leaf moisture retention capacity (-0.46*) and single leaf weight (-0.48*); length of the longest shoot was positively associated with total shoot length (0.69**) and leaf yield per plant (0.51**); total shoot length showed a positive association with leaf yield (0.52**); internodal distance was positively associated with leaf moisture content (0.55**), Leaf moisture retention capacity (0.59**) and single leaf weight (70**); leaf moisture content showed positive relationships with leaf moisture retention capacity (0.77**), leaf yield per plant (0.46*) and single leaf weight (0.76**); leaf moisture retention capacity showed positive relationships with leaf yield per plant (0.53**) and single leaf weight (0.91**). Similar observations on association of different traits with leaf yield was also reported by Sarkar *et al.*, (1987), Tikader and Roy (2001), Tikader and Dandin (2005) and Vijayan *et al.* (1997).

Divergence Analysis

The genetic diversity analysis among the mulberry accessions was carried out and the accessions were grouped into 7 clusters following divergence

TABLE 2
Mean performance of indigenous mulberry accessions

Sl. No.	Accessions	NB	LLS	TSL	INTD	MC	MRC	LYD	SLW
1	MI-0029	14.39	154.62	1718.60	5.08	73.53	63.50	818.42	2.22
2	MI-0080	9.35	122.43	891.26	5.15	75.28	71.12	686.10	5.77
3	MI-0154	11.92	166.81	1521.28	4.41	71.33	65.83	866.92	2.36
4	MI-0252	10.11	174.56	1257.08	6.96	74.47	72.20	828.87	6.80
5	MI-0290	12.61	170.88	1605.33	5.35	73.28	63.12	796.38	2.59
6	MI-0296	14.27	143.28	1611.64	5.19	71.81	61.83	790.23	2.55
7	MI-0301	10.08	142.69	1120.67	5.09	73.92	68.17	598.83	3.49
8	MI-0308	11.31	165.98	1434.08	5.18	74.96	72.36	906.57	4.44
9	MI-0310	11.21	150.40	1338.80	5.57	74.56	72.32	993.35	5.46
10	MI-0312	10.27	154.28	1225.83	4.44	70.74	61.49	496.68	1.88
11	MI-0313	13.67	161.67	1677.55	4.43	71.78	61.00	788.86	2.16
12	MI-0324	11.81	152.00	1365.14	5.96	73.57	70.21	820.70	4.80
13	MI-0326	11.65	153.07	1297.08	5.59	73.66	70.08	746.93	4.86
14	MI-0346	12.73	151.62	1515.06	4.19	70.22	63.45	725.63	1.82
15	MI-0349	11.93	147.00	1355.28	4.78	72.49	63.29	612.02	2.51
16	MI-0369	12.13	139.00	1347.68	5.14	74.19	69.91	861.65	3.80
17	MI-0370	9.30	135.10	970.08	4.30	72.07	65.12	526.07	2.54
18	MI-0376	13.10	176.38	1715.08	6.06	75.24	70.80	902.59	4.12
19	MI-0388	10.92	127.45	1085.68	4.98	73.29	67.46	657.16	3.73
20	MI-0400	10.88	133.34	1145.50	5.28	74.21	67.82	719.67	4.19
21	MI-0415	11.28	145.64	1294.93	5.52	70.75	67.60	709.45	3.42
22	MI-0416	11.60	174.45	1552.23	4.92	73.37	70.66	1071.08	5.18
23	MI-0431	10.86	150.07	1278.90	5.15	74.13	69.69	860.20	4.61
24	MI-0437	10.74	145.81	1244.30	5.03	75.79	72.75	950.21	5.59
25	MI-0439	9.95	160.01	1239.05	5.35	73.18	69.05	725.85	4.53
Mean		11.52	151.94	1352.32	5.16	73.27	67.63	778.42	3.82
Min		9.30	122.43	891.26	4.19	70.22	61.00	496.68	1.82
Max		14.39	176.38	1718.60	6.96	75.79	72.75	1071.08	6.80
SE		0.28	2.92	44.58	0.12	0.31	0.76	27.75	0.28
CV%		10.03	9.60	16.48	11.74	2.08	5.61	17.83	36.32
F-test									
Accession		**	**	**	**	**	**	**	**
Season		NS	**	**	**	**	**	**	**
Acc x sea		NS	**	**	**	**	**	**	**

**, Significant at 1% level, NS = Non-significant
NB = Number of branches per plant, LLS= Length of the longest shoot (cm), TSL = Total shoot length (cm), INTD=Internodal distance (cm), MC= Leaf moisture content (%), MRC=Leaf moisture retention capacity (%), LYD= Leaf yield per plant (g), SLW= Single leaf weight (g)

analysis (Table 4). Cluster I consisted of 6 accessions followed by 5 accessions each in clusters II and V and one accession each in cluster III and VI. The accessions grouped in cluster I showed the highest number of branches per plant (13.27) and total shoot length (1608.24 cm) (Table 5). Cluster II showed the highest leaf moisture content (74.78%) and leaf yield per plant (964.76g). Cluster III showed longer internodal distance, though a negative trait, it can be improved through suitable breeding approaches. The high single leaf weight was directly proportional to a longer internodal distance as reported by Tikader and Roy (2001). The other accessions that are grouped in clusters with traits having minimum values provide scope for further selection and improvement. All the materials collected from different geographical regions grouped in the same cluster or nearby cluster showed no relationship between genetic divergence and geographical diversity. Similar results were also highlighted by various authors

TABLE 3
Correlation matrix of different growth and yield traits in mulberry accessions

Traits	X1	X2	X3	X4	X5	X6	X7	X8
X1	——							
X2	0.33	——						
X3	0.90**	0.69**	——					
X4	-0.07	0.29	0.01	——				
X5	-0.23	-0.01	-0.19	0.55**	——			
X6	-0.46*	0.05	-0.33	0.59**	0.77**	——		
X7	0.35	0.51**	0.52**	0.33	0.46*	0.53**	——	
X8	-0.48*	0.04	-0.37	0.70**	0.76**	0.91**	0.48*	——

*, Significant at 5% level **, Significant at 1% level
X1 = Number of primary branches, X2 = Length of the longest shoot (cm),
X3 = Total shoot length (cm), X4 = Internodal distance (cm),
X5= Leaf moisture content (%), X6 = Leaf moisture retention capacity (%),
X7 = Leaf yield per plant (g), X8 = Single leaf weight (g)

TABLE 4
Distribution of indigenous mulberry accessions in different clusters

Clusters	No. of mulberry accessions	Accession details with States of India
I	6	MI-0029 - Kollegal (Karnataka) MI-0296 – Acc. 16 (Karnataka) MI-0290 - *Morus alba* (Karnataka) MI-0313 - Seekupari (Tamil Nadu) MI-0346 – Tingari local (Assam) MI-0154 – UP – 4 (Uttar Pradesh)
II	5	MI-0310 - Chak Majra (Uttarakhand) MI-0437- Baragarh –2 (Uttarakhand) MI-0308- V1 (Karnataka) MI-0416 – Keeraithodu (Kerala) MI-0376 – Kunjagao –2 (Uttarakhand)
III	1	MI-0252 – Kalimpong local (West Bengal)
IV	3	MI-0312- Gulikadava (Kerala) MI-0349- Garobodha (Meghalaya) MI-0370- Resham Majri – 7(Uttarakhand)
V	5	MI-0324- ERRC – 101 (Kerala) MI-0326- ERRC-73 (Kerala) MI-0439– RSRS, Sahaspur (Uttarakhand) MI-0369- Resham Majri –6 (Uttarakhand) MI-0431- Saharanpur Road (Uttarakhand)
VI	1	MI-0415- Guhanathapuram (Kerala)
VII	4	MI-0388 - Herbertpur (Uttarakhand) MI-0400- Krishnaswami –2 (Karnataka) MI-0301 - Acc. 1 (Karnataka) MI-0080 - BC259
Total	25	

TABLE 5
Cluster mean of growth traits of indigenous mulberry based on D2 values

	Clusters						
Traits	I	II	III	IV	V	VI	VII
X1	13.27	11.59	10.11	10.50	11.28	11.28	10.31
X2	158.15	162.60	174.56	145.46	150.83	145.64	131.48
X3	1608.24	1456.90	1257.08	1183.73	1305.57	1294.93	1060.78
X4	4.77	5.35	6.96	4.51	5.44	5.52	5.12
X5	71.99	74.78	74.47	71.76	73.75	70.75	74.17
X6	63.12	71.78	72.20	63.29	69.79	67.60	68.64
X7	797.75	964.76	828.87	544.92	803.07	709.45	665.44
X8	2.28	4.96	6.80	2.31	4.52	3.42	4.29

X1 = Number of branches per plant,
X2 = Length of the longest shoot (cm),
X3 = Total shoot length (cm),
X4 = Internodal distance (cm),
X5 = Leaf moisture content (%),
X6 = Leaf moisture retention capacity (%),
X7 = Leaf yield per plant (g),
X8 = Single leaf weight (g)

TABLE 6
Average inter and intra-cluster D2 values in 7 clusters

Clusters	I	II	III	IV	V	VI	VII
I	**2.37**	4.67	6.58	4.04	3.98	3.43	5.04
II		**2.64**	3.90	5.66	2.71	4.20	4.34
III			**0.00**	6.78	3.77	4.94	5.08
IV				**2.51**	4.12	3.04	3.64
V					**1.70**	2.57	2.77
VI						**0.00**	3.19
VII							**2.06**

Normal values indicate inter cluster distance
Bold values indicate intra-cluster distance

(Rajan *et al.*, 1997; Tikader and Roy, 2002; Tikader and Rao, 2003).

The average inter and intra-cluster distance presented in Table 6, reveals that a single accession in cluster III, has minimum intra-cluster distance. The maximum intra-cluster distance was in cluster II (2.64) and the accessions grouped in this cluster are diverse. The inter-cluster distance ranged from 2.71 to 6.78. The minimum inter-cluster distance was observed between cluster II and cluster V (2.71) which revealed close relationship among the accessions while the maximum inter-cluster distance was observed between clusters III and IV (6.78) thus revealing maximum diversity among the accessions.

CIMMYT Selection Index

The mulberry accessions are grouped based on the overall performance through CIMMYT selection index to select the high performing accessions for further use (Table 7). Mulberry accession MI-0416 performed better than all the other test materials while MI-0370 and MI-0312 were the poorest performers among the mulberry accessions tested. Due to the need for further utilization, other suitable mulberries may be selected from the pooled accessions.

TABLE 7
Listing of selected mulberry germplasm accessions (CIMMYT selection index)

Sl no.	Acc. no	Index	NB	LLS	TSL	INTD	MC	MRC	LYD	SLW
1	MI-0416	15	11.60	174.45	1552.23	4.92	73.37	70.66	1071.08	5.18
2	MI-0376	17	13.10	176.38	1715.08	6.06	75.24	70.80	902.59	4.12
3	MI-0308	17	11.31	165.99	1434.08	5.18	74.96	72.36	906.57	4.44
4	MI-0437	19	10.74	145.81	1244.30	5.03	75.79	72.75	950.21	5.59
5	MI-0310	19	11.21	150.40	1338.80	5.57	74.56	72.32	993.35	5.45
6	MI-0431	21	10.86	150.07	1278.90	5.15	74.13	69.69	860.20	4.61
7	MI-0369	21	12.13	139.00	1347.68	5.14	74.19	69.91	861.65	3.80
8	MI-0029	21	14.39	154.62	1718.60	5.08	73.53	63.50	818.42	2.22
9	MI-0326	22	11.65	153.07	1297.08	5.59	73.66	70.08	746.93	4.86
10	MI-0324	22	11.81	152.00	1365.14	5.96	73.57	70.21	820.70	4.80
11	MI-0290	22	12.61	170.88	1605.33	5.35	73.28	63.12	796.38	2.59
12	MI-0154	22	11.92	166.81	1521.28	4.41	71.33	65.83	866.92	2.36
13	MI-0313	23	13.67	161.67	1677.55	4.43	71.78	61.00	788.86	2.16
14	MI-0439	24	9.95	160.01	1239.05	5.35	73.18	69.05	725.85	4.53
15	MI-0252	24	10.11	174.56	1257.08	6.96	74.47	72.20	828.87	6.80
16	MI-0296	24	14.27	143.28	1611.64	5.19	71.81	61.83	790.23	2.55
17	MI-0400	25	10.88	133.34	1145.50	5.28	74.21	67.82	719.67	4.19
18	MI-0346	26	12.73	151.62	1515.06	4.19	70.22	63.45	725.63	1.82
19	MI-0349	26	11.93	147.00	1355.28	4.78	72.49	63.29	612.02	2.51
20	MI-0301	26	10.08	142.68	1120.67	5.09	73.92	68.17	598.83	3.49
21	MI-0415	27	11.28	145.64	1294.93	5.52	70.75	67.60	709.45	3.42
22	MI-0388	27	10.92	127.45	1085.68	4.98	73.29	67.46	657.16	3.77
23	MI-0080	28	9.35	122.43	891.26	5.15	75.28	71.12	686.10	5.77
24	MI-0312	31	10.27	154.28	1225.83	4.44	70.74	61.49	496.68	1.88
25	MI-0370	31	9.30	135.10	970.08	4.30	72.07	65.12	526.07	2.54

NB = Number of primary branches, LLS = Length of the longest shoot (cm),
TSL = Total shoot length (cm), ID = Internodal distance (cm),
MC= Leaf moisture content (%), MRC = Leaf moisture retention capacity (%),
LYD = Leaf yield per plant (g), SLW = Single leaf weight (g)

CONCLUSIONS

The mulberry accessions were selected from a field gene bank after preliminary characterization. The evaluation of mulberry accessions in this particular statistical design, interaction of accession and season have provided relevant data to estimate their ability to establish and express their potential. The analysis of variance, correlation, genetic diversity and selection indices have shown that the selected accessions have performed equally well with the existing high yielding genotypes. Some mulberry accessions performed better (MI-0416) and at par (MI-0376) with the check (MI-0308). These accessions with desirable traits may be selected and exploited for crop improvement in future mulberry breeding programmes.

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Properties of Pyrethroids-Treated Particleboards Manufactured from Rubberwood and Oil Palm Empty Fruit Bunches (EFB)

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ABSTRACT

The incorporation of small amounts of pyrethroid-based preservatives (Organic solvent-based permethrin (OSP), Water-based permethrin (WP) and deltamethrin) into particleboards made from latex timber clone rubberwood (RRIM 2002) *(Hevea brasiliensis)*, empty fruit bunches (EFB) of oil palm *(Eleasis guineensis)* and rubberwood + EFB (70:30 parts) blend through spraying during blending of furnish significantly increased the resistance of the boards to white rot *(Pycnoporous sanguineus)* and subterranean termite *(Coptotermes curvignathus)* attacks. This paper discusses the physical and mechanical properties of the pyrethroid-treated particleboards. These properties were compared with those of boric acid-treated and untreated particleboards. The preservative treatment did not significantly affect the internal bond (IB) and modulus of rupture (MOR) of rubberwood and rubberwood + EFB blend particleboards. However, the modulus of elasticity (MOE) of these boards increased when treated with either WP, or OSP. For the EFB boards, all pyrethroid compounds reduced the IB of the board whereas the MOR and MOE were either reduced or unaffected. However, the thickness swelling (TS) of rubberwood particleboard, improved when treated with all the pyrethroid-based preservatives, whilst the other two types of boards were less stable when treated with OSP or WP. Among the three types of particleboards, the mechanical properties of EFB particleboards were affected most by the pyrethroid treatments. The mechanical and physical properties of treated particleboards from rubberwood and admixture were comparable or better than those of the untreated particleboards. Boric acid treatment reduced the MOR of all particleboards but other properties were either improved or unaffected.

Keywords: Boric acid, empty fruit bunches, particleboard, pyrethroids, rubberwood

INTRODUCTION

Fabricating particleboards from rubberwood and rubberwood blended with oil palm empty fruit bunches (EFB) have been shown to have a huge potential (Chew and Ong 1985; Rahim *et al.,* 1994). Research has been conducted on fabricating homogenous and heterogenous layered particleboards from admixtures of these materials (Rahim *et al.*, 1994; Abdul Karim *et al.*; 1994, Zaidon *et al.*, 2007). The results indicated that most of the properties are satisfactory and meet the minimum Japanese Industrial Standards of particleboard (JIS A 5908) (JIS 2003). In general, rubberwood composites are less susceptible to rotting fungus and subterranean termite compared to solid wood (Zaidon *et al.,* 2002; Zaidon *et al.,* 2003). The higher resistance of the composites towards the deteriorating agents was attributed to the utilization of formaldehyde based adhesives (Behr, 1972). Due to the environmental impact caused by formaldehyde emission from the binder of the composites, improvements to these products are being made through the use of low formaldehyde

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emission adhesives such as the E1-grade melamine urea formaldehyde (maximum formaldehyde emission < 0.01 ppm).

This type of adhesive, however, has relatively inferior bonding strength, hence is more susceptible to biodegradable agents. Research have shown that the resistance of rubberwood composites to fungal and termite attack can be enhanced through incorporation of small amounts of boron compounds and chromated copper arsenate (CCA) (Zaidon *et al.*, 1998; Zaidon *et al.*, 2002; Zaidon *et al.*, 2003). Nevertheless, these compounds are not preferred nowadays due to their toxicity hazards and potential environmental pollution. Thus, new and more environment friendly preservative formulations are sought to preserve particleboards. Pyrethroids are a potential group of preservatives that can serve as an alternative to boron and CCA. Pyrethroids are a synthetic form of pyrethrin that has insecticidal properties. They are one of the least poisonous insecticides to mammals (Ray, 1991; Tomlin, 1994). Some of the pyrethroids that have been formulated into preservatives include permethrin, cypermethrin and deltamethrin and they are available in the market under different trade names.

Previous studies revealed that treatment of low formaldehyde emission MUF-bonded particleboards made from blending juvenile rubberwood, EFB and rubberwood-EFB through soaking of particles with 0.02% deltamethrin and 2% boric acid solutions enhanced the resistance of the product against white rot fungus (*Pycnoporous sanguineus*) and termite (*Coptotermes curvignathus*) attacks (Zaidon *et al.,* 2007). Most of the strength and physical properties, however, were reduced except for particleboards fabricated from empty fruit bunches. Improper curing of adhesive was identified as the cause for the poor performance of boards. This was attributed to the interference caused by the dry salt retention during manufacture of the board. It was thought that the pressing time used in the previous study, as recommended by the resin supplier was not sufficient to fully cure the resin. In another study, the incorporation of small amounts of pyrethroid-based and boric acid preservatives into particleboards made from rubberwood (clone RRIM 2002), empty fruit bunch (EFB) and rubberwood-EFB blend through spraying during blending resulted in significant increase in resistance to white rot and subterranean termite attacks (Zaidon *et al.*, 2008). However, the effects of these treatments on the mechanical and physical properties of the particleboards are unknown.

This paper reports the mechanical and physical properties of pyrethroid-treated rubberwood, EFB and rubberwood + EFB blend particleboards. The change in properties over untreated and boric acid-treated boards is also discussed.

MATERIALS AND METHODS

The materials used in this study were four-year-old latex timber clone rubber tree, *Hevea brasiliensis* Müll Arg. (clone RRIM 2002) which was extracted from Besut Terengganu and empty fruit bunches (EFB) from oil palm trees (*Elaeis guinensis* Jacq.) supplied by SABUTEK Sdn. Bhd, Telok Intan, Perak. Both materials were chipped, flaked and screened into 0.5-2.0 mm sized particles. The particles were dried to 5% moisture content (MC). The adhesive used in this study was a low formaldehyde (E1-grade resin) (MUF-E1, maximum permissible formaldehyde emission < 0.1 ppm) supplied by Malaysian Adhesive Company, Sdn. Bhd., Shah Alam. New formulations of pyrethroid-based preservatives (water-based permethrin, organic-solvent-based preservative, and deltamethrin) were used as treatment solutions. The concentration of the solution was calculated and the amount of active ingredients to be loaded in each board was maintained at the level recommended by the producers (Data sheet 2000, 2003 and NA). The active ingredients and concentration of each chemical compound are listed in Table 1. OSP is an organic solvent based preservative, while WP and deltamethrin are water-based preservatives. Analytical grade boric acid (orthoboric acid, H_3BO_3) was also used for comparison purposes.

Manufacture of Particleboard

Single layered particleboards with dimensions 340 mm x 340 mm x 10 mm with target density of 650 kg m^{-3} and final MC of 12% were fabricated. The parameters used for manufacture of the particleboards are summarized in Table 2. Boards from each treated and untreated rubberwood, EFB and rubberwood + EFB blend (70 parts rubberwood and 30 parts EFB) were made. Preliminary work showed that a higher composition of rubberwood in the admixture

TABLE 1
Chemical composition of preservatives used

Preservative	Composition	Concentration
Organic solvent-based permethrin (OSP)	Tributyltin napthenate	3.5%
	Permethrin	0.2%
	Dichlofuanid	0.1%
	Solvent Organic	96.2%
	(Total active ingredient)	(3.8%)
Water-based permethrin (WP)	Disodium octaborate	10%
	Benzalkonium chloride	2.0%
	Permethrin	0.2%
	Water	87.8%
	(Total active ingredient)	**(12.2%)**
Deltamethrin	Deltamethrin	0.2%
	Water	99.8%
	(Total active ingredient)	**(0.2%)**
Boric acid	Orthoboric acid	100% (solid)
	(Total active ingredient)	**(100%)**

[1]Data sheet 2000, [2]Data sheet 2003, [3]Data sheet NA

resulted in superior quality particleboard. The preservative solutions of various concentrations were sprayed onto the furnish, which was first blended with 11% MUF-EI and 1% wax. The concentrations of the solution were calculated to obtain loading of active ingredients in each board as recommended by the producers (Data sheet 2000, 2003, NA). For boric acid treatment, a pre-weighed solid salt was first dissolved in distilled water to produce a solution. The treated furnish was blended for 15 min to ensure uniform distribution of active ingredients in the boards. The furnish was then formed in a former, pre-pressed and subsequently pressed in a hot press at 160^0C. The pressing time for each individual treatment varied from 370 s to 520 s. Variations in pressing time for different pyrethroid treatments has been reported by Zaidon *et al.* (2007). For each treatment combination, a total of 6 boards were produced. Prior to cutting into testing blocks, all boards were conditioned at 20^0C and 65% relative humidity until equilibrium, i.e. 12% EMC.

TABLE 2
Parameters used for manufacture of the particleboards

Raw materials	Rubberwood (Clone RRIM 2002) Empty fruit bunches (EFB) Rubberwood-EFB blend (70:30 parts)
Target board density	650 kg m^{-3}
Target board MC	12%
Board size	(340 x 340 x 10) mm^3
Adhesive	
1. MUF-E1 grade (55.8% solid)	11% (w/w of particles)
2. Industrial grade Hardener, (NH_4Cl)	1% (w/w of solid resin)
3. Wax	1% (w/w of particles)
Preservatives	
1. OSP	5% solution (w/w of particles) or 0.19%a.i
2. WP	5% solution (w/w of particles) or 0.61% a.i
3. Deltamethrin	5% solution (w/w of particles) or 0.01% a.i
4. Boric acid	0.5% solution (w/w of particles) or 0.5% a.i.

Evaluation of Mechanical and Physical Properties

The boards were trimmed at the edges and cut into required dimensions for physical and mechanical tests. The tests conducted were static bending, internal bond, thickness swelling and water absorption. The static bending and internal bond tests were carried out using Universal Testing Machine (INSTRON, 50 kN). These were conducted according to Japanese Industrial Standard of Particleboard (JIS A 5908) (JIS 2003).

Statistical Analysis

Statistical analyses were performed on the physical and mechanical properties data to test if there are significant changes between the treated and untreated boards. Since a wide variation of density was found on the boards (570-740 kg m^{-3}), an analysis of covariance (ANOCOV) was performed to correct for expected differences in physical and mechanical properties due to density. Thus, density was chosen as a concomitant variable. Adjusted means were separated using Least Significant Difference (LSD) method.

RESULTS AND DISCUSSION

The adjusted values for mechanical and physical properties of treated and untreated particleboards are summarized in Table 3. For modulus of rupture (MOR) and modulus of elasticity (MOE) values, the negative signs indicate a reduction in the value of properties, while for thickness swelling and water absorption, the negative sign reflects an improvement in dimensional stability.

Properties of Treated Rubberwood Particleboards

There was no difference in internal bond (IB) on deltamethrin, OSP-treated and the untreated rubberwood particleboards. The IB values of these boards ranged from 0.91 to 1.05 N mm^{-2}. These values were comparable to the IB value found for boric acid-treated boards (0.98 N mm^{-2}). A significant reduction of 7.0% was found for the average IB values of OSP-treated boards. The MOR and MOE pyrethroid-treated boards were significantly higher than the untreated boards. The MOR increased by 8-15% from the original strength of 16.8 N mm^{-2}, while the MOE increased by 6-15% from the original stiffness of 1719 N mm^{-2}. Boric acid treatment significantly reduced the MOR by 21.9% to 13.1 N mm^{-2}, but this treatment did not affect the MOE of the boards. With regards to physical properties, all pyrethroid-treated boards exhibit lower thickness swelling (11.5-11.6%) compared to untreated boards (17.2%) and boric acid-treated boards (19.2%). Thickness swelling (TS) measures the dimensional stability of the boards. Lower TS indicate a more stable board. The results show that the dimensional stability of pyrethroids-treated rubberwood particleboard improved by about 33%. The water absorption (WA) value varied with treatments. deltamethrin treatment reduced the water absorption of the board and this value was comparable to boric-acid-treated board, i.e. 49.1% and 44.1%, repectively. The other two treated boards had relatively higher WA (between 72.3% and 76.7%) compared to the untreated board (71.9%), but this difference was not significant. In general, the IB, MOR and TS of pyrethroid-treated particleboards surpass the requirement level for Type 13 boards as indicated in the Japanese Industrial Standard (JIS 2003). Only boards treated with OSP passed the standard requirement of MOE. It is also interesting to note here that only the untreated particleboards passed the IB and MOR tests.

The IB and MOR recorded for the untreated rubberwood particleboard in this study were respectively 12.5% and 5.6% lower than those found for particleboard made from particles of rubberwood derived from mature trees. Zaidon *et al.* (2001) reported that particleboards produced from 25 years-old rubberwood had IB of 1.04 N mm^{-2} and MOR 17.8 N mm^{-2}. This was anticipated due to the higher content of hemicellulose rather than cellulose in the juvenile wood which contributes to fiber strength. On the contrary, when treated with a boron compound, a significant reduction in IB (9.3%) and MOR (17.8%) was recorded for boards of juvenile rubberwood compared to those fabricated from particles of mature rubberwood. The IB and MOR for the latter were 1.08 N mm^{-2} and 15.93N mm^{-2} (Zaidon *et al.*, 2001). The higher reduction in the properties was probably attributed to thermal degradation of juvenile cellulose of the rubberwood. The presence of boric acid in the board coupled with strong heat (160^0C) from the hot press lowers the pH in the MUF resin system and accelerates hydrolysis in the resin with more formaldehyde being released from the resin polymer, which results in

TABLE 3
Adjusted mean mechanical and physical values and percent change in properties for pyrethroids and boric-acid treated particleboards compared with untreated

Properties/ a.i. w/w)	Preservative treatments					
	Deltamethrin 0.01%	OSP 0.19%	WP 0.61%	Boric acid 0.5%	Untreated 0.0%	JIS Type 13 board 0.0%
			Rubberwood particleboard			
IB (N mm^{-2})	1.05^{A1}±0.23	0.96^{A}±0.18	0.85^{B}±0.30	0.98^{A}±0.26	0.91^{A}±0.23	0.2
Change (%)	15.4	5.2	-7.0	7.7		
MOR (N mm^{-2})	18.2^{A}±2.40	18.6^{AB}±2.96	19.3^{A}±1.30	13.1^{C}±3.10	16.8^{B}±2.68	13.02
Change (%)	8.3	11.1	14.9	-21.9		
MOE (N mm^{-2})	1820^{B}±204	1972^{A}±394	2189^{A}±258	1751^{C}±89	1719^{B}±453	2000
Change (%)	5.9	14.7	27.3	1.9		
TS (%)	11.5^{B}±1.80	11.6^{B}±3.0	11.6^{B}±1.60	19.2^{A}±1.05	17.2^{A}±4.94	12
Change (%)	-32.9	-32.5	-32.6	11.4		
WA	49.1^{B}±4.50	76.7^{A}±5.40	72.3^{A}±5.60	44.1^{B}±8.10	71.9 ^{A} ±14.0	
Change (%)	-31.8	6.7	0.6	-38.8		
			EFB particleboard			
IB (N mm^{-2})	0.60^{BC}±0.28	0.67^{BC}±0.19	0.56^{C}±0.15	1.02^{A}±0.27	0.80^{B}±0.27	0.2
Change (%)	-24.9	-16.1	-29.9	27.7		
MOR (N mm^{-2})	26.9^{A}±4.64	16.5^{C}±3.78	17.4^{BC}±5.50	19.6^{B}±3.12	22.0^{B}±3.62	13.0
Change (%)	22.1	-25.2	-20.9	-9.0		
MOE (N mm^{-2})	898^{C}±110	1003^{BC}±144	1191^{A}±250	1083^{BA}±225	1276^{A}±188	2000
Change (%)	-29.6	-21.4	-6.7	-15.1		
TS (%)	10.6^{A}±0.78	11.6^{A}±2.03	11.6^{A}±1.60	8.90^{B}±0.98	11.3^{A}±2.8	12
Change (%)	-6.9	6.7	2.7	-28.4		
WA	51.3^{B}±3.40	81.8^{A}±8.0	74.0^{A}±8.0 0.6	57.2^{B}±8.13	75.3^{A}±18.0	
Change (%)	-32.0	9.3	-1.3	-24.0		
			Rubberwood + EFB (70:30) particleboard			
IB (N mm^{-2})	0.97^{B}±0.28	1.0^{B}±0.15	0.96^{B}±0.13	1.21^{A}±0.12	0.80^{B}±0.20	0.2
Change (%)	21.3	25.0	20.0	51.3		
MOR (N mm^{-2})	19.9^{A}±2.49	20.4^{A}±3.34	18.3^{A}±2.94	18.2^{A}±2.04	19.4^{A}±1.76	13.0
Change (%)	4.7	7.5	-5.3	-4.2		
MOE (N mm^{-2})	1467^{BC}±87	1319^{C}±394	1674^{B}±206	1716^{A}±92	1392^{C}±56	2000
Change (%)	5.4	-5.2	20.3	23.3		
TS (%)	12.0^{B}±1.23	9.38^{C}±1.25	13.7^{B}±2.30	17.1^{A}±2.40	12.1^{B}±1.23	12
Change (%)	-0.7	-25.6	15.7	-41.2		
WA	48.4^{BC}±4.43	41.4^{C}±7.16	51.1^{AB}±8.17	41.8^{C}±2.11	56.4^{A}±5.82	
Change (%)	-13.6	-26.8	-8.9	-25.3		

[1]Means within a row followed by the same alphabets are not significantly different at p≤0.05, [2]Percent change over untreated and ± are standard deviations

degradation of IB, thus affecting the MOR. The loss of formaldehyde due to these factors was reported by Pizzi (2003).

Properties of Treated EFB Particleboards

The IB for these boards were substantially reduced by 16-30% when treated with pyrethroids. On the contrary the bonding quality of boric-acid treated boards increased by 28%. The mean IB value for untreated boards was 0.80 N mm^{-2}. The significant reduction of IB in pyrethroid-treated EFB boards may be attributed to insufficient curing of the resin as indicated by the lower IB values. The incorporation of pyrethroids into the EFB furnish during blending may have changed the curing rate of the adhesive. Pizzi (2003) reported that melamine urea formaldehyde (MUF) resin requires an

acidic condition (pH 4.5) to cure. Since EFB fiber is slightly alkaline in nature, the pH of glueline may change which subsequently slows down the polymerization reaction rate. Thus prolonged higher temperature is required to fully cure the resin. The gel time of admixture of MUF and pyrethroid preservatives has been reported to vary from 370 s to 520 s (Zaidon *et al.*, 2007) but the curing time of the adhesive when blended with EFB particles is not known and worth investigating. However, the IB values for all the treated and untreated boards conform with Type 13 board specified in JIS. With the exception of deltamethrin, other treatments reduced the MOR of the board. The MOR values for boards treated with WP and OSP were reduced by 21% and 25%, respectively, from the untreated with a value of 22.0 N mm^{-2}. Nevertheless, deltamethrin treatment increased the MOR of the board by 22% (26.9 N mm^{-2}). Pyrethroid treatments reduced the MOE of the boards even though the reduction was not significant for boards treated with WP. The mean MOE value for untreated board was 1276 N mm^{-2}. MOE was lowered by 29.6% (898 N mm^{-2}) for deltamethrin-treated board, by 21.4% (1003 N mm^{-2}) for OSP-treated board and by 6.7% (1191 N mm^{-2}) for WP-treated board. The MOE value for boric acid-treated board was reduced by 15.1% (1083 N mm^{-2}). Overall, the MOR of both treated and untreated EFB boards meet the minimum requirements of JIS standard for Type 13 board.

In terms of dimensional stability, the TS of deltamethrin-treated (10.6%), OSP-treated (11.6%) and WP-boards did not differ significantly from the untreated board (11.3%). and boric acid-treated boards had dimensional stability higher by 26.4% and 18.2%, respectively compared to untreated boards. TS of these boards achieved the standard requirement of JIS type 13 board. With respect to water absorption, only deltamethrin and boric acid treatments reduced the water absorption of the boards, i.e, by 32% and 24%, respectively from the untreated board absorption of 75.3%. For other treatments, however, the WA values did not differ significantly ($p \leq 0.05$).

Properties of Treated Rubberwood + EFB Blend Particleboards

Most of the properties for both untreated and treated boards showed values that ranged between those of rubberwood and EFB particleboards, and in many cases, the properties were more similar or leaned toward those of rubberwood particleboards. The IB of untreated blended board was similar to EFB board with a mean value of 0.80 N mm^{-2} whereas the IB values for pyrethroid-treated boards (0.96-1.0 N mm^{-2}) were comparable or slightly better than treated rubberwood boards (see Table 3). Similar to EFB boards, boric acid treatment significantly increased the IB (1.21 N mm^{-2}) of the blended boards. Apart from OSP and WP-treated boards, the MOR of these boards was superior to rubberwood boards, but inferior to EFB boards. Preservative treatments did not significantly affect the strength of these admixture boards (MOR values ranged from 18.2 to 20.4 N mm^{-2}). The MOR value for untreated boards was 19.4 N mm^{-2}. Deltamethrin and OSP treatments did not affect the stiffness of the boards (MOE 1319 and 1467 N mm^{-2}, respectively), but WP treatment increased the MOE by 20.3% to 1674 N mm^{-2}. A comparison of rubberwood and EFB boards showed that the mean MOE of admixture board was lower than that of rubberwood, but markedly higher than EFB.

Thickness swelling in untreated blended boards (12.06%) was comparable to EFB particleboards (11.3%) but they were more stable than rubberwood particleboards (17.2%). Only OSP-treated blended boards showed a significant increase ($p \leq 0.05$) in thickness swelling (by 25.6%). Boric-acid treated boards showed a reduced dimensional stability of the board by 41.2%. In general, all preservative treatments reduced the water absorption of these boards. The WA values for treated boards were in the range of 41.4 to 51.1% as compared to the untreated board which had a higher WA value of 56.4%.

Among the three types of particleboards, the mechanical properties of EFB particleboards were affected most by the pyrethroid treatments. This was indicated by significant reductions found in IB, MOR (except for deltamethrin-treated board) and MOE. The mechanical and physical properties of rubberwood and admixture particleboards that were treated with pyrethroid preservatives were comparable or better than those of untreated boards. When treated with boric acid, all the three types of particleboards showed a reduction in strength as reflected by lower MOR values. Such reduction may be attributed to the thermal degradation of cellulose during hot pressing under acidic conditions.

CONCLUSIONS

The findings from this study revealed that the pyrethroid treatments did not significantly affect bonding quality of rubberwood and rubberwood + EFB blend particleboards, but significantly reduced the IB of EFB particleboards. Strength of rubberwood boards (as reflected by MOR values) was improved by the treatments, while for boards from rubberwood + EFB blend, the strength was not affected. In the case of EFB boards, only deltamethrin treatment increased the strength but organic solvent-based and water-based permethrin treatments reduced the strength. Stiffness (MOE values) of pyrethroid-treated rubberwood and admixture boards was either increased or equal to the untreated boards. However, the stiffness of treated EFB boards decreased. Deltamethrin treatment increased the strength of admixture board, while organic solvent-based permethrin treatment increased the stiffness. The presence of pyrethroid preservatives markedly increased the stability of rubberwood and EFB particleboards. The thickness swelling of admixture board decreased only when it was treated with organic solvent-based permethrin. As a whole, regardless of raw material, pyrethroid-treated particleboards achieved the minimum requirement for IB, MOR and TS of Type 13 board of JIS 5908.

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Cross Species Amplification of Ikan Kelah, *Tor tambroides* by Using *Mystus nemurus* Microsatellite Markers

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ABSTRACT

Thirty eight microsatellite markers developed from a Random Hybridising Microsatellite (RAMs) enrichment protocol created from the DNA of the river catfish, ikan baung, *Mystus nemurus* were screened to cross amplify ikan kelah, *Tor tambroides*. Only five primers which amplified bands at the expected allele size regions were used for characterizing this species. The observed heterozygosity values were higher than the expected heterozygosity values for the bands amplified by primer pairs MnSC4-3B, MnLR2-1-52A and MnRmC3-1 across the three populations but the bands amplified by primer pairs MnSC4-1A and MnLR2-1-17B showed lower observed heterozygosity values than the expected heterozygosity values. The mean FIS value across the three populations was negative, indicating no deficit in heterozygosity. The mean value of FST was low indicating no gene was fixed within populations relative to the total population. The high value of Nm suggested high gene flow among the three populations. Both the (x^2) chi-square and the (G^2) likelihood ratio tests showed significant differences ($P<0.05$), indicating deviations from Hardy-Weinberg equilibrium in most loci except for one locus (MnSC4-1A) in the Negeri Sembilan population and two loci (MnLR2-1-52A and MnSC4-1A) in the Kelantan population. The genetic distance values generated ranged from 0.1053 to 0.1960. The UPGMA dendrograms constructed from the genetic distances based on the microsatellite markers showed that the Negeri Sembilan and Kelantan populations shared a similar cluster while the Pahang population was on its own.

Keywords: *Tor tambroides*, DNA microsatellite, cross species amplification

INTRODUCTION

The Malaysian mahseer, *Tor tambroides* locally known as kelah, is one of the most sought-after local freshwater fishes, both for food as well as being a highly priced sport fish. Kelah is classified under the sub-family Cyprinidae and sub-tribe Tores. This sub-tribe includes fishes belonging to the genera *Neolissocheilus / Acrossocheilus hexagonolepis, Probarbus* and *Tor* (Rainboth, 1996). Although generally kelah only refers to the large scaled barbs of the sub-tribe i.e. *Neolissocheilus / Acrossocheilus*, it is also being referred to those belonging to the genus *Tor*. Differences in colour patterns and geographical locations have led to various vernacular names for this fish of probably the same species. In Pahang and Kelantan, the kelah, *Tor tambroides*, is also referred to as kelah padi, kecau, kelah emas. These names have been sometimes confused with tengas (*Neolissocheilus* or *Accrossocheilus hexagonolepis*), which inhabits the same habitats (Eddy, 1997). There is also the kelah merah, which probably refers to *Tor duoronensis*, but the species is difficult to locate according to the local tribes in Kelantan. The distribution of this fish includes clear and clean rivers in West and East Malaysia and spawn in the upper parts of the river system.

In fisheries, the use of biochemical and molecular genetic markers has increased over the past years particularly in identification of species or hybrids, and in population characterization. Later, with the discovery of various types of DNA level genetic markers such as Restriction Length Polymorphism (RFLP),

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Randomly Amplified Polymorphic DNA (RAPD) and microsatellites, they have become the favourite choice in aquaculture research (Kesher *et al.,* 1998).

Microsatellite DNA, or short sequence repeat (SSR), is a PCR-based and co-dominant DNA marker (Tautz, 1989). This highly polymorphic SSR has great potentials as genetic tag for use in aquaculture. It has been proven to be particularly valuable for parentage studies, stock discrimination, population genetics and genome mapping because of its high levels of polymorphisms (O'Connell and Wright, 1997). Presently, there are very few studies and documentation on the use of molecular markers for the classification of kelah. Molecular markers as a tool for facilitating confirmation of taxonomy, examination of genetic variation as well as for molecular breeding strategies have gained importance and popularity among fish geneticists, conservationists and taxonomists. Thus, the aim of this study was to investigate the genetic relationships among three populations of *Tor tambroides* using newly developed microsatellite markers of *M. nemurus*.

MATERIALS AND METHODS

Kelah samples were collected from three locations in Malaysia. The samples were taken from the upper reaches of streams by rods and hooks. As many samples as possible were taken due to difficulties in catching and restrictions in accessing the sampling areas. The geographical regions, sampling sites and number of individuals taken together with the range of weight, total length and standard length are shown in Table 1.

A total of 38 microsatellite primer pairs designed for *M. nemurus* by Chan (2003) and Usmani (2002) were screened for possible cross-species amplification and for use in the population structure analysis of *T. tambroides*. Of these, five primers were selected based on polymorphic bands which amplified at the expected allele size regions (Table 2) for the T. *tambroides* samples.

Microsatellite-PCR Amplification

The PCR amplification mixture of 10 µl reaction volume consisted of 2mM $MgCl_2$, 10X buffer (Promega), 400µM of each dNTP, 0.5µM of each of the forward and reverse microsattelite primer, 30 ug of DNA samples, appropriate amount of ddH_20 and 3 units of *Taq* polymerase (Promega). The amplifications were carried out in a Peltier Thermal Cycler DNA Engine-DYAD™ (MJ Research) with an initial predenaturing step of 3 minutes at 96°C, denaturation step of 30 seconds at 95°C, annealing step of 30 seconds at varied temperatures, extension step of 40 seconds at 68°C followed by 39 repeated cycles of the previous steps, and a final extension of 5 minutes at 68°C. The final step was held at 4°C.

Optimization of the microsatellite was made on the adjustment of annealing temperatures which ranged from 45°C to 60°C. Optimization to omit stutter bands was made by increasing the annealing temperature. Bands that formed below 100bp were likely to be primer dimers and were

TABLE 1
Geographical region, location and sample size of kelah used in the study

Geographical Region	Location	Sample Size (n)	Range of Weight (g)	Range of Total Length (cm)	Range of Standard Length (cm)
Central Part of Peninsular Malaysia	Pahang (Sungai Sia)	26	35.8- 148.2	10.9- 18.9	8.8 - 13.8
Western Part of Peninsular Malaysia	Negeri Sembilan (Sungai Kampung Esok)	26	3.1- 5.7	5.5 - 8.8	4.2 - 7.0
Eastern Part of Peninsular Malaysia	Kelantan (Sungai Nenggiri)	11	25.0 - 1800	14.5-50.7	11.0-40.0
TOTAL		63			

TABLE 2
Microsatellite primer pairs used in the population structure study of kelah

Locus	Primer Sequence (5'-3')	Genebank Accession Number	Product Size	Annealing Temperature °C (based on primer list)	Optimized Annealing Temperature °C
*MnSC4-3B	F: GCCAAGGAGCTATGAACTGG R: GACGCAACTATGTCCACCAC	AF458324	208 bp	F: 59.84 R: 59.01	50
*MnLR2-1-52A	F: TCCCCTTTTTATTGCCATTC R:GGAACGAGGAGGGCTCTCTCT	AF425680	189 bp	F: 58.87 R: 63.53	60
£MnRmC3-1	F: AGTGCGAGGTGTGTGTGTG R: GGTGGACCAGTGCCTCTAGT	AF462254	251 bp	F: 59.30 R: 60.53	45
*MnSC4-1A	F: GCCAGCAACAAGGGGCCA R: CCTTGGATCGGAACTGGTC	AF458322	173 bp	F: 67.93 R: 60.46	55
*MnLR2-1-17B	F: GCAGTTTCCTTCTCTTCACT R: GGGGGGCGCGCAACTCTCTC	AF360982	132 bp	F: 55.82 R: 72.67	45

* Microsatellite markers developed by Chan (2003), £ microsatellite markers developed by Usmani (2002) designed for *Mystus nemurus*.

omitted from scoring. Bands that amplified within the range of the expected allele size were further optimized by making adjustments on the concentrations of $MgCl_2$, DNA, dNTP's and Taq polymerase.

Microsatellite-gel Electrophoresis

The PCR products were loaded onto metaphore gels containing 4.0% metaphore, 1X TBE buffer, 0.1µl/ml ethidium bromide, together with standard DNA ladders (20bp ladder). The gels were electrophoresed in 1X TBE buffer for 4 hours at 78 V/cm and photographed under UV light using an Alpha Imager 2200.

Data Analysis

Data collected were analyzed using the Popgene 1.31 (Yeh and Boyle 1997) computer software. The allelic frequencies were estimated from the genotypes assuming codominance. The measurement of genetic variability calculated for all the populations included mean number of allele per locus and the mean heterozygosity. Chi-square goodness of fit tests and the G log-likelihood ratio test were used to determine whether the observed genotypic numbers were consistent with the Hardy-Weinberg expectations for each population. The F-statistics was calculated according to Wright (1978). An unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal 1973) dendrogram based on Nei's (1978) genetic distance estimates was constructed using Pop Gene 1.31 and the multivariate analysis software (NT-SYS) of Rohlf (1989).

RESULTS AND DISCUSSION

Of the 38 *M. nemurus* microsatellite primer pairs screened for possible cross-species amplification and population studies of kelah, five were selected based on band amplifications at the expected allele size regions. These selected primer pairs produced clear and distinct bands that were polymorphic at the expected allele sizes.

The microsatellite primer pair MnSC4-3B with an expected allele size of 208 bp, produced the bands at 160 bp, 170 bp, 180 bp, 190 bp, 200 bp and 210 bp and 220 bp. The microsatellite primer pair MnLR2-1-52A with an expected allele size of 189 bp, produced the bands at 220 bp and 230 bp. The microsatellite primer pairs MnRmC3-1a and MnRmC3-1b, with the expected allele size of 250 bp, produced two consistent bands at 280 bp and 320 bp, which were homozygous across the three populations (Table 3). The microsatellite primer pair MnSC4-1A, with an expected allele size of 173 bp, produced bands ranging from 160 bp to 220 bp (*Fig. 1*) with a common band shared among all individuals from the three populations at 180 bp. The microsatellite primer pair MnLR2-1-17B, with an expected allele size of 132 bp, produced clear bands at the expected regions ranging from 150 bp to 180 bp. However, clear distinct bands were also shown at higher base

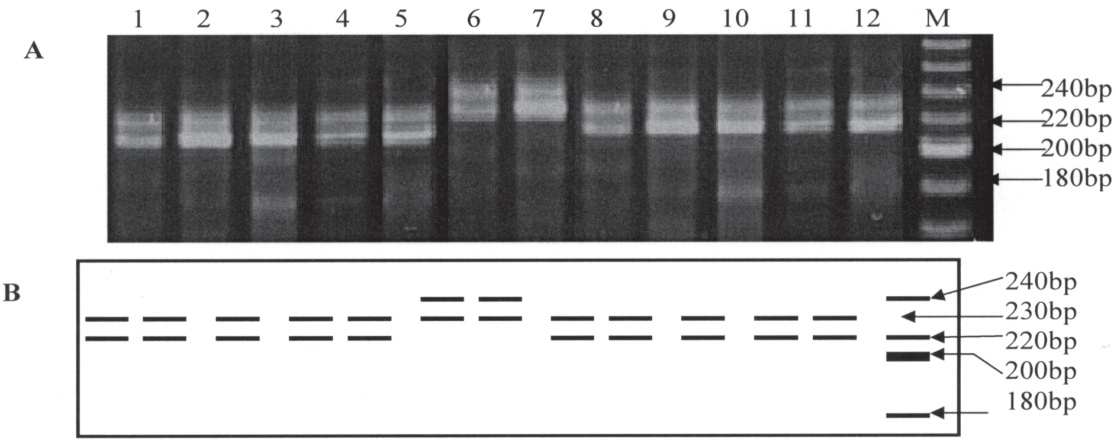

Fig. 1: Microsatellite banding profile of kelah samples from Pahang using primer pair MnLR2-1-52A. (B): A diagrammatic representation of the microsatellite bands in (A). Lane M: 20bp ladder. Lane 1-12: Individuals from Pahang

pairs but they were not considered for band scoring (*Fig. 2*).

Allele Frequency, Levels of Heterozygosity and F-statistics

The highest allele frequency among the populations studied was found in primer pair MnSC4-1A of the Negeri Sembilan population with a value of 0.788. The lowest allele frequency was found in primer pair MnLR2-17B of the Negeri Sembilan population with a value of 0.04. The highest allele frequency value for all the three populations was found in primer pair MnSC4-1A with a value of 0.718. The lowest allele frequency was found in the same primer pair with a value of 0.032 (Table 4). The common alleles for primer pair MnSC4-3B in the three kelah populations were found at 170 bp, 180 bp and 210 bp. The diagnostic alleles were found at 160 bp, 190 bp and 200 bp shown only in the Pahang population. The common alleles for primer pair MnLR2-1-52A in the three kelah populations were found at 220 bp and 230 bp. Primer pair MnRmC3-1 was homozygous for all the 63 samples and being considered as two separate loci. The most common allele for primer pair MnSC4-1A in the three kelah populations was found at 180 bp. The diagnostic alleles were found at 160 bp and 170 bp shown only in the Pahang population. The common alleles for primer pair MnLR2-17B in the three kelah populations were found at 160 bp and 170 bp. The diagnostic alleles were found at 150 bp and 180 bp shown only in the Pahang population while 155 bp was shown in the Negeri Sembilan population.

The number of observed and effective alleles per locus ranged from 2 to 7 (an average of 5) and 1.9 to 5.4 (an average of 3.1), respectively.

TABLE 3
Locus, allele size and allele frequency in the three kelah populations determined by using five microsatellite loci

Locus	Allele	Allele size (bp)	Allele frequency			
			Pahang	Negeri Sembilan	Kelantan	Overall
MnSC4-3B	A	160	0.115	0	0	0.048
	B	170	0.308	0.25	0.364	0.294
	C	180	0.039	0.231	0.136	0.135
	D	190	0.115	0	0	0.048
	E	200	0.192	0	0	0.079
	F	210	0.192	0.519	0.364	0.357
	G	220	0.039	0	0.136	0.039
MnLR2-1-52A	A	220	0.5	0.46	0.7	0.517
	B	230	0.391	0.46	0.3	0.405
	C	240	0.109	0.08	0	0.078
*MnRmC3-1a	A	280	1.0	1.0	1.0	1.0
*MnRmC3-1b	A	320	1.0	1.0	1.0	1.0
MnSC4-1A	A	160	0.08	0	0	0.032
	B	170	0.12	0	0	0.048
	C	180	0.64	0.788	0.727	0.718
	D	200	0.1	0.058	0	0.065
	E	210	0.06	0.077	0	0.057
	F	220	0	0.077	0.273	0.08
MnLR2-17B	A	150	0.083	0	0	0.033
	B	155	0	0.1	0	0.042
	C	160	0.25	0.24	0.273	0.25
	D	165	0.063	0.4	0	0.192
	E	170	0.208	0.04	0.091	0.117
	F	175	0	0.22	0.636	0.208
	G	180	0.396	0	0	0.158

* These two loci MnRmC3-1a and MnRmC3-1b were homozygous for all 63 samples

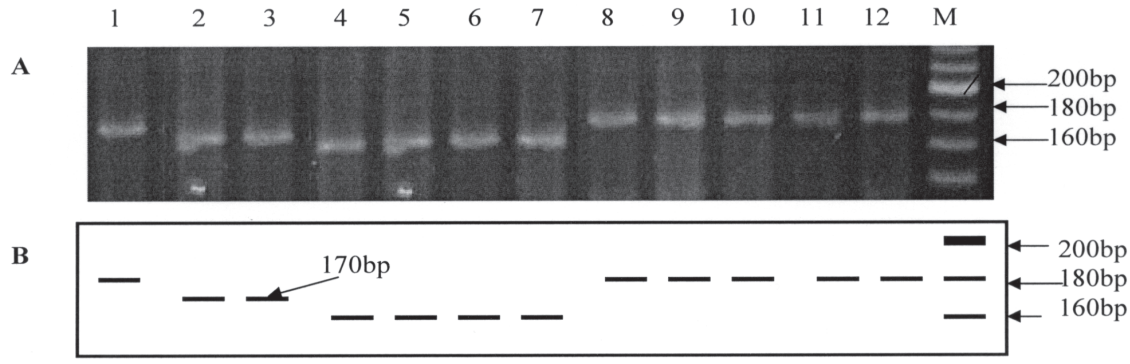

Fig. 2: Microsatellite banding profile of kelah samples from Pahang using primer pair MnLR2-1-17B. (B): A diagrammatic representation of the microsatellite bands in (A). Lane M: 20bp ladder. Lane 1-12: Individuals from Pahang

Whilst, the highest mean number of observed and effective alleles were found for the Pahang population with a value of 4.4 and 3.1, respectively. This was followed by the Negeri Sembilan and Kelantan populations with values of 3.4 and 2. 4, 2.6 and 2.1, respectively (Table 4).

Across the three populations, higher observed than expected heterozygosity values were observed for primer pairs MnSC4-3B, MnLR2-1-52A and MnRmC3-1, indicating excess of heterozygosity (Table 4). A check on the F_{IS} (Wright, 1978) values of the three primer pairs showed negative values of -0.328, -0.6 and -1.0, respectively indicating no deficit in overall heterozygosity and no inbreeding across the three populations (Table 4). However, the primer pairs MnSC4-1A and MnLR2-17B showed lower observed than the expected heterozygosity values with positive F_{IS} values of 0.082 and 0.688, respectively indicating deficit in heterozygosity across the three populations.

The mean value of F_{ST} or fixation index was low (0.075), indicating little genetic differentiation among the sample populations (Lowe *et al.*, 2004). The N_m estimate was 3.0627, a value indicating relatively high ongoing gene flow (Table 5).

Hardy-Weinberg Equilibrium

Both the (x^2) chi-square and the (G^2) likelihood ratio tests showed the presence of significant differences ($P<0.05$), indicating deviation from Hardy-Weinberg equilibrium for most loci except for one locus (MnSC4-1A) in the Negeri Sembilan population and two loci (MnLR2-1-52A and MnSC4-1A) in the Kelantan population.

Genetic Distance and Cluster Analysis

The values of genetic distance ranged from 0.1053 to 0.1960. The highest genetic distance was found between the Pahang and Kelantan populations with a value of 0.1960 followed by Pahang and Negeri Sembilan with a value of 0.1500. The lowest genetic distance was found between the Negeri Sembilan and Kelantan populations with a value of 0.1053 (Table 5). The UPGMA dendrogram constructed showed that the Negeri Sembilan and Kelantan populations were clustered into the same group while the Pahang population was away by itself (*Fig. 3*). The newly designed DNA microsatellites primer pairs for *Mystus nemurus* by Chan (2003) and Usmani (2002) were screened and only five of these primer pairs were selected on the basis of reproducibility and locus specificity. The ability of this newly designed microsatellite primer pairs to cross amplify on kelah suggested that certain sequences flanking the tandem repeats are highly conserved in Cypriniformes. Several studies have shown that flanking sequences of microsatellites may be conserved well enough throughout evolution to serve as primer-annealing sites for closely related species (Primmer *et al.*, 1996; Tong *et al.*, 2002). Reports of cross-species amplification of microsatellite primers designed from common carp, *Cyprinus carpio*, being used for the population studies of *Tor putitora* (Mohindra *et al.*, 2004), *Hypophthalmichthys molitrix* (silver carp) and *Aristichthys nobilis* (big head carp) (Tong *et al.*, 2002) are common and have produced reliable and satisfactory results. According to O'Connell and Wright (1997), microsatellite loci having numbers of allele

TABLE 4
Microsatellite variations, (χ^2) chi-square and (G^2) likelihood ratio tests among the three populations of kelah

Location	Locus	Observed number of alleles	Effective number of alleles	Observed heterozygosity	Expected heterozygosity	Degrees of freedom	Chi-square test		Likelihood ratio test	
							χ^2 value	Probability	G^2value	Probability
Pahang	MnSC4-3B	7.0	5.0	0.846	0.818	6	49.95	0.00*	65.39	0.00*
	MnLR2-1-52A	3.0	2.4	1.0	0.598	2	22.0	0.00*	30.87	0.00*
	MnRmC3-1	2.0	2.0	1.0	0.51	1	25.0	0.00*	35.03	0.00*
	MnSC4-1A	5.0	2.3	0.24	0.567	4	56.43	0.00*	35.86	0.00*
	MnLR2-17B	5.0	3.7	0.292	0.742	4	72.44	0.00*	47.24	0.00*
Mean		4.4	3.1	0.676	0.647					
Standard Deviation		1.9	1.3	0.38	0.128					
Negeri	MnSC4-3B	3.0	2.6	0.962	0.627	2	21.36	0.00*	27.94	0.00*
Sembilan	MnLR2-1-52A	3.0	2.3	0.92	0.582	2	17.73	0.00*	23.73	0.00*
	MnRmC3-1	2.0	2.0	1.0	0.510	1	25.0	0.00*	35.03	0.00*
	MnSC4-1A	4.0	1.6	0.423	0.370	3	1.67	0.9468	2.71	0.8432
	MnLR2-17B	5.0	3.6	0.32	0.737	4	48.04	0.00*	41.69	0.00*
Mean		3.4	2.4	0.725	0.565					
Standard Deviation		1.1	0.8	0.326	0.137					

Effective number of alleles [Kimura and Crow (1964)], Expected heterozygosity as computed using Levene (1949), *significant at 0.05 level.

Table 4 (continued)

Location	Locus	Observed number of alleles	Effective number of alleles	Observed heterozygosity	Expected heterozygosity	Degrees of freedom	Chi-square test		Likelihood ratio test	
							χ^2 value	Probability	G^2value	Probability
Kelantan	MnSC4-3B	4.0	3.3	1.0	0.731	3	31.0	0.00*	27.11	0.00*
	MnLR2-1-52A	2.0	1.7	0.6	0.442	1	1.48	0.2232	2.22	0.1359
	MnRmC3-1	2.0	2.0	1.0	0.524	1	10.0	0.00*	14.22	0.00*
	MnSC4-1A	2.0	1.7	0.546	0.416	1	1.25	0.2635	1.92	0.1649
	MnLR2-17B	3.0	2.0	0	0.537	2	33.9	0.00*	21.41	0.00*
Mean		2.6	2.1	0.629	0.53					
Standard Deviation		0.9	0.7	0.412	0.124					
Overall	MnSC4-3B	7.0	4.1	0.921	0.762	6	146.61	0.00*	119.7	0.00*
	MnLR2-1-52A	3.0	2.3	0.896	0.567	2	36.11	0.00*	43.04	0.00*
	MnRmC3-1	2.0	2.0	1.0	0.504	1	62.0	0.00*	86.32	0.00*
	MnSC4-1A	6.0	1.9	0.371	0.471	5	130.01	0.00*	48.05	0.00*
	MnLR2-17B	7.0	5.4	0.25	0.823	6	250.86	0.00*	146.49	0.00*
Mean		5.0	3.1	0.688	0.625					
Standard Deviation		2.3	1.6	0.349	0.158					

Effective number of alleles [Kimura and Crow (1964)], Expected heterozygosity as computed using Levene (1949), *significant at 0.05 level.

TABLE 5
The value of F-statistics for all the loci across the three populations based on data generated by utilizing five microsatellite loci

Locus	F_{IS}	F_{ST}	N_m
MnSC4-3B	-0.328	0.058	4.044
MnLR2-1-52A	-0.6	0.032	7.531
MnRmC3-1	-1.0	0.0	*****
MnSC4-1A	0.082	0.053	4.515
MnLR2-1-17B	0.688	0.181	1.133
Mean	-0.196	0.075	3.063

N_m = Gene flow estimated from $F_{ST} = 0.25(1 - F_{ST})/F_{ST}$

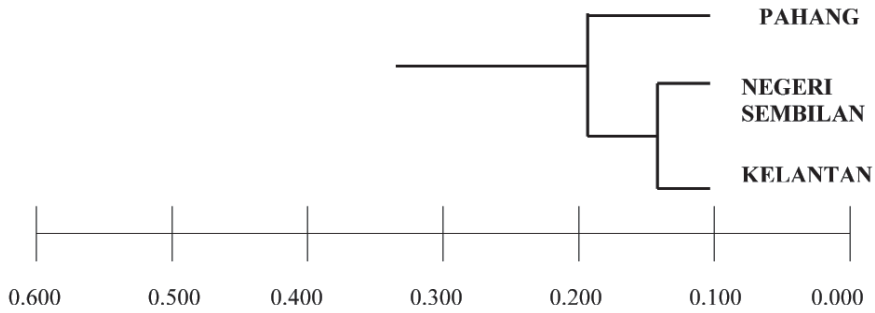

Fig. 3: Dendrogram constructed based on Nei's (1978) genetic distance values clustered by UPGMA for the three populations of kelah utilizing data from five microsatellite loci

ranging from 5 to 10 were sufficient to provide reliable results in a population study.

Generally, the observed heterozygosity was more than the expected heterozygosity across the three populations for primer pairs MnSC4-3B, MnLR2-1-52A and MnRmC3-1. A check on the F_{IS} (Wright 1978) values of the three primer pairs showed negative values of -0.328, -0.6 and -1.0, respectively indicating no deficit in heterozygosity across the populations. The observed heterozygosity was more than the expected heterozygosity across the three populations in the present study. This was expected as outcrossing occurred naturally in the wild (Chan, 2003). However, the primer pairs MnSC4-1A and MnLR2-1-17B showed lower observed heterozygosities than the expected heterozygosities across the three populations. A check on the F_{IS} (Wright 1978) values of the two primer pairs showed positive values of 0.082 and 0.688, respectively indicating deficit in heterozygosity. Similar situations were observed by Mohindra *et al.* (2004), Lal *et al.* (2004) and Mei *et al.* (2003) in their population studies of *Tor putitora*, *Cirrhinus mrigala* and *Zacco pachycephalus*, respectively. This could probably be due to methodological bias called stutter-related scoring errors. However, stutter bands were avoided during preliminary experiments. Secondly, such results could come from null alleles, leading to the scoring of heterozygous bands as homozygotes. Jarne and Lagoda (1996) reported that null alleles in fish might be quite common, and Pyatskowit *et al.* (2001) and Rodzen and May (2002) also reported the influence of null alleles in the inheritance of microsatellite loci in lake and white sturgeons. Founder effect during introduction might also be one of the reasons (Mei *et al.*, 2003) for the above observation.

The N_m measuring the movement of individuals per generation between populations obtained in this study was high (3.062), suggesting high genetic flow between the

TABLE 6
Genetic distance (below diagonal) and identity (above diagonal) based on data generated from five microsatellite loci

Populations	Pahang	Negeri Sembilan	Kelantan
Pahang	******	0.8607	0. 8220
Negeri Sembilan	0.1500	******	0.9001
Kelantan	0.1960	0.1053	******
		Nei's (1978)	

populations studied. As indicated by Lowe *et al.* (2004), Nm values greater than 1 suggest that populations are expected to retain genetic connectivity. This may have been due to human intervention as there is no connection between the rivers system where the fishes were obtained. However, only through an understanding of how organisms disperse their genes and the ecological requirements for propagate establishment, can one predict the likely effects of contemporary environmental change on genetic diversity.

Both the (x^2) chi-square and (G^2) likelihood ratio tests showed significant differences ($P<0.05$), indicating deviation from Hardy-Weinberg equilibrium in most loci except for one locus (MnSC4-1A) in the Negeri Sembilan population and two loci (MnLR2-1-52A and MnSC4-1A) in the Kelantan population. Many hypotheses could explain deviation from Hardy-Weinberg equilibrium. This phenomenon could probably be due to Wahlund effects (Silverstein *et al.*, 2004), founder effects (Mei *et al.*, 2003) and small sample sizes (Das *et al.*, 2005; Mohindra *et al.*, 2004). A sample size of 50 is large enough to disregard the effect of population size for deviation from Hardy-Weinberg equilibrium (O'Connell and Wright, 1997). Another reason could probably be the effect of the lower observed heterozygosity than the expected heterozygosity (Mei *et al.*, 2003). A deficit in heterozygosity (F_{IS} statistics) may cause deviation from Hardy-Weinberg equilibrium (Zhao *et al.*, 2005) but this is unlikely to be the reason as a negative F_{IS} value was observed across the three populations in this study suggesting no deficit in heterozygosity. In this study, the deviations from Hardy-Weinberg equilibrium were most likely caused by small sample sizes. Hence, in terms of population studies, these results should only be considered as preliminary since the small sample sizes may be insufficient to observe the whole range of alleles.

CONCLUSIONS

The newly developed primer pairs of *M. nemurus* were able to amplify the DNA of a fish from a different family and were useful for characterizing and differentiating the population structure of *T. tambroides*. The low range of genetic distances indicated that the *T. tambroides* from the three locations were genetically similar and were of the same species.

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Population Fluctuation of *Helopeltis antonii* Signoret on Cashew *Anacarcium occidentalle* L., in Java, Indonesia

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ABSTRACT

Population fluctuation of *Helopeltis antonii* was studied in a cashew smallholding in Wonogiri, Indonesia for two years beginning March 2004 to May 2006. Observation of *H. antonii* population was carried out systematically on 60 sample plants in 2 ha cashew smallholding every two weeks for 2 years in relation to the number of shoots and inflorescence, number of damaged shoots, inflorescence and fruits. Local rainfall, temperature and relative humidity, number of natural enemies, and fruit yield harvested were also considered. The *H. antonii* population fluctuated in a cyclical pattern with the peak population in July. The population began to increase at the end of the rainy season and was high during periods of low and intermittent rainfall. No insects were found during high rainfall. Number of shoots and inflorescences of cashew significantly influenced the number of *H. antonii* population. This trend of population abundance was not directly associated with rainfall, but rainfall influenced the physiology of shoot flushes and inflorescence production. Results of correlation and regression analysis showed that rainfall is not significantly correlated to *H. antonii* population and does not significantly contribute to the number of *H. antonii* population on cashew. However, the availability of food in the form of number of shoots and inflorescence positively correlated with the abundance of *H. antonii* population. The analysis between rainfall and number of shoots and inflorescence revealed that these parameters were negatively correlated. This indicated that rainfall did not directly influence the number of *H. antonii* population, but appears to influence the number of shoots and inflorescence.

Keywords: Population, fluctuation, *Helopeltis antonii*, cashew

INTRODUCTION

Helopeltis antonii Signoret is one of the most important insect pests of cashew in most cashew growing areas in Indonesia. It is also a major pest on cocoa, tea and neem (Stonedahl, 1991; Sundararaju and Babu, 1996; Onkarappa and Kumar, 1997). Both the nymph and adult feed on young and succulent parts of cashew such as the shoots, young leaves, inflorescence and fruits. Their feeding causes the drying up of new flushes resulting in a scorched appearance to the trees, shrivelling and abortion of immature nuts (Singh and Pillai, 1984).

An insect population always fluctuates according to the dynamic condition of its environment. Both physical (abiotic) and biotic factors are believed to be the factors responsible for the change in a population. Andrewartha and Birch (1954) stated four components of the environment that influenced animal or insect populations, namely weather conditions, food, other insects and organisms causing disease, and a place in which to live. Climatic factors such as rainfall and humidity have been known to greatly influence the population change of *Helopeltis* spp. (Roepke, 1916 in Geisberger, 1983; Swaine, 1959; Pillai *et al.*, 1979; Muhamad, 1990; Muhamad and Chung, 1993; Karmawati *et al.*, 1999). Other factors include natural enemies (Roepke, 1916 in Geisberger, 1983; Karmawati *et al.*, 1999; Peng *et al.*, 1999), temperature (Pillai *et al.*, 1979), and food supply (Swaine,

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1959; Pillai *et al.*, 1979). Knowledge of the seasonal abundance and trends in the population build up of pest has become important for effective control schedules. This study reports the seasonal population fluctuation of *H. antonii* and determines the influence of various environmental factors on its population in a cashew smallholding.

MATERIALS AND METHODS

Study Site

The study was carried out from March 2004 to May 2006 in a pesticide free cashew smallholding in Wonorejo, Ngadirojo District, Wonogiri, Central Java, Indonesia. The cashew trees were 12 - 15 years old and spaced 10 x 8 m apart. In general not much care was given to cashews. Manuring was done only on the intercrops such as groundnut (*Arachis hypogaea*), soybean (*Glycine max*), corn (*Zea mays*) and cassava (*Manihot utilisima*) which were planted during the wet season, while in the dry season only a few old cassava plants were intercropped. The observation plot was chosen in the centre of the cashew grove of 2 ha and consisted of trees of homogenous size and age. The plot was divided into 60 area units (15 x 4 m) and one sample plant was chosen per sample unit. Therefore, a total of 60 sample plants were selected for the study. Observations were done at 2 weekly intervals for two consecutive production cycles of two years. The number of *H. antonii,* specifically the eggs, nymphs and adults on each sample plant were recorded from the lower canopy up to about 2.5 m height. The number of healthy shoots and inflorescence, and number of shoots, inflorescence and fruits damaged by *H. antonii* were also recorded. Data on local rainfall, temperature and relative humidity were obtained from the local/nearest meteorological station. Other parameters observed were the number of possible natural enemies of *H. antonii,* other dominant insect pests and number of fruits produced by the sample plants. Observations were done between 0630 to 1100 hr. Data representation was done by plotting the data for parameters observed against time. Correlation among the parameters measured was statistically compared using Pearson Correlation Coefficients and Stepwise regression analysis was also performed between *H. antonii* population (dependent variable) and the environmental parameters measured using PC-SAS (Anonymous, 1999).

RESULTS AND DISCUSSION

The presence of *H. antonii* population on cashews coincided with the abundance of food supply in the form of shoots and inflorescence. *Fig.1* shows population abundance of *H. antonii* on cashews in relation to the numbers of shoots and inflorescence and other environmental factors between March 2004 and May 2006. *Helopeltis antonii* numbers began to increase soon after rainfall ceased and reached its peak during low and intermittent rainfalls. The number began to drop at the inception of a new rainy season and then completely disappeared at the height of the rainy season. This cycle of population rise and fall was repeated during the second year of the study.

Based on cashew plant phenology or crop season, the flushes of shoots and inflorescence correlated positively with the abundance of *H. antonii* except during the first post-flowering season (September 2004 - March 2005). Other factors were not significantly correlated except for the temperature which was negatively correlated during the first flushing-flowering season (Table 1). Pooled data for the two seasons revealed that *H. antonii* population correlated negatively with rainfall and temperature, but correlated positively with shoots and inflorescence. Stepwise regression analysis (Table 2) confirmed that the shoots and inflorescence were the contributing factors to the population fluctuation of *H. antonii* except for the post-flowering season. A higher temperature also contributed to the presence of *H. antonii* population in the first flushing-flowering season, while an increased relative humidity was a contributory factor during the second flushing-flowering season. However, their contribution was comparatively low. Pooled season data revealed that only shoots and inflorescence were the significant contributors to the population fluctuation and abundance of *H. antonii* in cashew.

The trend of population fluctuation of the mirid bug was not directly associated with rainfall, but the physiology of the cashew plant to produce flushes/shoots and then inflorescence was in response to increase in rainfall. Correlation and regression analyses showed that rainfall did not significantly contribute to *H. antonii* population abundance on cashews. However, the availability of food in the form of increased number of shoots and inflorescence positively correlated

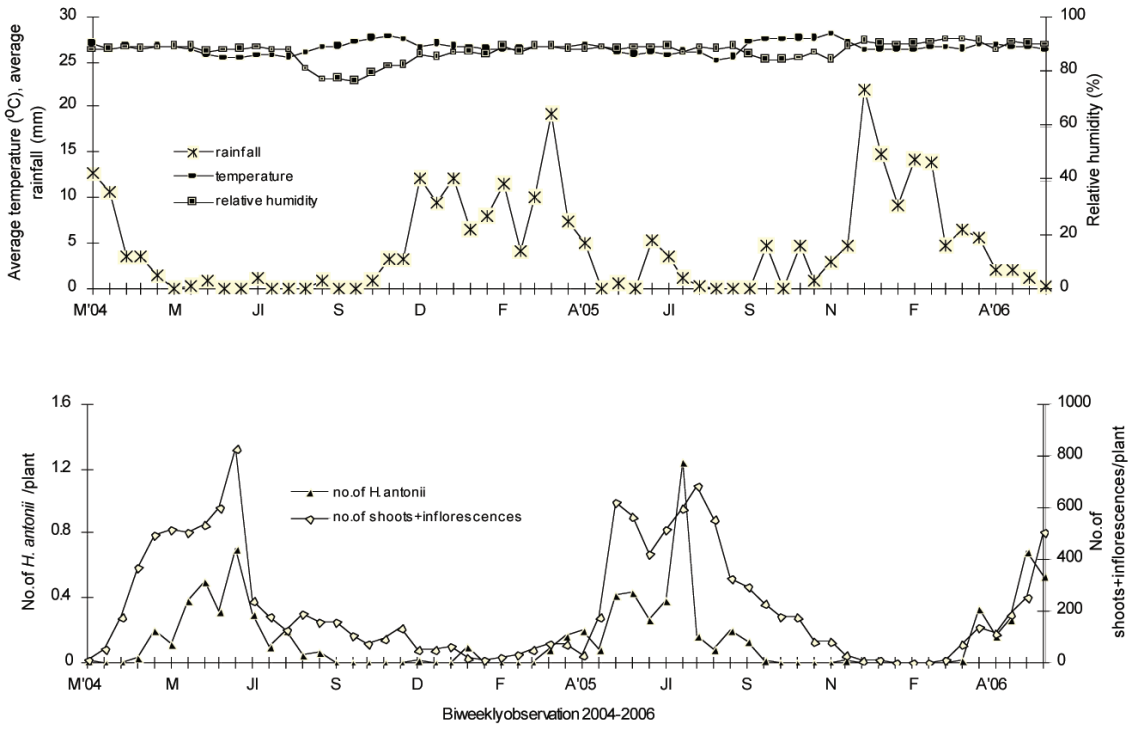

Fig. 1: Population abundance of Helopeltis antonii *on cashews in relation to the number of shoots and inflorescence, and rainfall, temperature and relative humidity between March 2004 and May 2006*

with the abundance of *H. antonii* population (Table 1). On the contrary, correlation analysis between rainfall and number of shoot and inflorescence revealed that these parameters produced negative correlation (r= -0.55*), indicating that rainfall influenced the growth of shoots and inflorescences but did not directly influence the number of *H. antonii* population. Regression analysis, however, suggested that the number of shoots and inflorescence of cashew plants (R^2 = 0.40) were the contributors to the increase in *H. antonii* population.

During the rainy season between October to March, with a relative humidity of 79-91.5%, temperature of 26.3-28.1°C, rainfall of 0.69-21.77mm/day and reduced number of shoots and inflorescence, the population of *H. antonii* became very low or absent. This trend of population fluctuation of *H. antonii* on cashew plants appeared to be cyclical with the population peaking in July. The increase of *H. antonii* population positively correlated with the availability of shoots and inflorescence as this was their food source. Mujiono (1987) also observed a cyclical population fluctuation of *H. antonii* on cocoa in East Java, Indonesia and it was related to the abundance of cocoa pods. The reduction or absence of *H. antonii* population was also reported by other researchers (Giesberger, 1983; Mujiono, 1987; Karmawati *et al.*, 1999). It is known that *H. antonii* is a polyphagous insect, with a wide range of host plants (Devasahayam and Nair, 1986; Stonedahl, 1991). However, in this study, there was no observation of insects feeding on weeds and other plants in the vicinity of the cashew smallholding except on cocoa. Cocoa is also known as one of the major host plants of *H. antonii* which are usually sparcely planted in farmer's homestead. During the dry season some new flushes could still be found together with a few cherelles and young pods. The presence of *H. antonii* on cocoa coincided with the abundance of the insect on cashew. When the insects were absent on cashew, they were also not found on other plants.

Predators did not play a significant role in influencing the number of *H. antonii*. Observations in the field showed that the population of the black ant, *Dolichoderus thoracicus*

TABLE 1
Coefficient of correlation (r) between *Helopeltis antonii* population and rainfall, temperature and relative humidity during two consecutive flushing-flowering stages of 2004-2005 and 2005-2006

Planting stage	Month	Rainfall	Temperature	Relative Humidity	Shoots+ Inflorescence	Predator
Flushing-flowering	March-Sept. 2004	(ns)	-0.61 *	(ns)	0.73 **	(ns)
Post flowering	Sept-March 2004-2005	(ns)	(ns)	(ns)	(ns)	(ns)
Flushing-flowering	March-Nov 2005	(ns)	(ns)	(ns)	0.50 *	(ns)
Post flowering	Nov-April 2005-2006	(ns)	(ns)	(ns)	0.78 **	(ns)
Pooled stages	March-May 2004-2006	-0.37 **	-0.41 *	(ns)	0.63 **	(ns)

ns not significant
* significant at P≤0.05
** significant at P≤0.01

TABLE 2
Stepwise regression for *Helopeltis antonii* population against rainfall, temperature and relative humidity during two consecutive flushing-flowering stages of 2004-2005 and 2005-2006

Planting stage	Month	Regression parameter			
		Intercept (a)	Gradient (b) of variable selected	R^2	Significance Pr>F
Flushing-flowering	March-Sept 2004	199.48	1. shoot+inflorescence: 0.0004	0.540	0.0018
			2. temperature: -7.550	0.132	0.0484
Post flowering	Sept-March 2004-2005	-	-	-	-
Flushing-flowering	March-Nov 2005	-0.145	1. shoot+inflorescence: 0.0007	0.250	0.0411
Post flowering	Nov-April 2005-2006	-118.99	1. shoot+inflorescence: 0.002	0.603	0.0030
			2. relative humidity : 1.299	0.187	0.0196
Pooled stages	March-May 2004-2006	-49.73	1. shoot+inflorescence: 0.0006	0.396	<.0001
			2. relative humidity : 0.577	0.023	0.1405

was always high, with an average of 314 ants per plant (46 - 596). The role of *D. thoracicus* to control *Helopeltis* spp. has been extensively studied and well understood (Giesberger, 1983; Way dan Khoo, 1991). The predatory ant, *Oecophylla smaragdina*, was also found in high numbers for each observation and no *H. antonii* was found on cashew plants occupied by this ant. In Northern Australia, *O. smaragdina* has been used to control *H. pernicialis* on cashews (Peng *et al.*, 1995; 1997a,b; 1999a,b). Other predators frequently found in quite high numbers were arachnids and to a lesser extent mantids and coccinellids. Apart from *H. antonii*, other pest species such as aphids (*Toxoptera* sp.), leaf miners (*Conopomorpha* sp.) and chrysomellid beetles were also found but in relatively low numbers.

Previous observations by Karmawati *et al.* (1999) showed that relative humidity and the presence of predators influenced *H. antonii* population with $R^2 = 0.35$. A study by Pillai *et al* (1979) in India suggested that the population build up of *H. antonii* was negatively correlated with minimum temperature, minimum relative humidity and rainfall but was positively correlated with sunshine. However, the most favourable

period for rapid multiplication and population build up was during abundant supply of succulent plant parts, which is similar to the results of this study. Swaine (1959) observed that *H. anacardii* was absent during rainy season but increased in number during the flushing period after rainy season. This also suggests that the number of *H. antonii* population was positively correlated with cashew damage, although the population did not influence cashew yield since it was very low with mean yields less than one *H. antonii* per tree (Table 3).

TABLE 3
Coefficient of correlation (r) between cashew yield and rainfall, temperature and relative humidity during two consecutive flushing-flowering stages of 2004-2005 and 2005-2006

Planting stage	Month	Rainfall	Temperature	Relative Humidity	*H.antonii* population	*H.antonii* damage
Flushing-flowering	March-Sept 2004	(ns)	-0.64 **	0.53 **	(ns)	(ns)
Post flowering	Sept-March 2004-2005	-0.70 **	0.73 **	-0.88 **	(ns)	(ns)
Flushing-flowering	March-Nov 2005	(ns)	(ns)	-0.57 *	(ns)	(ns)
Post flowering	Nov-April 2005-2006	(ns)	0.75 **	-0.66 *	(ns)	(ns)
Pooled stages	March-May 2004-2006	-0.39 **	(ns)	-0.41 **	(ns)	(ns)

ns = not significant
* = significant at P≤ 0.05
** = significant at P≤ 0.01

CONCLUSIONS

The population fluctuation of *H. antonii* on cashew plants was cyclical reaching a peak in July. The population increased just after the end of rains and reached a peak when rainfall was intermittently low. The population dropped when the rainfall was persistently low, and absent with increased rainfall until peak rainfall. Number of shoots and inflorescence of cashew plants showed a significant influence on the population of *H. antonii*.

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Wooden Furniture Purchase Attributes: A Malaysian Consumers' Perspective

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ABSTRACT

Consumers evaluate a product based on various attributes when making a purchase decision. In this paper, the types of attributes important to Malaysian consumers when purchasing wooden living room furniture were investigated. Data were gathered from a questionnaire survey of 115 mall-intercepted adult respondents on the perceived importance of five products- and two store-related attributes. It was found that (in rank order) quality, design, and price of the wooden furniture were important product-related purchase attributes for Malaysian consumers. These consumers are expected to limit their purchase to neighbourhood outlets as the location of retail outlets is perceived as an important store-related attribute. Demographic characteristics of the respondents have an influence on the perceived importance of the attributes.

Keywords: Consumer behaviour-evaluative criteria-furniture-wood product

INTRODUCTION

Consumers make various purchase decisions daily. Some of these decisions, at one end of the continuum, are made quickly and with little mental effort especially when purchasing low-priced, frequently bought goods. While on the other end, consumers would normally go through an extensive decision-making process as considerable time and effort are spent in searching information about the product, seeking alternatives, and then to finally decide which of the more expensive and less-frequently bought product to purchase (Mowen and Minor, 2001). Faced with a multitude of products, deciding on a purchase may sometimes not be a simple matter to the consumer. How would the consumer then choose from the many available alternatives?

Consumers would normally evaluate the alternatives based on a myriad of attributes like price, brand name, country of origin, quality and design. These attributes, with different importance in the consumer's mind (Wickliffe and Pysarchik, 2001) form the selection criteria in purchase decision-making (Gustav *et al.*, 1999; Williams, 2002; Zhiming *et al.*, 2002). It is through this alternative evaluation that consumers gain the information needed to make a final choice. Usually, consumers would compare less-frequently purchased goods like furniture, based on suitability, quality, price, style and functionality (Armstrong and Kotler, 2003).

Several empirical studies on attributes of wooden furniture that are important to consumers in making a purchase decision are available in the literature. Kozak *et al.* (2004), for example, reported that consumers in Western Canada would consider attributes like quality, design and price when deciding on the purchase of value-added wood products including furniture. While similar attributes are mentioned by consumers in British Columbia, price is the second most important after quality (Forsyth *et al.*, 1999). Pakarinen and Asikainen (2001) concluded that quality and design are important attributes for the Finnish wooden household furniture market. The consumers in Croatia and Slovakia would pay more attention to quality

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and price when buying furniture items (Motik *et al.*, 2003). While quality, durability, functionality and comfort are the main concerns to consumers in the European Union (Anon., 2000).

New Zealanders buying wooden outdoor furniture were most interested in the origin, source and management status of the wood material and length of the warranty period (Bigsby and Ozanne, 2002). Even though price is noted as least important, some of the respondents in their survey consider it as the key attribute in making a purchase decision. Based on a study of advertisements placed in newspapers and distributed by furniture stores, Karki (2000) concluded that consumers in southern Germany perceived design, wood species and price as important purchase attributes. Meanwhile, Jen-lan *et al.* (2000) reported a totally different set of attributes are of importance to consumers in Taipei City, Taiwan - namely comfort, durability and size.

The various studies mentioned above shows that some purchase attributes are common to consumers worldwide. While there are common (and different) attributes used in making a wooden furniture purchase decision, the importance placed on each attribute by consumers could vary in different markets. In this context, this study seeks to identify the choice criteria used by Malaysian consumers when purchasing wooden household furniture.

METHOD

The type of wooden furniture considered in this study is wooden living room furniture. Based on a preliminary survey, five product-related attributes were identified, which are believed to be of concern to Malaysian consumers when deciding on the purchase of furniture items. These attributes are quality, design, price, origin of the product and brand name. Two store-related attributes, namely location of the retail outlet and availability of credit terms, were also included as the respondents frequently mentioned these attributes during the survey.

A questionnaire was then designed, which sought to obtain data on the perceived importance of the attributes. The respondents were asked to indicate the extent to which each of the attributes were important in their purchase of wooden living room furniture based on a 5-point Likert scale ranging from 1 = not at all important to 5 = very important. Demographic information was also collected, whereby the respondents were asked to indicate their gender, age, ethnic group and monthly gross household income at the time of completing the questionnaire.

The self-administered questionnaire was distributed to systematically sampled adults in a shopping mall. These adults were selected randomly based on a pre-determined criterion - every fifth adult who passed the interviewer was to be solicited. However, only those who indicated their willingness to participate in the survey were given the questionnaire. In addition, only those who are 20 years of age and above were included in the survey with the assumption that they are or would be buyers of furniture items. One hundred and fifteen completed and usable questionnaires were obtained at the end of the survey period. A summary of the respondents' characteristics are presented in Table 1.

RESULTS AND DISCUSSION

Relative Importance of the Attributes

The following analysis concerns the evaluation of relative importance of the seven attributes based on their mean scores. As the scale used in this study ranges from 1 to 5, a score above 3 (the midpoint) indicates that the attribute is important, while a score below it indicates that the attribute is not important. Table 2 shows the mean and standard deviation of the scores for the attributes for all respondents.

The respondents placed an importance on three product-related attributes when deciding on a purchase of wooden living room furniture. These attributes (in rank order) are quality, design, and price. The mean scores of the three attributes are above 4, which mean that they are highly important to all respondents. There is also less variation in opinions on the importance of these attributes, with the largest standard deviation being 0.72, when compared with other attributes.

The importance of quality in a purchase decision of a product was not only emphasised by the respondents in this study but also in others. Hult *et al.* (2002) concluded that the Malaysian consumers indicated that quality is of utmost importance when deciding on purchases of groceries, clothing and automobiles. It has also been reported that Malaysian consumers have a strong preference for quality when choosing banking services (Dusuki and Abdullah, 2007). Respondents in a study conducted by

TABLE 1
Demographic characteristics of the respondents

Variables		Frequency (%) (n = 115)
Gender:		
	Male	47.0
	Female	53.0
Age:	20 – 29 years	30.4
	30 – 39 years	33.0
	40 – 49 years	27.6
	50 years and above	9.6
Ethnic group:		
	Malay	43.5
	Chinese	39.1
	Indian	17.4
Monthly gross household income:		
	Less than RM1,000	7.0
	RM1,000 – RM2,999	39.1
	RM3,000 – RM4,999	38.3
	RM5,000 and above	15.7

TABLE 2
Distribution of respondents' response and relative importance of wooden living room and store attributes

	Level of importance						
	1	2	3	4	5	Mean	SD
Product-related attributes:							
Quality	0	0	3	36	76	4.63	.53
Design	0	1	12	44	58	4.38	.71
Price	1	1	10	59	44	4.25	.72
Origin of product	8	33	46	24	4	2.85	.95
Brand name	14	62	23	13	3	2.38	.93
Store-related attributes:							
Location of outlet	4	20	45	34	14	3.29	1.01
Availability of credit terms	24	28	28	26	9	2.72	1.25

Note: The scale used was 1 = "not at all important" to 5 = "very important"

Ong and Phillips (2007) on older consumers in Malaysia placed a very high importance on quality (and durability) in their purchase decision making. While the notion of quality differs in these situations as they involve different products or services, these findings provide evidence that quality is an important purchase criterion for consumers in Malaysia.

Design or appearance of the product has a propounding influence on consumer evaluation and choice (Creusen and Schoormans, 2005). Consumers in north-eastern United States of America, for example, are reported to be willing to purchase wooden furniture made from low-grade hardwoods provided they are well-designed (Wang *et al.*, 2004). In general, the respondents of the study placed design as a second most important attribute in deciding on a purchase. However, it is not possible to determine the design of wooden living room furniture preferred from the data collected in this study.

It is rather surprising that price was relatively less important than quality and design. Consumers in developing countries are expected to place a relatively higher importance on price than other product attributes (Zhang *et al.*, 2002). Furthermore, the furniture retailers seem to perceive that price is the most important factor in consumers' purchase decision as the many advertisements placed by these retailers have almost always emphasised on price, with minimal details on other attributes of the wooden furniture items advertised. The most probable explanation is that the respondents may have been reluctant to admit that they are price-sensitive thus understating the importance of price in their responses.

Of the two store attributes included in this study, the respondents perceived the location of stores as the only important factor. Most furniture outlets in Malaysia are located in the business section of a residential area or in the proximity of several residential areas, especially the larger outlets. In areas where households are more interspersed, furniture outlets are usually situated in town centres which are readily accessible by most consumers. As location of the store outlet is important to the respondents, it is expected that they would limit the purchase to outlets within their neighbourhood as studies have shown that the likelihood for a consumer to shop in a location declines as distance increases (e.g. Davies, 1995; Darley and Lim, 1999; Baltas and Papastathopoulou, 2003).

Impacts of demographic factors on the importance of the attributes

Household furniture consumption has been found to be determined by demographic and economic characteristics of the population (International Trade Centre UNCTAD/GATT, 1982). As the above discussion was based on the entire sample, further analyses using ANOVA tests or independent *t*-tests were done to determine the impact of selected demographic variables of the respondents on the importance of the attributes.

The mean importance scores by demographic variables are shown in Table 3, and the results of the statistical tests are summarised in Table 4.

TABLE 3
Mean importance score for wooden living room furniture and store attributes

	Product-related attributes					Store-related attributes	
	Quality	Design	Price	Origin of product	Brand name	Location of outlet	Availability of credit terms
Gender:							
Male	4.57	4.17	4.28	2.81	2.41	3.26	2.78
Female	4.69	4.57	4.23	2.89	2.36	3.33	2.67
Age:							
20 – 29 years	4.71	4.46	4.26	2.86	2.34	3.20	2.89
30 – 39 years	4.66	4.21	4.21	3.05	2.34	3.26	3.08
40 – 49 years	4.55	4.42	4.45	2.61	2.13	3.55	2.42
50 years and above	4.55	4.64	3.82	2.85	3.36	3.00	1.82
Ethnic group:							
Malay	4.68	4.26	4.20	3.00	2.44	3.30	3.06
Chinese	4.58	4.49	4.18	2.56	2.62	3.29	2.13
Indian	4.65	4.45	4.55	3.15	1.70	3.30	3.20
Monthly gross household income:							
Less than RM1,000	4.75	3.88	4.88	3.50	2.13	3.88	3.63
RM1,000 – RM2,999	4.69	4.38	4.27	3.00	2.29	3.29	2.98
RM3,000 – RM4,999	4.57	4.36	4.27	2.77	2.27	3.16	2.52
RM5,000 and above	4.61	4.67	3.89	2.39	3.00	3.39	2.17

Note: The scale used was 1 = "not at all important" to 5 = "very important"

TABLE 4
Impacts of demographic variables on the importance of attributes

	Product-related attributes					Store-related attributes	
	Quality	Design	Price	Origin of product	Brand name	Location of outlet	Availability of credit terms
Gender	ns	*	ns	ns	ns	ns	ns
Age	ns	ns	ns	ns	*	ns	*
Ethnic group	ns	ns	ns	*	*	ns	*
Monthly gross household income	ns	ns	ns	*	*	ns	*

Note: *Significant at $p< 0.05$ level, ns = not significant at $p< 0.05$ level

Gender: The only significant impact of gender is on design. In general, women placed a significantly higher importance on design of the wooden furniture item than men. Similarly, a study on older consumers (55 years and older) in Malaysia showed that women placed higher importance on design than men (Ong and Phillips, 2007). Women tend to place emphasis on design (in this case for appearance/style) when evaluating and choosing a variety of products including living room furniture (William and Slama, 1995) and design-related elements like style, appearance and colour for apparels (Williams, 2002; Zhang *et al.*, 2002).

Age: Age of the respondents has significant impacts on the perceived importance of brand name and availability of credit terms. The respondents aged 50 years and above perceived brand name an important criteria in their purchase decision but not for the others. Moschis (2003) suggested that matured consumers (aged 55 years and above) tend to prefer hassle-free products that can be obtained by purchasing familiar or reputable brands or by patronising a well-established or well-known retail outlet. The younger aged respondents, most probably with lower income than the other groups, placed a higher importance on the availability of credit terms. As purchase of household furniture would normally require a substantial amount of expenditure, the ability to purchase on credit would be attractive to this group of consumers.

Ethnicity: Ethnicity has significant impacts on the perceived importance of origin of the product and availability of credit terms. In general, the Malays and Indians put some degree of importance on these product-related attributes but not the Chinese. While the impact of ethnicity on origin of product is hard to ascertain, the importance of credit terms can be attributed to the general economic well-being of the three ethnic groups in Malaysia. With an average monthly household income higher than other ethnic groups, the Chinese would have not perceived availability of credit terms very important compared to the Malays and Indians in deciding on the purchase of household furniture. In 2001, for example, the Chinese households were earning an average of RM3456 per month, while the Indian and Malay households were earning RM2704 and RM1984 respectively (Peng, 2002).

Household income: Household income has significant impacts on the importance of origin of product, brand name and availability of credit terms. Generally, respondents in the lower income group (monthly gross household income of RM2999 and less) placed a significantly higher emphasis on origin of the product and availability of credit terms. As discussed above, the ability to pay on credit for high expenditure consumer durables like furniture would be attractive for low income earners. It is not possible to explain, with the existing data, the importance of the product's origin to this group of respondents. Those in the higher income (monthly gross income of RM5000 and more) are concerned about brand name in making wooden household furniture purchase decision. Consumers with higher income have been reported to place a higher importance on brand name than lower income earners (Dickson *et al.*, 2004).

CONCLUSIONS

In this study, the importance of product- and store-related attributes in wooden household furniture purchase decision was investigated. The survey revealed that quality, design and price of the wooden household furniture matters to all consumers. Location of the retail outlet is the only store-related attribute perceived important by the consumers. In addition, there exist highly significant differences in perceived importance of the attributes by demographic variables which are useful when promoting wooden household furniture to specific segments of the population.

Considerations of how representative samples are must be kept in mind when evaluating the broader implications of the findings of this study. This study holds true only for the patrons of the mall where the study was carried out. The number of respondents was limited and may not be representative of the Malaysian population. Even so, this study should still be useful for manufacturers and retailers of wooden household furniture. There are also opportunities for further research in understanding consumer preferences for wooden household furniture based on the findings of this study. For example, while quality is important to the consumers, its meaning and measurement from the consumers' perspective is relatively unknown. The impact of a product's country of origin on consumer purchase decision also warrants further research. After a thorough understanding of the needs and desires of the consumers, marketing of wooden household furniture to a very diverse market will be successful.

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Comparison of Heavy Metal Concentrations (Cd, Cu, Fe, Ni and Zn) in the Shells and Different Soft Tissues of *Anadara granosa* Collected from Jeram, Kuala Juru and Kuala Kurau, Peninsular Malaysia

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ABSTRACT

The concentrations of Cd, Cu, Fe, Ni and Zn were analysed in different parts (shells, mantle plus gills and foot plus visceral mass) of the red blood cockle, *Anadara granosa* collected from an anthropogenic-receiving site at Kuala Juru and from relatively unpolluted sites at Jeram and Kuala Kurau. The metal concentrations (μg/g dry weight) in the total soft tissues of *A. granosa* ranged from 1.30-9.44 (mean: 4.69) for Cd, 91.9-203.5 (mean: 130.2) for Zn, 0.80-16.15 (mean: 7.67) for Ni, 455.91-1125.5 (mean: 715.3) for Fe and 5.41-7.39 (mean: 6.14) for Cu. The present study revealed that the *A. granosa* was a potential biomonitor for Cu and Zn as observed in the comparison with those metals in the sediment. Although the elevated concentrations of Cu and Zn were found in the soft tissues of Kuala Juru's cockles, these metal concentrations were still below the maximum permissible limits established by the Malaysian Food Regulations 1985 and the WHO standard guidelines but the Cd concentrations of cockles from Jeram were higher than the maximum permissible limits established by both guidelines. As suggested by many reported studies found in the literature, regular biomonitoring of heavy metal concentrations at these three sites is needed since the edible *A. granosa* is a popular commercial bivalve in Malaysia.

Keywords: *Anadara granosa*, biomonitoring, blood cockle, bivalve, different parts, heavy metals, soft tissues, shells

INTRODUCTION

Red blood cockle, *Anadara granosa* [Order: Arcoida; Family: Arcidae] is a filter-feeding bivalve that lives in muddy intertidal areas feeding on phytoplanktons and zooplanktons. Their biology and ecology (Broom, 1985) are very interesting from the biomonitoring point of view (Phillips and Rainbow, 1993). Like other bivalves, cockles fulfill most of the requirements for a good biomonitor of heavy metal pollution by having a wide geographical distribution and are easily collected, as well as having a sessile lifestyle (Lowe and Kendall, 1990). The cockles have been shown to be able to accumulate heavy metals to significant levels in their soft tissues (Mat *et al.,* 1994). Besides, the cockles are also an important protein source in the Southeast Asian region including Malaysia.

In the literature, the use of the soft tissues of cockles for biomonitoring studies of heavy metal pollution had been reported in *Cerastoderma edule* from the Moroccan Atlantic coastline (Cheggour *et al.,* 2001) and *A. granosa* from Malaysia (Ibrahim, 1994; Mat, 1994; Mat and Maah, 1994; Alkarkhi *et al.,* 2008). Phillips and Muttarasin (1985) reported concentrations of Cd, Cr, Cu, Fe, Ni, Hg and Zn in the commercial cockle *A. granosa* from Thailand and their findings indicated the potential use of the cockles to estimate the toxicological risks of heavy metals by determining the heavy metal concentrations in the soft tissues of cockles.

Besides, the shell had also been proposed as a biomonitoring material by a few researchers. Yap *et al.* (2003a) suggested that the shell of the bivalve, *Perna viridis* may act as a safe storage

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matrix for heavy metals, which is resistant to soft tissue detoxification mechanisms (Walsh *et al.*, 1995). The metals found in the shell could be explained on the basis that some trace metals are incorporated into the shells of the bivalves through substitution of the calcium ions in the crystalline phase of the shell or are associated with the organic matrix of the shell (Foster and Chacko, 1995; Yap *et al.*, 2003a).

The semi-culture of *A. granosa* is of considerable economic importance in Malaysia. According to Noordin (1988), there were about 4-5000 ha of mudflats along the west coast being utilized for this purpose. Contamination of the highly productive mudflats with heavy metals resulted in the accumulation of these metals in the filter-feeding cockles which often serve as important environmental sinks of heavy metals (Pringle *et al.* 1968).

Based on the aforementioned, *A. granosa* which are easily available in the coastal areas of Peninsular Malaysia (Noordin, 1988); and its ability to accumulate heavy metals (Pringle *et al.* 1968), it fulfils the criteria as a potential biomonitor.

Since the three sampling sites at Kuala Juru, Kuala Kurau and Jeram are located in high anthropogenic activity areas of the west coast of Peninsular Malaysia, the present study is therefore crucial in order to monitor the heavy metal status in the west coast besides the economical importance of *A. granosa*. Based on the sediment samples collected during 1999-2001 (Yap *et al.*, 2002a, 2003b, 2003c, 2005), it was revealed that the heavy metal pollution in the intertidal area of the west coast of Peninsular Malaysia are localized and near to anthropogenic sources (Yap *et al.*, 2008). This study was therefore important.

The objectives of this study were: (i) to determine the concentrations of Cd, Cu, Ni, Fe and Zn in the edible soft tissues of *A. granosa* (shells, mantle plus gills and visceral mass plus foot in addition to total soft tissues) collected from Kuala Juru, Kuala Kurau and Jeram. (ii) to ascertain the potential of *A. granosa* as a biomonitor by comparing metals found in the tissues with those in the sediment.

MATERIALS AND METHODS

The cockles and sediments were collected on 19-20th April 2005 from Kuala Juru, Kuala Kurau and Jeram (*Fig. 1*). Total soft tissues of cockles were dissected from the shells. The soft tissues were separated into two major parts (namely mantle plus gills and foot plus visceral mass).

The shells and the different parts of the soft tissues of cockles were digested in concentrated nitric acid (69%) while the dried and 63 μm sieved sediments were digested in a combination of nitric acid and perchloric acid in the ratio of 4 : 1. They were placed in a hot-block digestion first at low temperature for one hour and then they were fully digested at high temperature (140°C) for at least 3 hours. The digested samples were then diluted to 40 ml with double distilled water. After filtration, the prepared samples were determined for Cd, Cu, Fe, Ni and Zn by using an air-acetylene flame atomic absorption spectrophotometer (AAS) Perkin-Elmer Model AAnalyst 800. The data were presented in μg/g dry weight basis. The analytical procedures for cockles and sediments were checked with the Certified Reference Materials (CRM) for soils and dogfish and the recoveries of all metal were satisfactory (Table 1).

To avoid possible contamination, all the glassware and equipment used were acid-washed and the accuracy of the analysis was checked with the blanks and quality control samples made of standard solutions. The percentage recoveries for the heavy metal analyses were between 90-110%.

RESULTS AND DISCUSSION

The allometric variables which included the water contents, condition indices, shell lengths, shell heights, shell widths, total soft tissue dry weights and total shell dry weights, are presented in Table 2.

Fig. 2 and 3 depict the concentrations of heavy metal in the total soft tissues, mantle plus gills and foot plus visceral mass of the *A. granosa*. Among the three sites, the highest levels of Cd and Ni were found in the shells, total soft tissues and all the different parts of the cockles' soft tissues from Jeram. The highest levels of Zn were found in the shells, total soft tissues and parts of the cockles' soft tissues from Kuala Juru. The highest levels of Cu and Fe were also found in the total soft tissues and all parts of the cockles' soft tissues from Kuala Juru but not in the shells.

From Table 3, the metal concentrations (μg/g dry weight) in the total soft tissues of *A. granosa* ranged from 1.30-9.44 (mean: 4.69) for

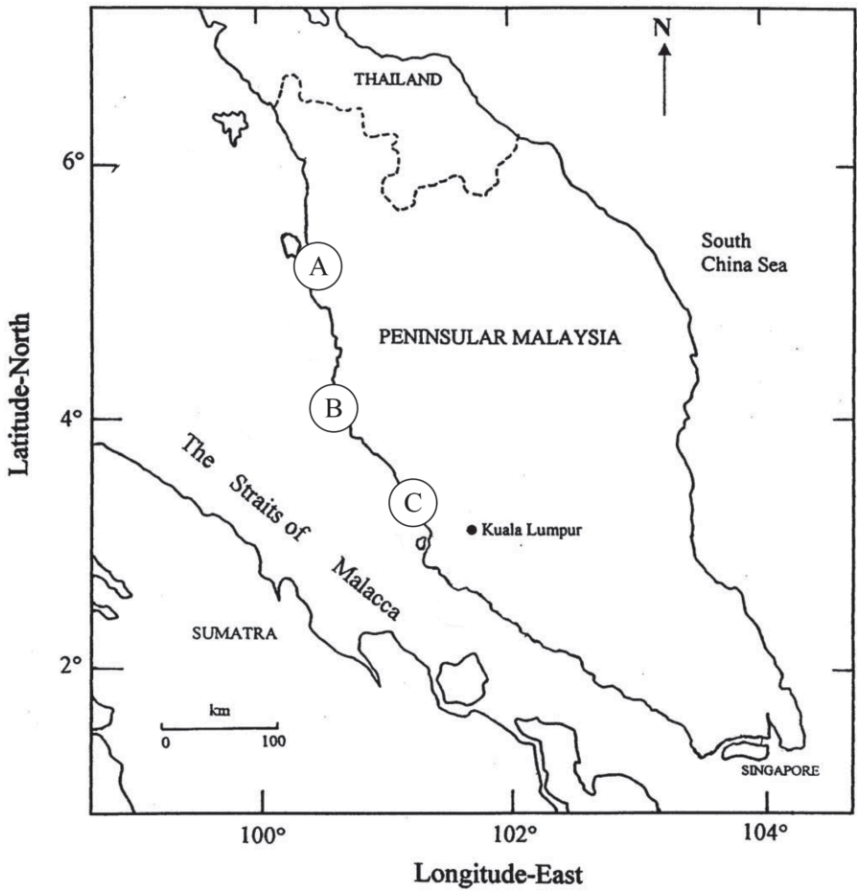

Fig. 1: Sampling sites for the cockle, Anadara granosa *at A) Kuala Juru, B) Kuala Kurau and C) Jeram*

Cd, 91.9-203.5 (mean: 130.2) for Zn, 0.80-16.15 (mean: 7.67) for Ni, 455.91-1125.5 (mean: 715.3) for Fe and 5.41-7.39 (mean: 6.14) for Cu. Based on the various soft tissues and total soft tissues, the metal abundance were Fe> Zn> Ni> Cu> Cd while the metal abundance based on the shells were Fe> Ni> Zn> Cd> Cu. The differences in metal abundance between the soft and hard tissues indicated that the metal accumulation and the metal binding capabilities of the two tissue types vary. Generally, the shells had higher concentrations of Cd and Ni than the soft tissues of the cockles while the soft tissues [including the two different soft parts] contained higher concentrations of Zn, Cu and Fe than in the shells. These results could be related to the differences in the accumulation and depuration of the hard tissues and soft tissues of the cockles. In the soft tissues, the metals were bound to metallothionein, which played an important role in metal detoxification (Roesijadi, 1980) while the metals in the shells were fixed in the crystalline lattices of the carbornate structures of the shells (Watson *et al.,* 1995). However, the questions that arise are 'Why did the soft tissues of cockles have higher levels of Zn, Cu and Fe than the shells?' and 'Why did the cockle shells have higher levels of Cd and Ni than the soft tissues?' Answers to these require future studies. Some authors (Carell *et al.,* 1987; Sturesson, 1976, 1978) suggested that the materials in mollusc's shell could be a permanent record of the environmental changes in heavy metal concentrations. Since the composition of the shell was strongly related to chemical mineralogy which included metals accumulated from the environment, this could be one of the reasons why shell metal concentrations related to the metal concentrations in its environment (Yap *et al.,* 2003a) besides the roles played by the calcium

TABLE 1
Analytical results for the reference material and its certified values for each metal (μg/g dry weight)

Metal	Sample	Certified reference material (CRM)	Measured value	Percentage % of recovery
Cd	DOLT-3 Dogfish-liver (National Research Council Canada)	19.4	20.0	103
	MESS-3 Marine Sediment (Vienna)	NA	NA	NA
Cu	DOLT-3 Dogfish-liver (National Research Council Canada)	31.2	32.0	103
	MESS-3 Marine Sediment (Vienna)	33.9	29.3	86.5
Fe	DOLT-3 Dogfish-liver (National Research Council Canada)	1484	1070	72.1
	MESS-3 Marine Sediment (Vienna)	4340	4013	92.5
Ni	DOLT-3 Dogfish-liver (National Research Council Canada)	NA	NA	NA
	MESS-3 Marine Sediment (Vienna)	46.9	37.6	80.1
Zn	DOLT-3 Dogfish-liver (National Research Council Canada)	86.6	100	116
	MESS-3 Marine Sediment (Vienna)	159	145	91.2

Footnote: NA = Not available

ions in the crystalline phase of the shell as discussed in the introduction.

In supporting the use of cockles as biomonitors of heavy metal pollution in the intertidal mudflats, a comparison of metal concentrations between the sediments and cockles was conducted as shown in Table 4. Based on Table 4, the highest levels of Cu and Zn were found in the surface sediments of Kuala Juru, which was in agreement with the high levels of Cu and Zn found in the soft tissues of cockles from the site. The highest level of Cd was also found in the sediments and cockles from Jeram. Yap *et al.* (2002b) also found a correlation for Cd, Cu and Pb between soft tissues of *Perna viridis* and sediments. However, the highest levels of Ni and Fe found in the cockles from Jeram and Kuala Juru, respectively, were not reflected in the high levels of these two metals found in the surface sediments from both sites. The results of this study indicate that *A. granosa* is a good biomonitor of Cu and Zn but not for Ni and Fe.

Further studies are required to confirm the utility of *A. granosa* as a biomonitor of Ni and Fe. Furthermore, the depletion of Ni and Fe in the surface sediments of Jeram and Kuala Juru could be possibly explained by the speciation chemistry, complexation reactions with cations and bioavailability of these micronutrients in the sedimentary environments. Several studies including those of Martin and Fitzwater (1998), Cullen (1995) and Martin (1990) explained that Fe is needed by phytoplankton to uptake nitrates for their metabolism and/or constituents of enzymes.

Among the three sites, Jeram has the highest bioavailability and contamination of Cd and Ni [as indicated by the hard and soft tissues] while Kuala Juru had the highest bioavailability and

TABLE 2
The allometric variables of *Anadara granosa* from Kuala Juru, Kuala Kurau and Jeram. All sites N= 20

Site	Variable	Mean	SE	Min	Max
Kuala Juru	Water Content [%]	81.01	-	-	-
	Condition index [g/cm^3]	20.05	0.60	14.48	24.21
	Shell length [cm]	2.57	0.05	2.18	2.99
	Shell height [cm]	2.40	0.04	2	2.7
	Shell width [cm]	3.36	0.07	2.84	3.97
	Soft tissue dry weight [g]	0.42	0.02	0.23	0.58
	Shell dry weight [g]	6.68	0.28	3.84	8.66
Kuala Kurau	Water Content [%]	81.14	-	-	-
	Condition index [g/cm^3]	27.71	0.67	22.09	33.24
	Shell length [cm]	2.46	0.04	2.19	2.98
	Shell height [cm]	2.35	0.04	2.06	2.76
	Shell width [cm]	3.29	0.04	2.95	3.63
	Soft tissue dry weight [g]	0.53	0.02	0.33	0.78
	Shell dry weight [g]	6.61	0.30	4.45	9.94
Jeram	Water Content [%]	80.74	-	-	-
	Condition index [g/cm^3]	25.35	0.76	19.55	30.65
	Shell length [cm]	2.62	0.03	2.42	2.92
	Shell height [cm]	2.28	0.02	2.14	2.48
	Shell width [cm]	3.39	0.03	3.18	3.69
	Soft tissue dry weight [g]	0.52	0.02	0.37	0.72
	Shell dry weight [g]	6.39	0.35	0.96	8.32

contamination of Zn [as indicated by the hard and soft tissues] and Cu [as indicated by the different soft tissues]. The highest bioavailability of Cd and Ni found in the cockles collected from Jeram is to our knowledge, inexplicable. Further studies are needed to identify the sources of Cd and Ni in Jeram. Based on the sediment data (Table 4), high levels of Zn and Cu were found in Kuala Juru and therefore supported the use of the shells and soft tissues of *A. granosa* as biomonitors of Cu and Zn. In the literature, Cheggour *et al.* (2001) found that Cu and Zn concentrations in edible cockle, *C. edule,* appeared to be regulated over the concentration ranges that were found in lagoon sediments. Based on our comparative results between the cockles and sediments, the regulative mechanisms for Cu and Zn were not significant since the concentrations of both metals [Cu and Zn] correlated positively between the cockles [whether shells or soft tissues] and the sediments. The highest Cd level found in Jeram was also supported by the high level of Cd in the sediment.

Kumaraguru and Ramamoorthi (1979) reported the Cu concentrations in *A. granosa* inhabiting the Vellar estuary. The Cu levels in the soft body tissues ranged from 7.56 to 11.08 μg/g dry weight in *A. granosa* [comparable to the 5.41-7.39 μg/g dry weight from this study]. Analysis for metals in the different organs (gills, mantle, hepatopancreas, foot and adductor muscles) showed that the gills and mantle contained elevated Cu concentrations, suggesting a binding capacity of the mucous protein to the Cu (Kumaraguru and Ramamoorthi, 1979). In a previous study, Kumaraguru and Ramamoorthi (1978) also reported the toxicity of Cu to *A. granosa* inhabiting the Vellar estuary in Porto Novo (southern India). These studies revealed that the cockle, *A. granosa*, is tolerant to heavy metal pollution in coastal waters. The tolerance exhibited by the cockle also may be due to the formation of metal-thiolate complex with the cysteine residues situated inside the lysosomes, which eventually reduce its toxicity by preventing the metal(s) from interfering with the cellular metabolism (Webb, 1987). Since the cockle is able to survive in the highly polluted waters, the heavy metals accumulated in the hard tissues and soft tissues should be monitored from time to time.

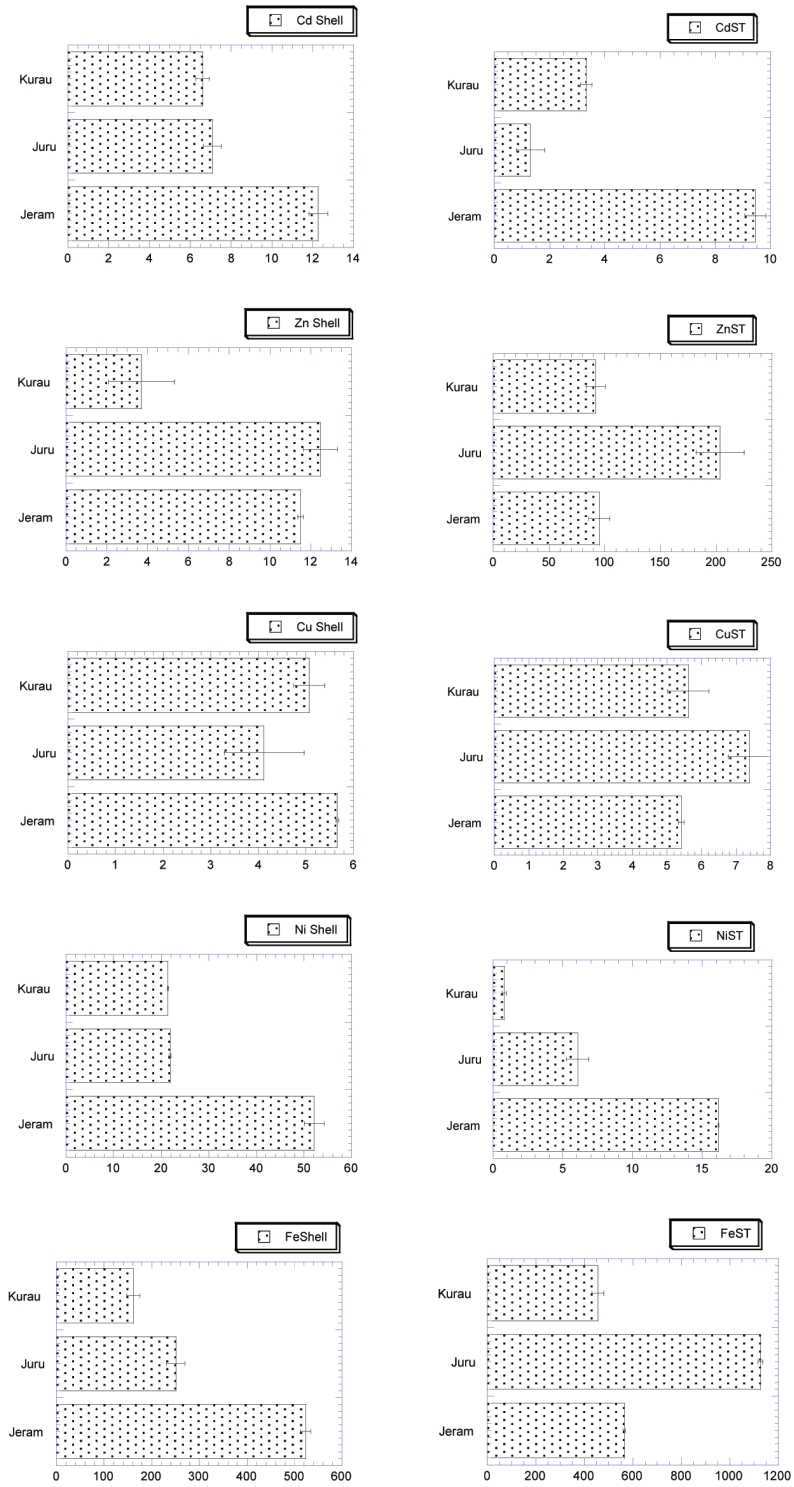

Fig. 2: The concentration (mean (μg/g ± SE dry weight) of heavy metals (Cd, Cu, Ni, Fe and Zn) in the shells and total soft tissues (ST) of the Anadara granosa *from Jeram, Kuala Juru and Kuala Kurau*

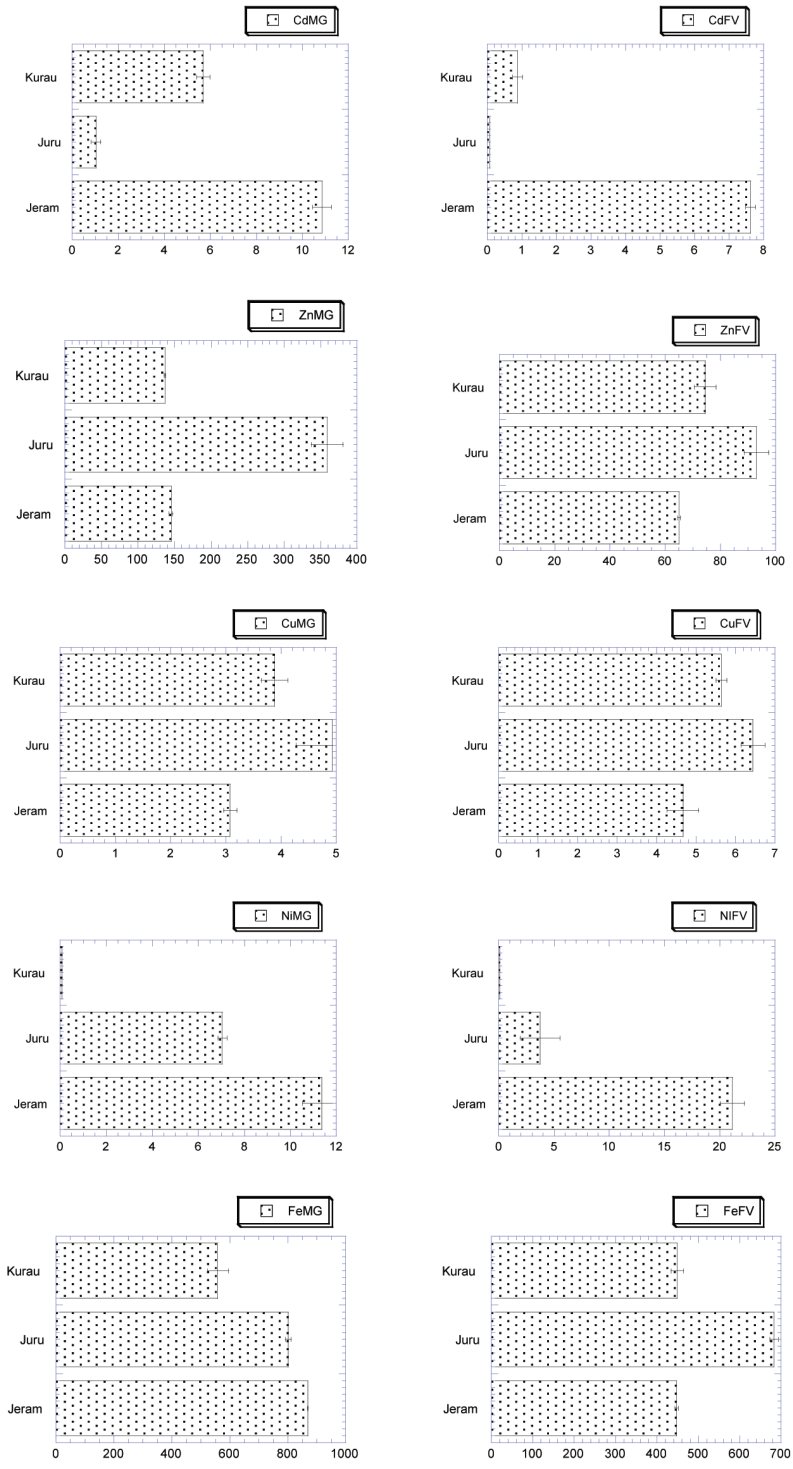

Fig. 3: The concentration (mean (μg/g ± SE dry weight) of heavy metals (Cd, Cu, Fe, Ni and Zn) in the pooled tissues of mantle plus gill (MG) and pooled tissues of foot and visceral mass (FV) of the Anadara granosa *from Jeram, Kuala Juru and Kuala Kurau*

TABLE 3
The concentration [μg/g dry weight] of heavy metals in different parts of the *Anadara granosa*. N= 3

Tissue	Metal	Minimum	Maximum	Mean	Std Error
Total soft tissue	Cd	1.30	9.44	4.69	2.45
	Zn	91.89	203.47	130.20	36.65
	Cu	5.41	7.39	6.14	0.63
	Ni	0.80	16.15	7.67	4.50
	Fe	455.91	1125.50	715.31	207.48
Mantle plus gills	Cd	1.04	10.85	5.86	2.83
	Zn	136.67	358.90	213.58	72.70
	Cu	3.08	4.92	3.96	0.53
	Ni	0.08	11.36	6.16	3.29
	Fe	559.48	868.82	743.19	93.90
Visceral mass plus foot	Cd	0.08	7.62	2.86	2.39
	Zn	65.07	93.05	77.54	8.22
	Cu	4.67	6.44	5.58	0.51
	Ni	0.08	21.13	7.78	6.70
	Fe	447.59	682.77	526.45	78.16
Shell	Cd	6.60	12.27	8.65	1.82
	Zn	3.70	12.47	9.22	2.78
	Cu	4.12	5.66	4.95	0.45
	Ni	21.43	52.15	31.80	10.17
	Fe	161.75	523.38	311.98	108.78

TABLE 4
The mean concentration (μg/g dry weight ± standard error) of heavy metals in the surface sediments collected from Kuala Kurau, Kuala Juru and Kuala Jeram. N= 3

	Kurau	Juru	Jeram
Cd	1.70 ± 0.10	1.24 ± 0.10	2.32 ± 0.08
Zn	74.64 ± 1.05	317.4 ± 2.48	98.97 ± 2.34
Cu	12.91 ± 0.25	32.91 ± 0.41	17.81 ± 0.64
Ni	114.1 ± 3.52	510.93 ± 45.86	24.01 ± 1.15
Fe	35365 ± 403	27595 ± 1795	47242 ± 2281

The heavy metal concentrations in the cockles found in this study were compared with data reported in the literature (Table 5). In general, the metal concentrations from the present study are well within those reported from Thailand (Huschenbeth and Karms, 1975; Phillips and Muttasasin, 1985; Hungspreud *et al.*, 1994), India (Patel *et al.*, 1985), and some previous studies from Malaysia (Jothy *et al.*, 1983; Devi, 1986; Mat *et al.*, 1994; Mat and Maah, 1994; Mat, 1994). Comparing the metal concentrations in the cockles with the permissible limits set by the USFDA, the results indicated no metal toxicolgical threat to consumers. Although high concentrations of Cu and Zn were found in the Kuala Juru cockles, these metal concentrations were still below the maximum permissible limits established by the Malaysian Food Regulations 1985 and the WHO standard guidelines. However, Cd concentrations of Jeram's cockles exceeded the maximum permissible limit as established by the Malaysian Food Regulations 1985 and should be avoided. The high Cd level found from this study [0.25-1.79 µg/g dry weight] was in agreement with those reported by Mat (1994). The Cd concentrations were comparatively higher than those reported by Huschenbeth and Harms (1975), Phillips *et al.* (1982) and Phillips and Mutarasin (1985) for *A. granosa* and *Anadara* sp.

TABLE 5
The comparison of the concentrations (μg/g dry weight) of Cd, Cu, Ni, Fe and Zn in the soft tissues of *Anadara granosa* from the present study with those other reported studies

Samples/locations	WB	Ni	Fe	Cd	Cu	Zn	Authors
Thailand	Wet	NA	NA	0.28	5.60	16.2	Huschenbeth and Karms (1975)
Penang and Perak coastal waters	Wet	NA	NA	1.91	0.51	19.2	Jothy *et al.* (1983)
Northern part of Peninsular Malaysia	Wet	NA	NA	0.20-0.79	0.80-1.60	14.6-23.1	Devi (1986)
Lekir, Perak	Dry	NA	NA	6.10	6.30	64.0	Mat *et al.* (1994)
Batu Kawan and K. Selangor	Dry	NA	NA	2.10-6.90	3.40-4.50	56.0-64.0	Mat and Maah (1994)
Bombay Harbour (1976-1980)	Dry	3.90-10.8	1400-2000	2.80-4.50	8.10-11.1	70.0-132	Patel *et al.* (1985)
Thailand coastal waters	Dry	1.30-2.00	442.1-1055.6	0.25-0.80	4.55-7.37	42.0-57.9	Phillips and Muttasasin (1985)
Retail outlets in Kuala Lumpur (4 sites)	Wet	0.29-0.54	NA	1.23-1.42	0.64-0.80	12.85-14.73	Mat (1994)
Thailand coastal waters	Dry	NA	NA	1.88-3.71	1.36-2.44	77.75-119.81	Hungspreug *et al.* (1994)
Jeram, Kuala Juru and Kuala Kurau	Dry	0.80-16.15 (7.67)	455.91-1125.5 (715.3)	1.30-9.44 (4.69)	5.41-7.39 (6.14)	91.9-203.5 (130.2)	This study
Jeram, Kuala Juru and Kuala Kurau	Wet*	0.15-3.07 (1.46)	86.6-213.9 (135.9)	0.25-1.79 (0.89)	1.03-1.40 (1.17)	17.5-38.7 (24.7)	This study
Permissible levels of metals on food	Wet	NA	NA	1.00	30.0	100	Malaysian Food Regulations 1985

*Wet weight basis was converted to dry weight basis based on a conversion factor of 0.19.
Note: NA= Not available

(Phillips *et al.*, 1982). However, it was of similar levels as those reported by Jothy *et al.* (1983). Although the degree of coastal water pollution is not serious in Malaysia, the presence of 'hot-spots' cannot be ruled out (Broom, 1985). Therefore, it is important to monitor the level of contamination of Cd in the muddy coastal environment. This is due to the extensive culture of cockles in this area. Contamination in seafoods, caused by industrialization and other anthropogenic activities in the vicinity of coastal areas is always positively associated with elevated levels of Cu, Zn, Ni and Cd in cockles (Phillips, 1978; Phillips *et al.* 1982). In the *A. granosa,* the concentrations of Ni varied from 0.80-16.15 μg/g dry weight. Ni could be found in various forms of alloy in heavy machineries such as turbine blades as well as its variety of uses as industrial catalysts. Therefore, the monitoring of Ni in the environment is also important.

CONCLUSIONS

In conclusion, *A. granosa* can be used as a biomonitor for Cu and Zn. Further studies, however, are necessary to explain the ability of *A. granosa* as a biomonitor of Ni and Fe. On the other hand, the depletion of Ni and Fe may be due to the chemistry speciation, complexation reactions with cations and bioavailability of those micronutrients in the sedimentary environments in Jeram and Kuala Juru. The Cd levels of the cockles from Jeram which exceeded the Malaysian Food Regulation 1985, suggested that the cockles should be avoided for human consumption.

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Cellular Structure and Related Physico-Chemical Changes During Ripening of *Musa* AAA 'Berangan'

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ABSTRACT

Ripening is the result of complex changes and it turns an unpalatable fruit to be palatable. The distinct changes in eating quality of ripening fruits are determined by changes in their cellular structure and composition. This work was carried out to relate structural changes of *Musa* AAA 'Berangan' with related physico-chemical quality characteristics during ripening. The cell structure of Berangan banana was examined using scanning electron microscopy while pulp firmness, soluble solids concentration and starch pattern were determined as fruit ripened from mature green stage (ripening stage 1) until full yellow (ripening stage 6) at 27°C. As ripening progressed, the cellular structure of Berangan banana showed dramatic changes and coincided with changes in the physico-chemical characteristics. The epicuticular wax and stomatal complex of peel degenerated while cell wall integrity was lost as fruit ripened. The starch began to degrade from the central core outward and concomitant with a decrease in starch granules sizes and increase of soluble solids concentration as ripening took place. The loss of cell wall integrity and conversion of starch into sugars as ripening progressed contributed to fruit softening. It is concluded that as ripening progressed, the increase in soluble solids concentration and decrease in firmness of Berangan banana fruit was closely related to the changes in the cellular structure.

Keywords: Epicuticular wax, firmness, soluble solids concentration, starch granules, starch distribution pattern

INTRODUCTION

In Malaysia, bananas are the second largest cultivated fruit crops, after durian (*Durio zibethinus*). The major commercial bananas cultivated by smallholders are 'Berangan', 'Mas' and 'Rastali', while the larger plantations grow Cavendish. Cavendish banana is the most commercially important banana in global trade; however, it does not degreen naturally under tropical temperatures such as in Malaysia (Ding *et al.*, 2007). To degreen Cavendish banana under Malaysian conditions, cool ripening rooms have to be built to provide temperatures of 18-20°C. This has resulted in increased production costs for smallholders who are the major banana producers in Malaysia. In light of these difficulties, smallholders prefer to plant traditional cultivars of bananas which can degreen naturally under tropical conditions although Cavendish banana is a major international trading banana.

Ripening is the result of complex changes. During ripening, green peel of banana will turn to yellow with the breakdown of chromoplast grana-thylakoid membranes (Ding *et al.,* 2007). The texture of fruit softens progressively. The relative firmness of the fruit is greatly determined by physical and chemical attributes such as peel thickness and starch content (Lizada *et al.,* 1990). The starch in the pulp of banana will be hydrolysed into sugar (Marriot *et al.*, 1981) and organic acids, which give desirable sugar-to-acid balance, will change. Malic acid will be the main acid during ripening, followed by oxalic and citric acids (Marriott, 1980).

Among local cultivars, Berangan banana is of highest popularity. The market potential of Berangan banana increased by 5% per annum

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to 39,970 metric tonnes in 2006 (Anon, 2006). Malaysia exports Berangan banana mainly to Singapore, Brunei, Germany and UAE. Unlike Cavendish banana whose genome make up is also AAA, the peel of the Berangan banana naturally changes from green to golden yellow as ripening progresses under tropical conditions. Information on the changes in structural aspects during ripening has importance in relation to fruit quality, storage and commercial value. There is very little information on the cellular structure of this Berangan banana cultivar during ripening. This study attempts to relate structural changes in the peel and pulp of Berangan banana using scanning electron microscopy with selected physico-chemical quality characteristics during ripening.

MATERIALS AND METHODS

Plant Materials

Mature green *Musa AAA* 'Berangan banana' bunch were obtained from a fruit distributor and transported to the laboratory. Hands of second and third from the proximal end of the peduncle of a bunch were used in the experiment. A total of 4 bunches of banana were used and each bunch served as a replicate. Each hand containing 16-18 fingers, with each finger weighing 80-90 g, were placed in a box and gassed with acetylene from CaC_2 (with an equivalent of 10 g CaC_2 kg^{-1} fruit) at room temperature of 27±2°C with relative humidity of 85% for 24 h. The fruits were allowed to ripen in the same environment after 24 h of ripening initiation. Fruit ripening was visually assessed based on the following ripening stages (RS) as established by the Federal Agricultural Marketing Authority: 1 = mature green; 2 = tinge of yellow; 3 = more green than yellow; 4 = more yellow than green; 5 = yellow with green tips and 6 = full yellow. Two fingers from each RS of fruit were analysed with each fruit taken from the upper and lower row of a hand.

Scanning Electron Microscopy (SEM) Studies

Samples blocks measuring 0.5 cm x 0.5 cm x 1 cm were taken from the mid region of fruit and fixed in Bouin's fixative for 4 h under vacuum. The samples were washed in 1% cacodylate buffer, post-fixed in 1% osmium tetroxide in 0.1 mol L^{-1} cacodylate buffer for 2 h, washed in 0.1 mol L^{-1} cacodylate buffer before dehydration through graded series of ethanol to absolute ethanol, and critical-point-dried using a Balzer CPD 030 (Balzer Union, Furstentum, Liechtenstein). The fruit tissues were mounted on stubs, sputter coated in gold and viewed in a JEOL 6400 SEM (JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 15 kV and at working distance of 15 mm. Dimension of cells at the five upper layers of peel and starch granules in pulp were measured using Semafore (digital slow scan image recording system) release 3.04. The length was the longest distance between two ends and the width was the distance transversely across at the middle of a cell and starch granule. Fifty cells at the uppermost layer of peel and 50 starch granules in pulp per replicate were determined from each RS and replicated twice.

Physico-chemical Characteristics

(i) Determination of flesh firmness

Flesh firmness was evaluated using the Bishop Penetrometer FT 327 (Alfonsine, Italy). The force required for an 11-mm probe to penetrate to the depth of 5 mm of the pulp was recorded. The penetration force was expressed in kg cm^{-2}. Two fingers of each RS were analyzed at each time and replicated four times.

(ii) Determination of soluble solids concentration (SSC)

One centimeter thick of banana pulp was cut transversely from mid region of a banana finger, and a total of 2 fingers for each RS were macerated with a chopping knife. Ten grams of the macerated tissue was homogenised with 40 mL of distilled water by using a kitchen blender. The mixture was filtered with cotton wool. A drop of the filtrate was then placed on the prism glass of a refractometer (Atago Co, Ltd., Model N1, Tokyo, Japan) to obtain the %SSC. The readings were corrected to a standard temperature of 20°C by adding 0.28% to obtain %SSC at 27°C. The determination was repeated four times.

(iii) Determination of starch distribution pattern

A 2-cm-thick cross-sectioned of pulp cut from the mid-region of a fruit was stained with 1% of KI/I_2 for 1 min. The intensity and area of stain was photographed with a camera (Canon EOS 888, Japan) and total of four replications were carried out.

Statistical Analysis

The experiments were conducted using a randomized complete block design with each RS replicated four times using 2 fingers at a time except for SEM studies which were repeated twice. Data were analyzed using analysis of variance (SAS Institute, Cary, NC) and means were separated with Duncan's multiple range test (DMRT) at $P \leq 0.05$.

RESULTS

The peel of Berangan banana fruit was bounded by epidermis. The epidermal cells were hexagonal in shape and covered by lamellae-like strands of wax that fused together into a compact mound forming papillae topography (*Fig. 1A*). Elliptical-shaped stomata were distributed randomly among epidermal cells and raised slightly above the surface. The stomata density and stomatal aperture have been reported by Ding *et al.* (2006). At RS 1, the lamellae-like strands of wax were distinct in appearance (*Fig. 1B*). When green fruit began to turn yellow, the wax started to degenerate and became completely degenerated in full yellow ripe fruit, RS 6 (*Fig. 2A*). The guard cells, subsidiary cell and extended wings of stomata were also deformed at RS 6 (*Fig. 2B*). The stomata were no longer found to be raised in later stage of ripening, especially at RS 6. This could due to the loss of guard cells turgidity and lead to the sunken appearance of the stomatal complex as senescence started to take place at the end of the ripening process.

The integrity of cell walls was intact in mature green RS 1 cells (*Fig. 3A*). As ripening progressed, the breakdown of cell wall was clearly noticed in the micrograph of the peel at RS 6 (*Fig. 3B*). The characteristic of the pulp region of Berangan banana was the starch granules containing cells. The granules were irregular in shape with spheroid and oval elongated form being predominant (*Fig. 4A*). In green bananas, the surface of the starch granules was smooth and the cell wall and membrane were well organized. As ripening advanced, the cell wall and membrane broke down. At RS 6, parallel striation appeared on the previously smooth surface of starch granules (*Fig. 4B*). The length and width of starch granules decreased significantly ($P \leq 0.05$) as ripening progressed (Table 1).

The firmness of Berangan banana flesh decreased significantly ($P \leq 0.05$) by 83% as fruits ripened from RS 1 to 3 (Table 2). Thereafter, there was no significant decrease in flesh firmness until RS 6. The SSC of fruits was closely associated with ripeness stage where it increased significantly ($P \leq 0.05$) as ripening progressed (Table 2). RS 1 had the lowest while RS 6 had the highest SSC value. The SSC values increased by 648% as fruits ripened from stage 1 to 6. Hydrolysis of starch into sugar increases the SSC value. The increase in SSC with hydrolysis of starch into sugar could be further explained by the starch iodine pattern (*Fig. 5*).

Before fruit ripening initiation (RS 1), the tissue was not well stained with iodine solution and showed lightly stained blue-black colour. Probably the exuded latex in green mature fruits prevented the starch from being stained by iodine solution. However, some areas of peel tissues was stained with iodine solution. Once the ripening had been initiated (RS 2), the iodine solution began to stain well with the banana pulp starch and some area of peel tissues. At this RS, the whole transversal surface was stained with blue-black colour. At RS 3, the blue-black

TABLE 1
Effects of ripening stage on pulp starch granules length and width of Berangan banana fruit

Ripening stage	Starch granules length[z] (μm)	Starch granules width[y] (μm)
1	29.26 a[x]	15.12 a
2	27.78 a	13.73 b
3	26.21 bc	12.76 bc
4	24.66 cd	12.17 c
5	23.61 de	12.00 c
6	22.79 e	11.50 c

[z,y] Mean of 100 observations

[x] Mean separation within column by DMRT at P ≤ 0.05.

TABLE 2
Effects of ripening stage on soluble solids concentration and firmness of Berangan banana fruit pulp

Ripening stage	Soluble solids concentration (%SSC)	Firmness (kg cm^{-2})
1	2.63 e[z]	10.60 a
2	10.25 d	2.97 b
3	14.00 c	1.78 c
4	16.61 b	1.44 c
5	17.53 b	1.27 c
6	19.67 a	1.16 c

[z] Mean separation within column by DMRT at $P \leq 0.05$.

stain at the central core of the fruits started to clear while the peel tissue was no longer stained by iodine solution. The stain clearing of the pulp central core became obvious as ripening advanced. At the end of evaluation (RS 6), the ovarian cavity of Berangan banana was not stained with iodine solution and only the peripheral of the transverse section was stained in blue-black colour. The pattern indicated that the starch degradation started at the central core of the fruits and advanced towards the outer borders as ripening progressed.

DISCUSSION

The surface morphology of Berangan banana was papillae with a well-organized pattern. Burdon *et al.* (1993) found that the surface sculpture of banana fruits differ among fruit genome. To the best of the author's knowledge, there is little literature reported in changes of epicuticular waxes as ripening progressed. Bally (1999) observed changes in epicuticular surface structure during fruit development of 'Kensington Pride' mango. However, there was no study on the changes of epicuticular surface as ripening progressed. The epicuticular wax with lamellae-like strands of Berangan banana degenerated as ripening progressed (*Fig. 2B*). Epicuticular waxes play an important role in regulating water loss, uptake or release of chemicals (Riederer and Schreiber, 1995) and act as barriers to insects and pathogens (Juniper and Cox, 1973). In cranberry fruit, the thicker wax accumulation at calyx end was related to microorganism entry retardation into the fruit during wet harvest (Ozgen *et al.*, 2002). The degenerated epicuticular waxes at RS 6 (*Fig. 2A*) of Berangan banana indicated the physical barrier had weakened and the fruits are prone to microorganism infection as compared to RS 1.

The loss of cell wall integrity and disappearance of starch granules as observed under SEM in Berangan banana was similar to the findings of Prabha and Bhagyalakshimi (1998) for 'Robusta' banana. Microscopic examination of jackfruit (*Artocarpus heterophyllus L.*) showed loss of cell wall integrity and starch granules as ripening progressed (Rahman *et al.*, 1995). A similar observation was also reported in ripening of 'Harumanis' mango where the cells lose their integrity and strach granules were degraded as ripening progressed (Muhammad and Ding, 2007). This indicated cellular structures of fruits undergo degradation as ripening progressed and this shortens the postharvest life of fruits if poor postharvest management is being practised.

As ripening progressed, the starch granules hydrolysed and sugar accumulated as reflected by high %SSC and fading of starch-iodine staining pattern. Hydrolysis of starch granules could be noticed with striation appearing on its surface as observed under SEM (*Fig. 4B*). This striated structure is due to the action of amylase during ripening which hydrolysed polysaccharides of starch granules into monosaccharides (Fuwa *et al.*, 1979). As a consequence of this action, the length and width of starch granules decreased significantly ($P \leq 0.05$) as ripening progressed from stage 1 to 6 (Table 2), indicating starch granules turned from large to small size. In addition, the density of starch granules in pulp cells decreased as ripening progressed from green to full yellow stage. Starch constituted 85-

95% of the dry matter in green banana pulp, which is reduced to 5-15% and consequently sugar content increased to 70-80% as fruits ripened (Hubbard *et al.*, 1990). The enzymatic transformation of starch granules to soluble sugars (e.g. sucrose, fructose and glucose) appeared as a reduction in granular size in ripe banana as evidenced in Table 1. Prabha and Bhagyalakshmi (1998) observed that more than 80% of the radio-activity of [^{14}C] starch was incorporated into soluble sugars in Robusta banana, indicating active sugar interconversions. The transformation of starch is a complex process involving several enzymes and more than one pathway as proposed by Beatriz and Lajolo (1995).

The loss of starch from the central core of banana has also been reported by Blankenship *et al.* (1993) and Garcia and Lajolo (1988) in Cavendish banana. This indicated that Berangan banana had the similar starch-iodine staining pattern as Cavendish banana. The starch degradation advanced towards the outer border of the peel as ripening progressed until the starch was completely degraded at RS 6. The blue-black stain was due to the presence of amylose. Garcia and Lajolo (1988) noted that banana starch consists of 16% amylose which is the unbranched chain polysaccharide component of starch and has a strong affinity for iodine to produce a deep blue-black complex.

As ripening progressed, fruit firmness of Berangan banana decreased markedly (Table 3). During ripening, pectins and hemicelluloses of cell wall undergo solubilization and depolymerization thus leading to softening (Redgwell *et al.*, 1992). The insoluble pectins of banana decreased from 0.5 to 0.2% fresh weight with a corresponding rise in soluble pectins (Marriot *et al.*, 1981). The interconversion of pectic substances is presumed to lead to cell wall breakdown and results in loss of turgidity and firmness of banana fruits during ripening. It is also reported that the softening of banana fruits during ripening is a result from the concerted action of at least four polygalacturonase genes (Asif and Nath, 2005). The decrease of fruit firmness is also related to starch hydrolysis. Starch could contribute to the structural properties of fruits (John and Marchal, 1995) and create resistance to the penetrating force. Seymour (1993) stated that the loss of starch likely plays a major role in textural changes in ripening bananas. Recently, Sane *et al.* (2007) found out that expression of multiple expansin genes in Dwarf Cavendish banana might be required for softening of monocot fruits too.

In conclusion, once ripening is initiated, Berangan banana showed an irreversible structural and physico-chemical change. The cellular structure changes observed under SEM were closely associated with physico-chemical changes. As ripening took place, fruits soften due to cell wall breakdown and conversion of starch into sugar. As a result, fruits become palatable with soft and sweet pulp. Senescence happening at the end of ripening will degenerate the structure of fruit cells and cause the fruit to lose quality.

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Antimicrobial Activities of Chitosan and Calcium Chloride on *in vitro* Growth of *Colletotrichum gloeosporioides* from Papaya

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ABSTRACT

The antimicrobial activities of chitosan and calcium chloride ($CaCl_2$) on the growth, spore germination, and hyphal morphology of *Colletotrichum gloeosporioides*, isolated from naturally infected papaya fruits, was investigated in in vitro studies. Chitosan was found to inhibit the radial growth and spore germination of the fungus significantly at higher concentrations. Radial growth was inhibited by 52 and 82% with 0.75 and 1% chitosan, respectively. Spore germination was completely inhibited in Potato Dextrose Agar (PDA) medium containing 0.5% and above chitosan concentrations. Light microscope observations showed that chitosan induced morphological changes, including abnormal branching, swelling hyphal tips, vacuolation and distortion. Calcium chloride at 1 to 4% had no pronounced effects on mycelial growth inhibition. However, PDA amended with 3 and 4% $CaCl_2$ significantly ($P \leq 0.05$) inhibited about 26% of spore germination relative to the control. Findings from these experiments demonstrated that chitosan has suppressive activity against *C. gloeosporioides* of papaya and could be used as part of a disease management program. Nevertheless, to a lesser extent, $CaCl_2$ can also be included as part of the program.

Keywords: Non-chemical control, natural compounds, postharvest pathogen, papaya

INTRODUCTION

Papaya (*Carica papaya* L.) is considered one of the most important fruit crops throughout the tropical and subtropical countries with high consumer demand worldwide. The fruits are very susceptible to diseases caused by many microorganisms especially fungi, as they are rich in moisture and nutrients (Sankat and Maharaj, 1997). At postharvest stage, many diseases greatly reduce the storage life and quality of papaya, out of which anthracnose caused by *Colletotrichum gloeosporioides* is the major disease of this fruits in tropical countries (Snowdon, 1990).

Anthracnose in papaya can be controlled by prochloraz or propiconazole (Sepiah, 1993) and hot water dip treatment (HWT) at 43-49°C for 20 min (Couey *et al.*, 1984). However, HWT affect the ripening process in papaya (Paull, 1990) and resistant strains of *C. gloeosporioides* have already been developed against some commonly used fungicides (Bautista-Banos *et al.*, 2003). Furthermore, residues of fungicides present on the fruit may be harmful to consumers. All of these issues prompted an investigation of potential safer approaches to disease management. Recently, biologically active natural products have become an effective alternative to synthetic fungicides as a means to control fungal decay (Spadaro and Gullino, 2004; Tripathi and Dubey, 2004).

Chitosan is a polycationic biopolymer that can be produced industrially by chemical deacetylation of chitin, which is found in arthropod exoskeletons and the cell walls of some plant pathogenic fungi (Hernandez-Munoz *et al.*, 2006). This natural compound is associated with its fungistatic or fungicidal properties against pathogens of various fruits and vegetables (Bautista-Banos *et al.*, 2003). Several studies have shown that growth of some important postharvest fungi such as *Alternaria alternate* and *Fusarium oxysporum* is inhibited on nutrient media

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amended with various concentrations of chitosan (Benhamou, 1992; Bhaskara Reddy *et al.*, 1998). In *in situ* studies, the fungicidal effect of chitosan on strawberries against *Botrytis cinerea* and *Rhizopus stolonifer* has also been reported (El Ghaouth *et al.*, 1992a).

Many organic and inorganic salts have been shown to be active antimicrobial agents against a range of phytopathogenic fungi. In particular, postharvest treatment with $CaCl_2$ has been proposed as safe and effective alternative means to control postharvest rot of fruits and vegetables (Smilanick *et al.*, 1999; Ippolito *et al.*, 2005). Prior studies have shown that the addition of $CaCl_2$ to nutrient media reduced spore germination and germ tube growth of *Rhizopus stolonifer, Penicillium expensum* and *Botrytis cinerea* (Wisniewski *et al.*, 1995; Narayanasamy, 2006). However, not much had been reported on using chitosan and $CaCl_2$ as postharvest treatment against anthracnose of papaya especially with cv. Sekaki caused by *C. gloeosporioides*. Therefore, the objectives of this study are to evaluate the effects of chitosan and $CaCl_2$ on *in vitro* growth, spore germination and hyphal morphology of *C. gloeosporioides* of papaya.

MATERIALS AND METHODS

Culture of Fungus and Study Site

Colletotrichum gloeosporioides, the causal agent of anthracnose was isolated from naturally infected papaya fruits following the procedures described by Bautista-Banos *et al.* (2003). Purified cultures were maintained on Potato Dextrose Agar (PDA) slant at room temperature (28±2°C). All the experiments were conducted at the Plant Protection Laboratory, Faculty of Agriculture, Universiti Putra Malaysia in 2007.

Preparation of Chitosan Solutions

One hundred milliliters of 0.1, 0.25, 0.5, 0.75 and 1.0% chitosan solutions were prepared, where 0.1, 0.25, 0.5, 0.75 and 1.0 g of chitosan (Shrimp shell chitosan, Chito-Chem (M) Sdn Bhd, Malaysia) were dissolved in 75 mL distilled water and 2 mL glacial acetic acid. The mixture was heated with continuous stirring for proper dissolution of chitosan. The final pH of the solution was adjusted to 5.6 with 2 N NaOH and volume made up to 100 mL with sterilized distilled water. To improve the wettability, 0.1 mL of Tween 80 was added to the solution (Jiang and Li, 2001).

Preparation of Conidial Suspension of C. gloeosporioides

Isolates of *C. gloeosporioides* were grown on PDA agar (Merck) at 28±2°C for seven days. Spores were subsequently harvested by flooding the surface of the media with distilled water containing 0.1% Tween 80 (v/v) and the plate was gently agitated with a bent sterilized glass rod to dislodge the spores. The resulting suspension was filtered through two layers of sterile muslin cloth. The concentration of conidia in the filtered suspension was adjusted to 1×10^5 conidia mL^{-1} with sterile distilled water using a haemacytometer (Obagwu and Korsten, 2003).

Effect of Chitosan on C. gloeosporioides

The effect of chitosan on the mycelial growth of *C. gloeosporioides* was studied using agar plates. The viscous stock solution of chitosan (1.25%) was autoclaved and subsequently diluted with sterile, molten PDA to obtain chitosan concentrations of 0.1, 0.25, 0.5, 0.75 and 1.0%. Twenty milliliters aliquots of this solution were immediately dispensed into 9-cm-diameter petri plates. Each of the amended PDA plate was inoculated with a 6-mm-diameter mycelial plug taken from the margin of 4-day-old culture of *C. gloeosporioides*. The plates were then incubated at 28±2°C for a maximum period of seven days. The radial measurements of growth were taken after seven days of incubation until the fungus reached the edge of the control plates. The percent inhibition of radial growth (PIRG) was calculated according to the formula described by Sivakumar *et al.* (2000).

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

where, R1 = Radial growth of *C. gloeosporioides* in control plate

R2 = Radial growth of *C. gloeosporioides* interacting with antagonistic bacteria.

Cultures of *C. gloeosporioides* in PDA without chitosan served as the control. To observe the morphological changes, hyphal strands from the end of the fungal colony were removed aseptically and examined under a microscope for abnormalities.

For spore germination test, 100 µl aliquots of the spore suspensions ($1x10^5$ conidia mL^{-1}) of *C. gloeosporioides* were pipetted onto each PDA plate amended with chitosan and spread with a

sterile bent glass rod. Control plates contained PDA only. Inoculated plates were incubated at 28±2°C for 13 h. Data on spore germination were recorded at every 2 h interval starting from 7 h after inoculation. Germination of 100 spores per plate was determined microscopically. A spore was considered to have germinated when the germ tube length equaled or exceeded the length of the spore (El-Ghaouth *et al.*, 1992a).

Effect of Calcium Chloride on C. gloeosporioides

The effect of $CaCl_2$ on mycelial growth and spore germination of *C. gloeosporioides* was also observed on PDA plates amended with different concentrations of $CaCl_2$ (1, 2, 3 and 4%). Experimental design and procedures were the same as described earlier for chitosan except that the incubation period was six days instead of seven days for the measurement of radial growth of the test fungus. The incubation period for spore germination was 7 h unlike 13 h with chitosan.

Experimental Design and Statistical Analysis

All experiments were arranged in a Completely Randomized Design (CRD) with five replications and were performed twice unless stated otherwise. Since most of the data were quantitative and there was no significant variability among the trials, they were pooled. All percentage data were arcsine transformed before subjected to analysis of variance (ANOVA) and means separation was done by the Tukey's Studentized Range (HSD) Test using SAS version 8.1.

RESULTS AND DISCUSSIONS

Effects of Chitosan on C. gloeosporioides

All chitosan concentrations tested significantly ($P \leq 0.05$) inhibited the radial growth of *C. gloeosporioides* after seven days of incubation, with a marked effect at higher concentrations (*Fig. 1*). The highest inhibition of mycelial growth (82%) was recorded with 1% chitosan solution followed by 0.75% chitosan with radial growth inhibition of 52.2%. Growth inhibition ranging from 2.6 to 26.8% was obtained with chitosan at concentrations ranging from 0.1 to 0.5%.

By visual observation, it was found that overall sporulation was lower on PDA plates amended with chitosan when compared with the control. However, no spore was formed on PDA amended with ≥ 0.75% chitosan concentration after seven days of incubation; whereas, numerous spores were formed on 0.1% chitosan amended medium and in control plates. Chitosan at higher concentrations markedly reduced spore germination of *C. gloeosporioides* (*Figs. 2* and *3*). Spore germination was completely inhibited with ≥ 0.5% chitosan concentrations even after 24 h of inoculation (*Fig. 3e*). Whereas, on PDA plates amended with 0.25% chitosan, germination was inhibited by 75% after 7 h of inoculation (*Fig. 3d*), which decreased considerably with incubation and reached 0.0% inhibition after 11 h of inoculation that was similar to the control. Lowest concentration of chitosan (0.1%) used in this experiment did not inhibit spore germination (*Fig. 3c*).

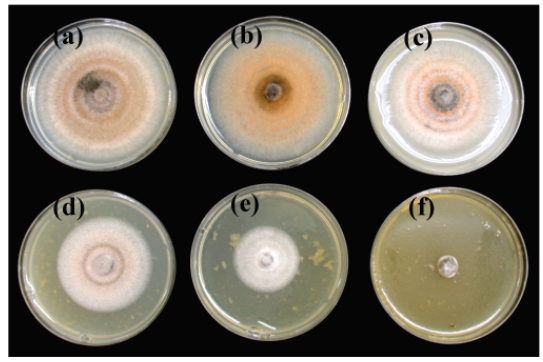

Fig. 1: Effect of different concentrations of chitosan on the radial growth of C. gloeosporioides *after seven days of incubation at 28±2°C.* ***(a)*** *= 0.0% (Control);* ***(b)*** *= 0.1%;* ***(c)*** *= 0.25%;* ***(d)*** *= 0.5%;* ***(e)*** *= 0.75% and* ***(f)*** *= 1.0% chitosan amended with PDA*

Chitosan showed a significant influence on growth and sporulation of *C. gloeosporioides* in these *in vitro* experiments. Results of this study showed that *C. gloeosporioides* was very sensitive to chitosan since growth was affected even at lower concentrations including 0.25%. Mycelial growth, spore germination and hyphal morphology were affected by chitosan indicating that it affected various stages of development. Similar results were found by Asgar *et al.* (2004), who worked on Eksotika variety and reported that mycelial growth, spore germination and germ tube elongation of *C. gloeosporioides* were markedly reduced by chitosan with greater effect at higher concentrations. It has been widely reported in the literature that the level of inhibition of fungi is highly correlated with chitosan concentration, indicating that chitosan performance is related

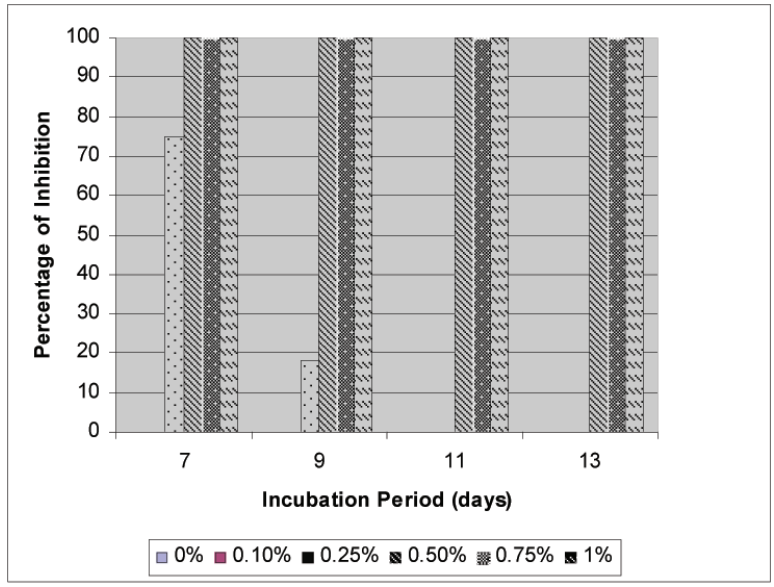

Fig. 2: Effect of different concentrations of chitosan on spore germination of C. gloeosporioides *after 7, 9, 11, and 13 h of inoculation at 28±2°C. Means separation was done on the arcsine transformed values at P≤0.05 according to Tukey's Studentized Range (HSD) Test*

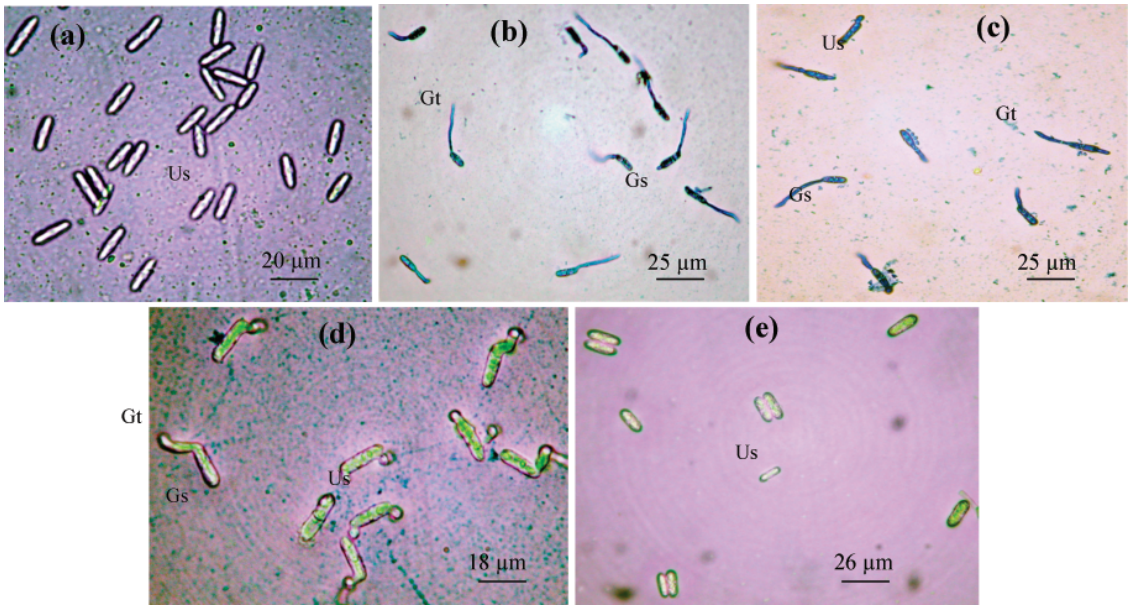

Fig. 3: Effect of different concentrations of chitosan on spore germination of C. gloeosporioides *on PDA after 7 h of incubation at 28±2°C.* ***(a)*** *= Typical ungerminated spores;* ***(b)*** *= Germinated spores in control plate;* ***(c)*** *= Germinated spores with 0.1% chitosan;* ***(d)*** *= Germinated spores with 0.25% chitosan and* ***(e)*** *= Ungerminated spores with 0.5 to 1.0% chitosan. Gs = Germinated spore; Us = Ungerminated spore; Gt = Germ tube*

to rate of application. There are strong evidences that mycelial growth can be inhibited or retarded when the growth media of fungi are amended with chitosan. El-Ghaouth *et al.* (1992a) reported that chitosan inhibited spore germination and radial growth of *Botrytis cinerea* and *Rhizopus stolonifer in vitro*. Inhibition of radial growth and spore germination of *B. cinerea* were 95.5 and 98.7%, respectively at chitosan concentration of 6 mg mL^{-1}.

Overall, sporulation of fungi treated with chitosan was generally reported to be lower than in untreated fungi. In this study, no spore was formed at higher chitosan concentrations (0.75 to 1.0%) amended plates, whereas, numerous spores were produced by *C. gloeosporioides* at lower chitosan concentration (0.1%) as well as in control plates. The inhibition of spore formation was also found by other researchers in *F. oxysporum, R. stolonifer, C. gloeosporioides, A. alternate* f. sp. *lycopersici* and *A. niger* (Bhaskara Reddy *et al.*, 1998; Bautista-Banos *et al.*, 2003; Plascencia-Jatomea *et al.*, 2003). Nevertheless, chitosan sometimes stimulates sporulation. It is reported that spore formation of *P. digitatum* when grown on 0.5 and 1.5% chitosan was significantly greater than the control treatment (Bautista-Banos *et al.*, 2006) and suggesting that this high sporulation could be due to a stress response induced by chitosan.

Spore germination of *B. cinerea* was reported to be inhibited by 50% even at lower concentrations of chitosan (20-30 μg mL^{-1}) with complete inhibition at 50 μg mL^{-1} (Ben-Shalom *et al.*, 2003). El-Ghaouth *et al.* (1992a) found that chitosan reduced the spore germination and germ tube elongation of *R. stolonifer* by more than 90 and 75% with 10 mg mL^{-1}chitosan concentration. Similarly, this study showed that spore germination of *C. gloeosporioides* was completely inhibited by chitosan at higher concentrations (0.5 to 1.0%). The long-term fungicidal effect of chitosan can also be related to concentration and incubation time. It was observed that the inhibition of spore germination decreased with incubation time, when grown on PDA amended with 0.25% chitosan. This finding is in agreement with Benhamou (1992) who reported that inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* grown at two of the lowest concentrations (1.0 and 2.0 mg mL^{-1}) decreased with increased incubation time. However, the differences regarding inhibition of mycelial growth and spore germination of fungal pathogens may be due to the molecular weight and concentrations of chitosan used and as well as fungal species response (El-Ghaouth *et al.*, 1992b).

Morphological Changes

Observations on inhibition of fungal growth *in vitro* was carried out further by light microscope investigations (*Fig. 4*). When fungal plugs were deposited on chitosan-free agar, mycelial growth was found to be regularly septate in which hyphae were branched at diverging angles (*Fig. 4a*). In contrast, noticeable morphological changes occurred in hyphae when the fungus was grown on chitosan amended agar medium. At all concentrations, except 0.1%, hyphae growth was abnormal in shape and many hyphal tips were swollen (*Fig. 4c*). A greater degree of contortion was observed at chitosan concentrations of 0.5 to 1.0%, where significant increase in hyphal vacuolation and shriveling (*Fig. 4d*), coiling of hyphae and hyphal tips (*Fig. 4e* & *4f*) and abnormal branching (*Fig. 4b*) were observed.

Previous studies have shown that chitosan is not only effective at halting the growth of pathogens, but also induces marked morphological changes, structural alterations and molecular disorganization of fungal cells (El Ghaouth *et al.,* 1992b; Ait Barka *et al.*, 2004). In this study, microscopic observation of *C. gloeosporioides* treated with chitosan showed that it can affect the morphology of the hyphae. Hyphal tips of the fungus became malformed, and hyphae were thickened and vacuolar compared with hyphae in the control plate. Many swellings occurred in the hyphae, whereas normal hyphal walls were smooth with no swellings or vacuolation. These findings are in agreement with El Ghaouth *et al.* (1992b) who reported that chitosan not only inhibited the radial growth of major postharvest pathogens, but also induced severe morphological alterations in *Rhizopus stolonifer* and *Botrytis cinerea*, as well as increased cellular leakage in both fungi, presumably by interfering with fungal plasma membranes. Other observations carried out on fungi such as *F. oxysporum* f. sp. *radicis-lycopersici, R. stolonifer* and *S. sclerotiorum* treated with chitosan, showed excessive mycelial branching, abnormal shapes, swelling, and hyphal size

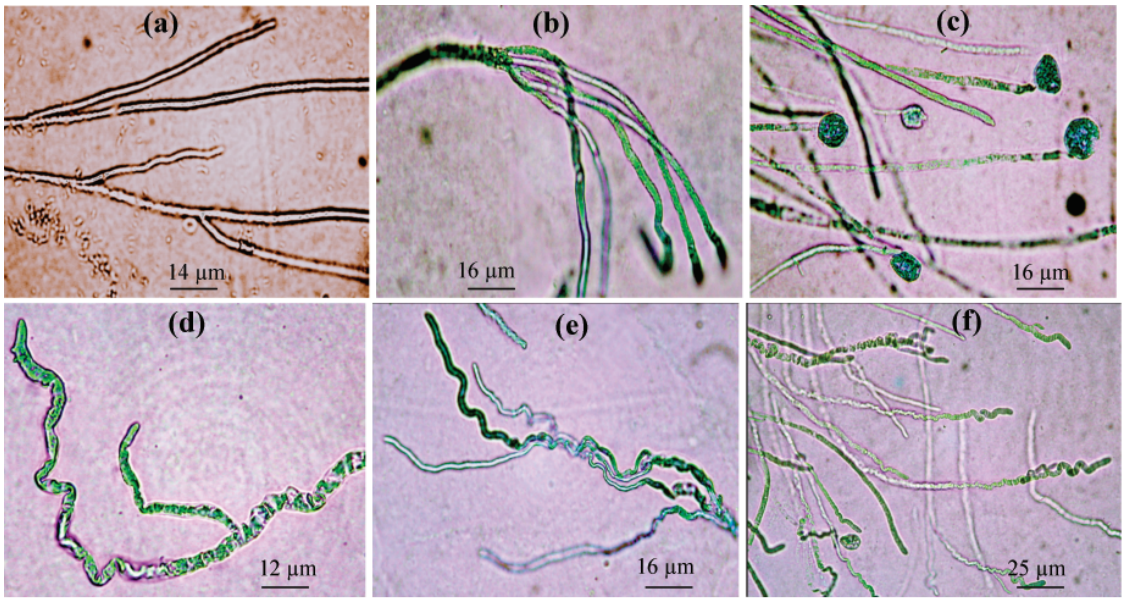

Fig. 4: Effect of chitosan on hyphal morphology of C. gloeosporioides, ***(a)*** *= hyphae with normal brancing (control);* ***(b)*** *= Hyphae with abnormal branching;* ***(c)*** *= swallowed hyphal tips;* ***(d)*** *= Shriveled hyphae;* ***(e)*** *and* ***(f)*** *= Coiling of hyphae and hyphal tips*

reduction (Cheah *et al.*, 1997; Benhamou, 1992; El Ghaouth *et al.*, 1992a, 1992b).

Effects of Calcium Chloride on C. gloeosporioides

Different concentrations of $CaCl_2$ showed significant effect on the radial growth of *C. gloeosporioides* (*Figs. 5* and *7a*). Radial growth (8.5 cm) was significantly ($P \leq 0.05$) higher for PDA amended with 1 or 2% $CaCl_2$ and by 3 and 4% after six days of incubation. The radial growth of the fungus in control plates was 7.6 cm, which was significantly lower than $CaCl_2$ amended plates at any concentration. The results of this study showed that not only was fungal growth uninhibited by $CaCl_2$ in the growth media, but concentrations of $CaCl_2$ up to 3% in the media actually proved to be somewhat stimulatory to the growth of the test fungus.

Although the mycelial growth of *C. gloeosporioides* was not inhibited by $CaCl_2$, spore germination was markedly reduced at higher concentrations (*Figs. 6, 7b* and *7c*). Spore germination (26%) was significantly higher ($P \leq 0.05$) when PDA was amended with 3 and 4% of $CaCl_2$ (*Fig. 7c*) followed by 2% with 6.3% inhibition. The lowest concentration of $CaCl_2$ (1%) did not inhibit the spore germination (*Fig. 7b*). However, spore germination in control and 1% $CaCl_2$ plates was 94 and 95%, respectively.

Calcium is an essential plant mineral, which plays major roles in plant functions. Postharvest treatment of fruits with low concentration of calcium salt has been found to reduce physiological disorders, mold growth and delay senescence (Conway, 1982; Poovaiah, 1986). In the present study, although $CaCl_2$ did not reduce mycelial growth of *C. gloeosporioides,* reduced germination of spores exposed to $CaCl_2$ has been observed. These findings are in agreement with Conway and Sams (1984) who reported that calcium ions *in vitro* did not reduce the fungal growth of *Penicillium expansum*, and there was also no growth reduction when the fungus was grown on juice extracted from calcium treated fruits. In the same work, the authors also found that calcium had a stimulatory effect on the growth of *P. expansum.* In another study, Biggs *et al.* (1997) reported that $CaCl_2$ did not inhibit the growth of *Monilinia fructicola* on PDA as strongly as some other salts. Nevertheless calcium salts have been shown to reduce mycelial growth of *C. gloeosporioides and C. acutatum,* which are causal agents of bitter rot of apple and *Leucostoma persoonii in vitro* (Biggs *et al.*, 1994;

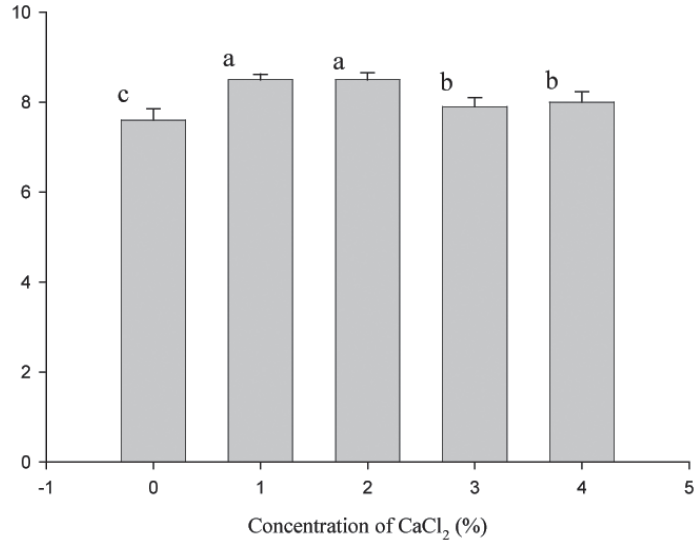

Fig. 5: Effect of different concentrations of calcium chloride on the radial growth of C. gloeosporioides *on PDA after six days of incubation at 28±2 °C. Means (Bars) followed by the same letter (s) are not significantly different according to Tukey's Studentized Range (HSD) Test at P≤0.05. Vertical bars represent the standard error of the means*

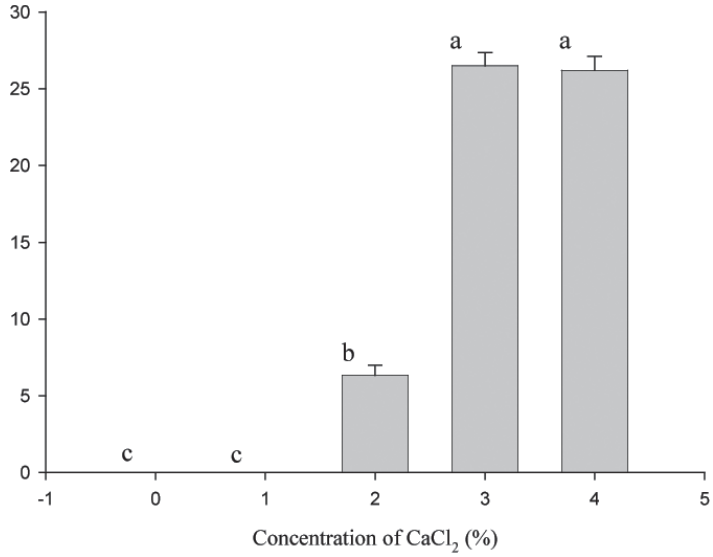

Fig. 6: Effect of different concentrations of calcium chloride on the spore germination of C. gloeosporioides *on PDA after 7 h of incubation at 28±2°C. Means (Bars) followed by the same letter are not significantly different according to Tukey's Studentized Range (HSD) Test at P≤0.05 on the arcsine transformed values. Vertical bars represent the standard error of the means*

Biggs, 1999). In this study, *in vitro* growth stimulation of C. *gloeosporioides* was significantly ($P \leq 0.05$) lower at $CaCl_2$ concentrations of 3 and 4% compared to 1 and 2%. Moreover, significant reduction of spore germination (26%) was found at 3 and 4% $CaCl_2$. This might be due to the toxicity of higher concentrations of calcium to *C. gloeosporioides* by affecting the osmotic balance in the fungal cells and inhibition of pectinolytic enzymes (Arras *et al.*, 1998). These results are

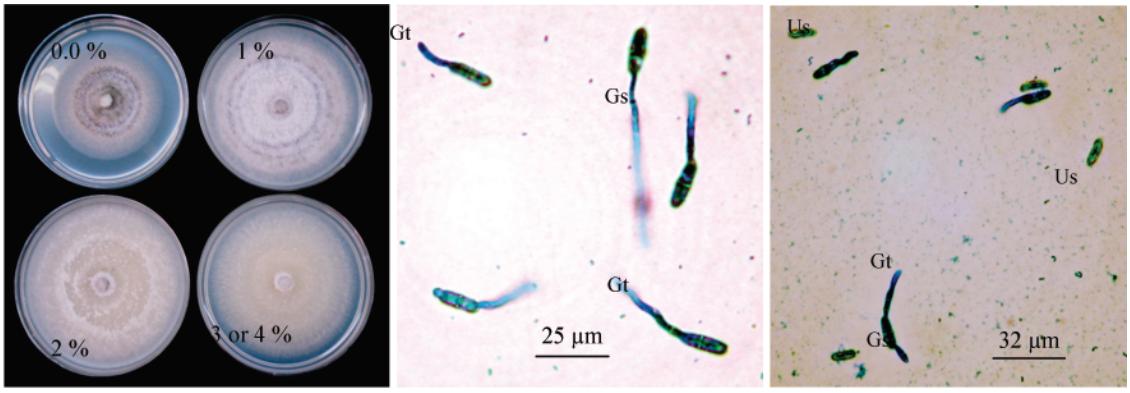

Fig. 7: Effect of different concentrations of calcium chloride on the radial growth and spore germination of C. gloeosporioides *after six days and 7 h of incubation, respectively at 28±2°C. (**a**) = radial growth of fungus on PDA amended with different concentrations of $CaCl_2$; (**b**) = germinated spores with 0.0, 1, or 2% $CaCl_2$; (**c**) = inhibition of spore germination with 3 or 4% $CaCl_2$. Gs = Germinated spore; Us = Ungerminated spore; Gt = Germ tube*

corroborated with the result reported by Narayanasamy (2006), who found that the addition of 2% calcium chloride reduced the spore germination and growth of germ tubes of *Rhizopus stolonifer in vitro*. Reduction of spore germination of *Penicillium digitatum* exposed to calcium chloride has also been observed (Droby *et al.*, 1997), as well as reduced germination and germ tube growth of *P. expansum* and *Botrytis cinerea* (Wisniewski *et al.*, 1995).

CONCLUSIONS

The findings of this *in vitro* study demonstrate that chitosan has significant effects on the growth, spore germination and hyphal morphology of *C. gloeosporioides* from papaya. On the contrary, $CaCl_2$ was not inhibitory but rather stimulates (to a small extend) radial growth of the fungus. However, $CaCl_2$ was ineffective in controlling anthracnose of papaya cv. Sekaki in particular and other benefits of $CaCl_2$ can still be exploited. It is hoped that various combinations of both could be used in postharvest treatments as an alternative to synthetic fungicide in preventing the occurrence of anthracnose in papaya caused by *C. gloeosporioides*.

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Determination of Soil Nutrient Levels for Maximum Yield of Okra (*Abelmoschus esculentum*) Using Sole and Amended Plant Residues

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ABSTRACT

The determination of critical or optimum level of nutrients for a particular crop (i.e. soil calibration) is very important. An investigation was carried out in Akure rainforest zone of Southwest Nigeria to determine the critical soil nutrient levels for pH, organic matter (OM), N, P, K, Ca and Mg for okra crop using sole plant residues, wood ash, cocoa husk, spent grain, rice bran and saw dust amended with pig, goat and poultry manure. Twenty organic fertilizer treatments were compared to control (no treatment) and a reference treatment 400kg/ha NPK 15-15-15 fertilizer in four experiments. Application of sole and amended plant residues at 2,4,6,8 and 10 t ha^{-1} to soil increased soil N, P, K, Ca, Mg, Na, pH and OM, growth and pod weight of okra relative to the control. The wood ash, spent grain and cocoa husk were the most effective in improving soil nutrient contents for okra crop. The NPK fertilizer decreased soil OM, pH, Ca and Mg. The pod weight increased up till 6 t/ha under the different plant residues with R^2 value of 0.907 and decreased at 8 and 10 t ha^{-1}. Spent grain + poultry manure treatment gave the best performance in increasing all soil properties as well as yield and leaf area. For instance spent grain + poultry manure treatment had the highest okra yield (4555.5 kg ha^{-1}) with critical or optimum nutrient combination 5.2% N, 51.03 mg kg^{-1} P, 0.78 mmol kg^{-1} soil K, 0.43 mmol kg^{-1} soil Ca, 0.0 mmol kg^{-1} soil Mg, 2.96 mmol kg^{-1} soil Na, 3.2% soil OM and 6.9 for soil pH respectively.

Keywords: Soil calibration, level of nutrients, *Abelmoschus esculentum*

INTRODUCTION

The efficacy of soil testing as a means for predicting the nutrient needs of crops to be grown has been established. Olson *et al.* (1982) reported that three major concepts were used by various organizations in U.S.A. and Britain doing soil testing programmes and they are, sufficiency level arising from soil calibration studies (soil critical level), maintenance concept and cation ratio concept.

However, soil calibration test refers to the determination of a relationship between laboratory soil test values and yield responses to fertilizer application and indicates at which soil test values responses are expected. Different scientists have worked on the determination of critical P levels in Southwest Nigeria. Agboola and Corey (1973) worked on the determination of critical P level (10 mg kg^{-1}P) for maize using Bray P_1 method, while Okeya (1977) obtained levels of 17 and 12 ug ml^{-1} for Bray P_1 and Olsen modified methods from green house calibration studies. Adeoye (1980) established critical range of 10-16 ppm for P, 2.0-2.6 meq/100g for Ca, 0.35 meq/100g for K, 3.0 mg kg^{-1} Zn and 12.0 mg kg^{-1} for Cu for maize on sedimentary soils of Southwest Nigeria.

The determination of critical or optimum levels is very useful and it is defined as the soil test value about which response is not expected and below which a large yield response can be obtained with adequate supply of a particular nutrient.

Folorunso *et al.* (1995) reported that using fractional recovery modified $NaHCO_3$ multi-purpose extractant, a critical level of 0.20 mmol kg^{-1} K was established for south west Nigeria using muriate of potash fertilizer. It is interesting to note that the determination of soil critical or

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nutrient levels for crop responses has been based on inorganic fertilizer elements from Single Superphosphate, Urea and $ZnSO_4$ for N, P, K and Zn nutrients respectively.

Therefore, the high cost of fertilizer purchases, scarcity of fertilizer to farmers and the degradation of soils on continuous use of inorganic fertilizers had necessitated the need to use cheap available organic residues by farmers; hence, this has created the need to establish critical levels for soil nutrients and crop responses for different organic residues. However, research information on the soil critical levels for N, P, K, Ca and Mg for okra crop using different plant residues is lacking.

Waugh *et al.* (1973) reported the use of multiple regression equations as a tool for establishing meaningful soil nutrient availability, while Monsoon and Nelson (1973) also reported that multiple regression analysis could be used to relate crop yields and soil test values.

The objective of this paper is to report soil critical levels for pH, OM, N, P, K, Ca, Mg Na for okra crop (*Abelmoschus esculentum* L) using various sole plant residues or amended with manures on Alfisols soil in Southwest Nigeria.

MATERIALS AND METHODS

Four field experiments were conducted for okra between 1998 and 1999 respectively on the same site in Akure (7° N 5 10 E) in the rainforest zone of South west Nigeria. The site had been continuously cropped for ten years.

The soil is sandy loam texture and belongs to Akure series, (Iwo Association) and is classified as an Alfisol (Oxic tropudalf), (Harpstead, 1972). The underlying geology is basement complex (Durotoye, 1972).

The surface (0-15cm, depth) soil had a pH_{water} of 5.1, organic matter 0.53%, N 0.2%, 4.6 mg kg^{-1} extractable P, 0.08 mmol kg^{-1} exchangeable K, 0.11mmol kg^{-1} exchangeable Ca and 1.12 mmol kg^{-1} exchangeable Mg (Folorunso, 1999).

There were twenty plant residue treatments namely wood ash (sole), wood ash + goat dung, wood ash + pig dung, wood ash + poultry manure, cocoa husk (sole), cocoa husk + goat dung, cocoa husk + pig manure, cocoa husk + poultry manure, rice bran (sole), rice bran + goat dung, rice bran + pig dung, rice bran + poultry manure, spent grain (sole), spent grain + goat dung, spent grain + pig dung, spent grain + poultry manure, saw dust (sole) sawdust + goat dung, sawdust + pig dung, sawdust + poultry manure, plus 400 kg ha^{-1} NPK 15-15-15 fertilizer as reference treatment and control (no treatment) applied to the soil. The rates of plant residues (wood ash, spent grain, sawdust, rice bran and cocoa husk) applied were at 2,4,6,8 and 10 t ha^{-1} as sole. Each rate of plant residues was amended with goat, pig and poultry manures at the rate of 1,2,3,4 and 5 t ha^{-1} (1.1 ratio plant residue: manure).

All treatments were replicated three times at the same site and arranged in a randomized complete block design. The size of each plot was 4 m x 4 m (16 m^2). The fertilizer and manure were incorporated into soil ten days before planting. Before planting, the residues and animal manures were processed to speed up decomposition in the soil and analysed for their chemical composition.

Two seeds of okra (NHAe 47-4 variety) were planted per stand at spacing of 60 x 30cm, germination took place five days after planting, and thinning to one plant per stand was done. The plots were hand weeded thrice starting from second, fifth and seventh weeks after planting. The insect pests were controlled by spraying Vetox 85 at a rate of 28g a.i in 9 L of water at second week after planting and this was repeated at the third and fourth weeks after planting.

The total plant population per 16 m^2 plot was eighty-eight (88) and 26 plants were randomly selected and tagged. At 2, 4 and 6 weeks after planting, measures of plant height (cm), leaf area (cm^2) and stem girth (cm) were carried out. Harvest of mature pods stated 40 days after planting. The fresh pod weight (yield) was recorded per treatment plot and the harvest continued at four days interval till senescence period. The total harvest of the fresh pod weight per 16m^2 plot was recorded in kg ha^{-1} for the different treatments.

At the end of each field experiment, composite soil samples were collected to 15cm depth in each plot. The soil samples were air dried and passed through 2mm sieve for chemical analysis.

The soils were extracted by a multi-purpose extractant modified $NaHCo_3$ pH 8.5 for P, K, Ca and Mg. The P in solution was determine using Murphy and Riley (1962) blue the exchangeable bases (K, Ca and Na), and Mg in solution were

determined using flame photometer and atomic absorption spectrometer respectively (Jackson, 1964).

The okra pod weights data recorded for the three replicates were averaged for the 2, 4, 6, 8 and 10 t ha^{-1} of sole and amended plant residues. The same procedure was applied for plant height, leaf area and stem girth. The data were subjected to ANOVA F-test and their means were separated and compared using Duncan Multiple Range Test (DMRT) at 5% level. The soil critical level determination was done using multiple regression and linear correlation between the okra pod and soil test values of N, K, Ca, Mg, soil pH and OM under different rates of plant residues added (2, 4, 6,8 and 10 t ha^{-1}).

RESULTS AND DISCUSSION

The plant residues (sole and amended) and NPK fertilizer increased okra pod weight (Table 1), leaf area (Table 2), plant height (Table 3), stem girth (Table 4), soil N (Table 5), soil P (Table 6), soil K (Table 7) and soil Ca (Table 8) significantly ($P<0.05$) relative to control.

The amendment of cocoa husk, rice bran, spent grain, wood ash and saw dust with goat, pig and poultry manures increased soil Mg (Table 9), soil Na (Table 10) and soil pH (Table 11) compared to NPK fertilizer. However, NPK fertilizer resulted in better plant height, leaf area and stem girth than wood ash, saw dust, rice bran and cocoa husk amended with goat, pig and poultry manure except for the amended spent grain + poultry manure which resulted in the best values of okra pod weight followed by wood ash, cocoa husk, rice bran and saw dust amended with poultry manures respectively.

Okra pod weight increased with number or rate of application till 6 t ha^{-1} residue and decreased slightly at 8 and 10 t ha^{-1} residues. The plant residues had cumulative effect on soil OM, N, P, K, Ca and Mg which increased with manure addition from 2 to 10 t ha^{-1}. The mean pod weight of okra for soil treated with sole and amended plant residences at 2, 4, 6, 8 and 10 t ha^{-1} were 1597, 1950.55, 2493.58, 2308.31 and 2199.8 kg ha^{-1} respectively. The equivalent values for soil OM were 2.86, 3.0, 3.12, 3.16 and 3.21

TABLE 1
The effect of different levels of plant residues plus manure on the fresh pod yield (kg ha^{-1}) of Okra

Treatments	2	4	6	8	10 t ha^{-1}
Woodash	594.56d	901.25d	1450.75d	1300.75d	1262.75d
Woodash + Goat dung	1801.44 l	2101.75m	2500.80m	2325.50n	2275.00o
Woodash + Pig dung	1944.69m	2170.25o	2831.25o	2516.75p	2490.75p
Woodash + Poultry manure	2195.63r	2281.00q	3301.25q	2532.00r	2751.75r
Cocoa husk	666.56h	932.00f	1450.50d	1403.75e	1395.50f
Cocoa husk + Goat dung	1747.06j	2123.00n	2781.50n	2282.00m	2267.00n
Coca husk+ Pig dung	2004.44p	2185.75p	3202.00p	2829.75q	3152.25q
Cocoa husk + Poultry manure	2223.88s	2448.50r	3775.25s	3300.00t	3152.25s
Rice bran	644.56f	781.00b	1050.75b	1025.75b	1002.33b
Rice bran + Goat dung	1960.63k	1978.00 l	2110.75h	2026.51j	2000.75
Rice bran + Pig dung	1972.81n	1995.25k	2150.50 l	2126.00k	2026.0k
Rice bran + Poultry manure	1563.69o	1981.50j	21.87.75j	2196.25 l	2070.25m
Spent grain	1923.75i	2450.25s	2475.25 l	2400.75o	2050.75 l
Spent grain + Goat dung	2481.38t	3001.75t	3502.00r	3275.00s	3201.25t
Spent grain + Pig dung	2532.69u	3750.75u	4253.00t	4051.75u	3575.25u
Spent grain + Poultry manure	3007.19v	4251.00v	4551.50u	4313.25v	4038.25v
Saw dust	624.38b	853.75c	1206.50c	1131.00o	1078.25c
Saw dust + Goat dung	613.44c	906.75e	1472.75e	1427.00 l	1325.25e
Saw dust + Pig dung	704.25e	939.50g	1676.25f	1625.75g	1552.00g
Saw dust + Poultry manure	731.75g	978.00h	1950.25g	1777.25h	1750.75h
Control (no fertilizer)	47.00a	47.00a	46.75a	46.75a	46.75a
NPK 15-15-15	2013.00q	2062.75 l	2420.25k	1962.75 l	1920.00 l

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 2
The effect of plant residues plus manure on leaf area (cm^2) of Okra

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	60.45e	67.44c	91.66de	122.76gh	130.5 i
Woodash + Goat dung	79.33ij	70.84cd	102.22f	137.33 l	140.1h
Woodash + Pig dung	80.07j	84.34g	117.08j	137.04 l	140.9h
Woodash + Poultry manure	101.25m	90.46h	144.20m	139.76j	141.5h
Cocoa husk (sole)	61.99ef	83.50fg	101.70f	120.34g	123.3g
Cocoa husk + Goat dung	76.19i	102.74i	129.26k	137.89 l	140.5h
Coca husk+ Pig dung	94.96 l	128.17k	144.67m	149.89k	150.3j
Cocoa husk + Poultry manure	120.73o	178.38m	186.14o	161.23m	176.1k
Rice bran (sole)	54.58cd	73.45c	86.24cd	92.79c	94.1c
Rice bran + Goat dung	64.44fgh	80.30f	104.37fg	110.23e	117.2f
Rice bran + Pig dung	75.61 l	83.97fg	106.20gh	115.16f	119.16f
Rice bran + Poultry manure	87.62k	87.59gh	110.85hi	119.12fg	122.12g
Spent grain (sole)	115.46n	121.39j	130.11kl	147.77 l	151.77j
Spent grain + Goat dung	125.45pq	165.97 l	172.38n	180.75n	185.75 l
Spent grain + Pig dung	127.35qr	178.50m	193.89p	179.52no	184.52m
Spent grain + Poultry manure	131.90rs	185.55n	198.01qr	189.68o	192.10n
Saw dust (sole)	27.27b	37.95b	49.36b	73.04b	75.9b
Saw dust + Goat dung	53.39c	75.35de	82.67c	93.12c	97.12d
Saw dust + Pig dung	59.78e	82.28fg	87.75d	99.42d	19.42e
Saw dust + Poultry manure	63.22fg	88.71gh	92.29e	110.73e	126.37h
Control (no fertilizer)	8.27a	9.71a	9.78a	9.53a	9.32a
NPK 15-15-15	121.96op	167.20 l	196.48pq	146.99 l	158.16r

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 3
The effect of different levels of plant residues plus manure on the plant height (cm) of Okra

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	23.09f	34.73hij	36.16gh	39.90ij	40.5hi
Woodash + Goat dung	30.25n	40.04n	43.09 l	47.60 l	48.2 l
Woodash + Pig dung	32.02o	41.13o	45.24lm	48.09lm	49.5m
Woodash + Poultry manure	35.29r	43.01p	48.08no	49.14mn	51.14n
Cocoa husk (sole)	20.56d	29.45e	30.48d	32.48d	34.2d
Cocoa husk + Goat dung	27.73j	32.10g	35.32fg	36.12fg	38.1f
Cocoa husk+ Pig dung	28.68m	34.05h	37.80i	38.52hi	36.8e
Cocoa husk + Poultry manure	32.56op	38.81m	39.22jk	40.65jk	39.8g
Rice bran (sole)	16.14b	22.45b	24.38b	25.41b	27.1b
Rice bran + Goat dung	22.82e	30.24f	35.16fg	37.10g	39.1g
Rice bran + Pig dung	24.99hi	34.49hi	37.28hi	38.42hi	42.4j
Rice bran + Poultry manure	26.64k	37.01kl	38.77ij	38.99l	45.2k
Spent grain (sole)	27.53l	36.08k	38.68ij	41.62j	43.6j
Spent grain + Goat dung	32.88pq	43.33pq	46.05mn	49.10mn	52.1o
Spent grain + Pig dung	35.84rs	45.81r	48.23no	51.03o	56.0p
Spent grain + Poultry manure	38.32t	47.01s	52.56p	54.58p	60.6q
Saw dust (sole)	18.78c	24.97c	26.37c	27.90c	29.9c
Saw dust + Goat dung	22.90e	27.32d	30.75de	34.75e	36.9e
Saw dust + Pig dung	23.57fg	28.36de	34.84f	35.20ef	38.2f
Saw dust + Poultry manure	24.77h	37.26kl	37.60l	37.92gh	40.6hi
Control (no fertilizer)	11.22a	9.11a	9.21a	9.20a	9.69a
NPK 15-15-15	43.83u	65.75t	67.33q	66.67q	70.1r

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 4
The effect of different levels of plant residues plus manure on the stem girth (cm) of Okra

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	1.63e	2.61e	5.32i	4.26f	4.5e
Woodash + Goat dung	2.20h	3.51i	6.35n	5.08hi	5.2gh
Woodash + Pig dung	2.40i	3.73j	5.47jk	4.47g	4.6ef
Woodash + Poultry manure	3.13m	3.85k	6.48o	6.48no	6.6m
Cocoa husk (sole)	1.37c	2.19c	4.55f	4.06d	4.2d
Cocoa husk + Goat dung	2.23h	3.44h	5.13h	5.19I	5.3 l
Cocoa husk+ Pig dung	2.67j	4.18 l	5.44j	5.44j	5.6k
Cocoa husk + Poultry manure	3.28n	5.20o	6.30n	6.30n	6.6m
Rice bran (sole)	1.34c	2.12bc	3.28c	3.42c	3.6c
Rice bran + Goat dung	1.53cd	2.43d	4.06d	4.16e	4.4e
Rice bran + Pig dung	1.76ef	2.83f	4.31e	4.31f	4.6ef
Rice bran + Poultry manure	2.17k	3.51	5.04gh	5.04h	5.4ij
Spent grain (sole)	1.67e	2.64ek	4.28de	4.28f	4.7ef
Spent grain + Goat dung	2.84kl	4.56m	6.14 l	6.14 l	6.3 l
Spent grain + Pig dung	3.11m	5.04n	6.24m	6.24m	6.7m
Spent grain + Poultry manure	3.44o	5.51p	6.54p	6.54o	6.8m
Saw dust (sole)	1.14b	2.06b	2.90b	2.90b	3.2b
Saw dust + Goat dung	1.58d	2.56de	4.28e	4.28f	4.6ef
Saw dust + Pig dung	1.80f	2.82f	4.92g	4.92h	5.0g
Saw dust + Poultry manure	1.96g	3.06g	5.08gh	5.07hi	5.1g
Control (no fertilizer)	0.51a	0.51a	0.52a	0.52a	0.52a
NPK 15-15-15	2.78k	4.53m	5.51k	5.57k	5.6k

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 5
The effect of different levels of plant residues plus manure on the soil Nitrogen (%) of Okra plot

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	0.136c	0.157bc	0.242c	0.367e	0.420g
Woodash + Goat dung	0.236e	0.252ef	0.315e	0.420g	0.446hi
Woodash + Pig dung	0.252ef	0.275f	0.378gh	0.430h	0.489j
Woodash + Poultry manure	0.315gh	0.331h	0.436i	0.483k	0.520k
Cocoa husk (sole)	0.131c	0.168c	0.26cd	0.3I5d	0.331cd
Cocoa husk + Goat dung	0.247ef	0.273f	0.373gh	0.420g	0.430h
Cocoa husk+ Pig dung	0.273f	0.315f	0.394h	0.446hi	0.451i
Cocoa husk + Poultry manure	0.331h	0.367j	0.451j	0470ij	0.509jk
Rice bran (sole)	0.121b	0.163c	0.210bc	0.260c	0.315c
Rice bran + Goat dung	0.189cd	0.220d	0.325ef	0.367ef	0.393e
Rice bran + Pig dung	0.193d	0.242e	0.341f	0.393f	0.399e
Rice bran + Poultry manure	0.294g	0.304fg	0.357g	0.399fg	0.409f
Spent grain (sole)	0.273f	0.273f	0.289d	0.315d	0.341d
Spent grain + Goat dung	0.304gh	0.336h	0.378gh	0.425gh	0.446hi
Spent grain + Pig dung	0.325h	0.357i	0.390h	0.451i	0.483i
Spent grain + Poultry manure	0.378 l	0.430j	0.450j	0.483k	0.499j
Saw dust (sole)	0.199d	0.147b	0.189b	0.215b	0.268b
Saw dust + Goat dung	0.199d	0.220d	0.267c	0.320d	0.341d
Saw dust + Pig dung	0.215de	0.252ef	0.289d	0.341de	0.357d
Saw dust + Poultry manure	0.273f	0.325h	0.346f	0.373ef	0.394e
Control (no fertilizer)	0.017a	0.017a	0.017a	0.017a	0.017a
NPK 15-15-15	0.26ef	0.282f	0.420k	0.478j	0.499j

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 6
The effect of different levels of plant residues plus manure on soil available P (mg kg^{-1}) of Okra plot

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	29.25c	28.13b	38.25cd	47.25g	50.07ef
Woodash + Goat dung	38.25i	41.07i	47.82 l	52.50h	43.88cd
Woodash + Pig dung	46.13k	51.75m	61.32k	63.00j	66.38gh
Woodash + Poultry manure	63.00o	72.00p	79.32o	83.25m	85.50i
Cocoa husk (sole)	35.45h	38.82h	41.63g	47.75g	44.45de
Cocoa husk + Goat dung	47.25 l	50.63 l	60.20k	61.32j	61.88g
Cocoa husk+ Pig dung	50.09m	56.82n	65.25m	66.38k	69.75h
Cocoa husk + Poultry manure	57.38n	69.20o	77.82n	80.45 l	87.75i
Rice bran (sole)	28.13b	30.08d	35.45c	36.57bc	38.25bc
Rice bran + Goat dung	29.82c	32.63f	38.25d	41.63de	44.45de
Rice bran + Pig dung	31.63e	34.32g	40.50f	43.88f	47.25ef
Rice bran + Poultry manure	34.34g	37.70h	43.88h	47.82g	51.20f
Spent grain (sole)	32.63f	32.34e	40.50f	43.88f	47.25ef
Spent grain + Goat dung	34.34g	38.25h	51.20j	57.38i	63.57g
Spent grain + Pig dung	38.05i	44.45j	64.13i	66.38k	70.88h
Spent grain + Poultry manure	39.95j	46.13k	86.63p	88.88n	93.95j
Saw dust (sole)	28.13b	29.25c	34.32b	35.45b	36.57b
Saw dust + Goat dung	29.82c	3.95de	35.45c	38.25c	40.50bcd
Saw dust + Pig dung	30.38d	30.38d	38.82d	41.07de	43.88cde
Saw dust + Poultry manure	32.53f	34.32g	39.95e	40.32d	45.57def
Control (no fertilizer)	2.10a	2.13a	2.12a	2.18a	2.16a
NPK 15-15-15	130.0p	141.50	146.50q	151.50o	101.82k

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 7
The effect of different levels of plant residues plus manure on the soil K (mmol kg^{-1}) of Okra plot

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	1.064m	0.80n	1.001m	0.920 l	0.928jk
Woodash + Goat dung	0.80k	0.88o	1.120m	1.04m	1.08k
Woodash + Pig dung	0.960 l	1.040q	1.200n	1.16n	1.160 l
Woodash + Poultry manure	1.120n	1.160r	0.84k	1.20n	1.24m
Cocoa husk (sole)	0.72j	0.761m	1.00m	0.88kl	0.92j
Cocoa husk + Goat dung	0.88kl	0.96p	0.975 l	1.04m	1.08k
Cocoa husk+ Pig dung	1.040m	1.120r	1.240p	0.64 l	1.320n
Cocoa husk + Poultry manure	1.120n	1.240s	1.320q	1.32q	1.320n
Rice bran (sole)	0.192c	0.24dc	0.28d	0.32d	0.32c
Rice bran + Goat dung	0.288ef	0.32f	0.360ef	0.72f	0.38e
Rice bran + Pig dung	0.332f	0.34g	0.368ef	0.38fg	0.384ef
Rice bran + Poultry manure	0.34g	0.372h	0.40f	0.44g	0.48f
Spent grain (sole)	0.360h	0.52i	0.50g	0.56h	0.64g
Spent grain + Goat dung	0.360h	0.60j	0.64h	0.72j	0.76h
Spent grain + Pig dung	0.64 l	0.64k	0.72i	0.80k	0.82i
Spent grain + Poultry manure	0.72j	0.74 l	0.760j	0.84k	0.82i
Saw dust (sole)	0.120b	0.132b	0.140b	0.168b	0.20b
Saw dust + Goat dung	0.20cd	0.22c	0.248c	0.28c	0.32c
Saw dust + Pig dung	0.24d	0.28d	0.30e	0.32d	0.36d
Saw dust + Poultry manure	0.260e	0.30e	0.32df	0.34e	0.38e
Control (no fertilizer)	0.013a	0.013a	0.013a	0.012a	0.012a
NPK 15-15-15	1.132a	1.146t	1.320n	1.21o	1.24m

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 8
The effect of different levels of plant residues plus manure on the soil Ca (mmol kg^{-1}) of Okra plot

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	0.246g	0.30g	0.45j	0.54h	0.54h
Woodash + Goat dung	0.30j	0.45j	0.54k	0.69j	0.75j
Woodash + Pig dung	0.39jk	0.54kl	0.60 l	0.75k	0.790 l
Woodash + Poultry manure	0.45k	0.60 l	0.69m	0.78 l	0.780k
Cocoa husk (sole)	0.45k	0.54k	0.60 l	0.63 I	0.75j
Cocoa husk + Goat dung	0.57 l	0.75m	0.78mn	0.81j	0.84m
Cocoa husk+ Pig dung	0.60 l	0.84n	0.84n	0.87k	0.87mn
Cocoa husk + Poultry manure	0.69m	0.87o	0.90o	0.98m	0.90o
Rice bran (sole)	0.075c	0.09c	0.120c	0.150c	0.150b
Rice bran + Goat dung	0.09d	0.150d	0.195d	0.210d	0.213d
Rice bran + Pig dung	0.150e	0.195ef	0.210e	0.24e	0.243e
Rice bran + Poultry manure	0.150e	0.21f	0.225f	0.255f	0.258f
Spent grain (sole)	0.180f	0.180de	0.195d	0.210d	0.216d
Spent grain + Goat dung	0.270h	0.30g	0.36h	0.45g	0.456g
Spent grain + Pig dung	0.285i	0.39h	0.42i	0.54h	0.56h
Spent grain + Poultry manure	0.30j	0.410i	0.54k	0.63 l	0.69 l
Saw dust (sole)	0.06b	0.09c	0.150d	0.156c	0.168bc
Saw dust + Goat dung	0.15e	0.18de	0.210e	0.216d	0.225e
Saw dust + Pig dung	0.15e	0.190e	0.225fd	0.24e	0.24ef
Saw dust + Poultry manure	0.180f	0.210h	0.240g	0.25f	0.267f
Control (no fertilizer)	0.01a	0.010a	0.010a	0.01a	0.01a
NPK 15-15-15	0.020a	0.024b	0.028b	0.020ab	0.20ab

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

(Table 15). The values of soil N were 0.24, 0.27, 0.32, 0.38 and 0.41. Soil P values were 37.83, 41.46, 51.03, 54.11 and 56.55 mg kg^{-1} . Exchangeable K values were 0.58, 0.63, 0.68, 0.70 and 0.75 mmo1 kg-1 respectively.

These results implied that okra pod weight responded positively to soil fertility and there were positive and significant ($P<0.05$) relationships between okra pod weight and N, P, K, Ca, Mg, Na and OM (Table 12).

Table 13 presents multiple regression analysis showing the relationship between okra pod weight and soil K, Ca, Mg, Na, N, P and OM. The regression coefficients of determination (R^2) values were significant ($P<0.05$) and high for the okra pod yield and soil properties for different application rates of plant residues.

For instance, R^2 values at 2, 4, 6, 8 and 10 t ha^{-1} plant residue were 0.828, 0.83, 0.907, 0.804 and 0.80 respectively. This implied that soil nutrient Na, K, Ca, Mg, OM., N, P, and pH accounted for 82.8, 83, 90.7, 80.4 and 80% yield variation in okra for different plant residues respectively.

Spent grain + poultry manure generally produced the best performance in increasing all soil properties as well as yield and leaf area. For-instance, to obtain highest okra yield (4555.5 kg ha^{-1}) for spent grain + poultry manure using the regression equation (Table 13), the following gave the best nutrient combination: 5.2% N, 51.03 mg/kg P, 0.78 mmol kg-1 soil K, 0.43 mmol kg^{-1} soil Ca, 0.0 soil Mg, 2.96 mmol kg^{-1} soil Na, 3.2% soil OM and 6.9 for soil pH respectively.

The increases in growth, pod weight of okra and soil nutrients content due to application of sole and manure amended plant residues is consistent with the initial soil organic matter content which was much lower than the critical values of 2% (Agboola and Corey, 1973) recommended for soils in Southwest Nigeria. The soil was quite acidic for good performance of okra (Aduayi, 1980). The application of wood ash, saw dust, spent grain, rice bran and cocoa husk increased soil K, Ca, Mg, Ca, OM, N, P, and pH which is consistent with the fact that the organic materials are sources of all plant nutrients

TABLE 9
The effect of different levels of plant residues plus manure on the soil Mg (mmol kg^{-1}) of Okra plot

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	0.28c	0.30bc	0.325c	0.362c	0.40c
Woodash + Goat dung	0.55a	0.58d	0.62de	0.63e	0.662ef
Woodash + Pig dung	0.615f	0.66e	0.70ef	0.718f	0.718f
Woodash + Poultry manure	0.675fg	0.70ef	0.775f	0.812gh	0.812g
Cocoa husk (sole)	0.287c	0.32bc	0.33c	0.362c	0.40c
Cocoa husk + Goat dung	0.32cd	0.38bc	0.40c	0.437cd	0.445c
Cocoa husk+ Pig dung	0.362d	0.385bc	0.41c	0.418cd	0.441c
Cocoa husk + Poultry manure	0.40de	0.411bc	0.437c	0.47d	0.51cd
Rice bran (sole)	0.676fg	0.70ef	0.762f	0.66ef	0.737f
Rice bran + Goat dung	0.587e	0.60e	0.662e	0.70f	0.775fg
Rice bran + Pig dung	0.625f	0.62e	0.775f	0.775fg	0.775fg
Rice bran + Poultry manure	0.55e	0.587d	0.68e	0.775fg	0.825g
Spent grain (sole)	0.587e	0.52d	0.605de	0.625e	0.651ef
Spent grain + Goat dung	0.70g	0.80f	0.70ef	0.662ef	0.737f
Spent grain + Pig dung	0.775h	0.81f	0.85g	0.85g	0.887gh
Spent grain + Poultry manure	0.675fg	0.77ef	0.812g	0.8l2gh	0.812g
Saw dust (sole)	0.475de	0.51bc	0.55d	0.65e	0.587d
Saw dust + Goat dung	0.51de	0.54bc	0.587d	0.587de	0.625e
Saw dust + Pig dung	0.55e	0.587d	0.617de	0.62e	0.636e
Saw dust + Poultry manure	0.587e	0.60d	0.625de	0.625e	0.660ef
Control (no fertilizer)	0.0023a	0.023a	0.023	0.023	0.023a
NPK 15-15-15	0.0625b	0.066b	0.070b	0.071b	0.0775b

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 10
The effect of different levels of plant residues plus manure on the soil Na (mmol kg^{-1}) of Okra plot

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	0.293b	0.309b	0.37b	0.43b	0.52c
Woodash + Goat dung	0.67ef	0.77fg	0.80ef	0.806ef	0.88de
Woodash + Pig dung	0.813ij	0.88ij	0.86fg	0.87fgh	0.78de
Woodash + Poultry manure	0.813ij	0.78fgh	1.03hi	1.07kl	1.09gh
Cocoa husk (sole)	0.28b	0.37c	0.376b	0.43b	0.35b
Cocoa husk + Goat dung	0.43c	0.43d	0.45c	0.49b	0.52c
Cocoa husk+ Pig dung	0.43c	0.43d	0.50cd	0.47b	0.53c
Cocoa husk + Poultry manure	0.450c	0.46d	0.53d	0.60c	0.65c
Rice bran (sole)	0.86kj	0.92j	0.89fg	0.95hij	0.99efg
Rice bran + Goat dung	0.763hi	0.80gh	0.88fg	0.86fgh	1.05fgh
Rice bran + Pig dung	0.793i	0.82hi	1.01hi	1.03jkl	1.05fgh
Rice bran + Poultry manure	0.68ef	0.77fg	0.97h	1.05kl	1.11gh
Spent grain (sole)	0.73gh	0.79fgh	0.82efg	0.823ef	0.88d
Spent grain + Goat dung	0.906k	0.88ij	0.86fg	0.84fg	0.88efg
Spent grain + Pig dung	1.013 l	1.09 l	1.06i	1.10m	1.16h
Spent grain + Poultry manure	0.860jk	1.013k	1.07i	0.99ijk	1.05fgh
Saw dust (sole)	0.60d	1.61e	1.75e	0.71d	0.82d
Saw dust + Goat dung	0.66e	0.62e	0.77e	0.73def	0.86de
Saw dust + Pig dung	0.71fg	0.75f	0.77e	0.823ef	0.86de
Saw dust + Poultry manure	0.77hi	0.77fg	0.82efg	0.80ef	0.92def
Control (no fertilizer)	0.018a	0.018a	0.020a	0.021a	0.020a
NPK 15-15-15	0.788i	0.88ij	0.86fg	0.92ghi	1.07gh

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 11
The effect of different levels of plant residues plus manure on the soil pH_{water} of Okra plot

Treatments	2	4	6	8	10 t ha^{-1}
Woodash (sole)	7.10h	7.10 l	7.40 l	7.30k	7.20h
Woodash + Goat dung	7.20 l	7.30k	7.50m	7.40 l	7.30 l
Woodash + Pig dung	7.30j	7.40 l	7.50m	7.20j	7.40j
Woodash + Poultry manure	7.40k	7.50m	7.10 l	7.30k	7.60 l
Cocoa husk (sole)	7.20i	7.20j	7.20j	7.20j	7.30 l
Cocoa husk + Goat dung	7.30j	7.30k	7.40 l	7.30k	7.40j
Cocoa husk+ Pig dung	7.10h	7.40 l	7.30k	7.40 l	7.50k
Cocoa husk + Poultry manure	7.40k	7.50m	7.50m	7.50m	7.60 l
Rice bran (sole)	5.80b	5.90b	5.90c	6.00c	5.80c
Rice bran + Goat dung	6.10c	6.10c	6.30d	6.30d	6.50e
Rice bran + Pig dung	6.30d	6.30e	6.30d	6.40e	6.50e
Rice bran + Poultry manure	6.40e	6.20d	6.50e	6.60g	6.60c
Spent grain (sole)	6.10c	6.10c	6.30d	6.50f	6.30d
Spent grain + Goat dung	S7.10h	7.10 l	7.10 l	7.20j	7.30 l
Spent grain + Pig dung	7.20i	7.30k	7.20j	7.40 l	7.50k
Spent grain + Poultry manure	7.50 l	7.50m	7.40 l	7.50m	7.60 l
Saw dust (sole)	6.10c	5.90b	5.80b	5.80b	5.880c
Saw dust + Goat dung	6.60f	6.40f	6.60f	6.60g	6.80f
Saw dust + Pig dung	6.70g	6.60g	6.80g	6.80h	7.10g
Saw dust + Poultry manure	6.70g	6.70h	6.90h	6.90 l	7.10g
Control (no fertilizer)	5.30a	5.30a	5.32a	5.29a	5.29b
NPK 15-15-15	5.35ab	5.32a	5.34a	5.30a	5.10a

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

TABLE 12
Correlation coefficients (r0 values between the yield parameters of okra and soil N, P, K, Ca, Mg, Na OM and pH at different levels of plant residues plus manures

Parameters	2	4	6	8	10 t ha^{-1}
Fresh pod yield vs soil OM.	0.473**	0.51**	0.55**	0.48**	0.48**
Fresh pod yield vs soil K	0.66**	0.543**	0.541**	0.52**	0.587**
Fresh pod yield vs soil Ca	0.47**	0.48**	0.48**	0.46**	0.472**
Fresh pod yield vs soil N	0.832**	0.875**	0.90**	0.856**	0.818**
Fresh pod yield vs soil P	0.433**	0.524**	0.64**	0.667**	0.825**
Fresh pod yield vs soil pH	0.408**	0.404**	0.46**	0.48**	0.51**
Fresh pod yield vs soil Na	0.216*	0.245*	0.26*	0.37*	0.38*
Fresh pod yield vs soil Mg	0.36*	0.54**	0.6**	0.63**	0.68**

** - Significant at 1% level.
* - Significant at 5% level.

(Table14) which NPK fertilizer can not supply (Swift and Anderson, 1993).

The poultry, pig and goat manures resulted in higher values of N and P nutrients and least C/N ratio of 6.93, 6.72 and 7.93 respectively compared to the agricultural by products (wood ash) spent grain, rice bran, saw dust and cocoa husk). The wood ash had the highest K, Ca and Mg followed by cocoa husk and spent grain while rice bran and Na with the highest C/N ratio of 23.3 and 19.05 respectively (Table 14). The increase in growth parameters, such as plant height, leaf area and stem girth by NPK fertilizer could be associated with quick release of the nutrients for assimilation (Ojeniyi, 1984).

TABLE 13
Standardized regression coefficients between soil properties and okra yield under different levels of plant residues

Levels of plant residues	Regression equation	R^2
	$Y = abo + b_1 x_1 + b_2 x_2 + b_3 x_3$ .....+ $b_8 x_8$	
t ha^{-1}		
2	$Y = 1206.383 + 0.612 x_1 + 0.447 x_2 + 1.174 x_3$ + 0.561 x_4 + 0.642 x5 - 0.555 x_6 + 0.750 x_7 + 0.528 x_8	0.828
4	$Y = 2111.963 + 0.240 x_1 + 1.239 x_2 + 0.408 x_3$ + 0.197 x_4 + 0.629 x_5 + 0.199 x_6 + 0.443 x_7 + 0.750 x_8	0.834
6	$Y = -2737.64 + 0.190 x_1 + 0.508 x_2 + 0.064 x_3$ + 0.292 x_4 + 0.422 x_5 + 0.170 x_6 + 0.393 x_7 + 0.708 x_8.	0.907
8	$Y = -1268.803 - 0.483 x_1 - 0.217 x_2 + 0.735 x_3 + 0.576 x_4$ + 0.771 x_5 + 0.128 x_6 + 0.206 x_7 + 0.792 x_8	0.804
10	$Y = -2998.745 + 0.086 x_1 + 1.695 x_2 + 0.691 x_3$ - 0.720 x_4 - 0.120 x_5 + 0.644 x_6 + 0.386 x_7 - 0.924 x_8	0.80

X_1 = Soil Na, X_2 = Soil K, X_3 = Soil Mg, X_4 = Soil Ca, X_5 = % OM, X_6 = Soil pH, X_7 = Soil P and X_8 = % Soil N.

TABLE 14
Chemical characteristics of organic fertilizers used for the field experiments

Organic materials	% C	%N	C/N ratio	Available P (mg kg^{-1})	Na	Ca	K	Mg	Fe	Mn	Cu	Zn
					%					mg kg^{-1}		
Poultry manure	30.0	4.33	6.93	385.0	5.65	3.20	9.72	4.1	37.85	1.66	0.15	1.26
Pig manure	25.0	3.72	6.72	312.0	5.22	3.10	14.45	4.8	34.0	1.62	0.17	1.34
Goat manure	20.0	2.52	7.93	167.5	6.30	2.90	9.97	4.5	34.5	1.60	0.16	1.30
Cocoa pod Husk	16.0	1.44	11.1	100.0	4.41	9.34	20.59	7.1	50.4	8.64	0.55	1.69
Wood ash	18.0	1.53	11.76	86.0	8.26	9.40	23.02	8.52	65.51	11.92	0.66	1.83
Spent grain (Brewery waste)	10.0	0.78	12.82	76.0	4.57	0.13	7.86	3.10	3.39	0.99	0.1	0.7
Rice bran	14.0	0.6	23.33	56.0	4.43	0.12	7.93	1.8	6.25	1.78	0.18	0.49
Saw dust	8.0	0.42	18.96	10.0	4.39	0.10	5.12	1.3	4.01	1.69	0.16	0.40

The continuous use of NPK fertilizer for okra decreased soil pH, OM., Ca, Mg and Na nutrients and this could be traced to the ammonium (NH_4^+) component which reduced soil uptake. Besides, the high P and K contents of NPK could lead to nutrient imbalance P/Mg, K/Mg, K/Ca and K/Na (Bear, 1950).

The effectiveness of amended plant residues with manures compared to the sole treatments could be adduced to their high N, P, K, Ca, Mg, Na contents and lower C/N ratio of the manure which would aid decomposition and release of nutrients.

However the inferior performance of rice bran and saw dust in improving the soil pH could be due to their high C/N ratio and consequent immobilization of soil nutrients especially cations. The best crop performance associated with the use of spent grain is also attributed to possible improvement in other attributes of soil, such as bulk density for all the plant residues (Folorunso, 1999).

TABLE 15
The effect of different amount of plant residues plus manure on soil organic matter (%) of okra plot

Treatments	2	4	6	8	10 t ha-1
Wood ash (sole)	2.80k	2.86k	2.90g	2.96h	3.00f
Wood ash + goat dung	3.130	3.24k	3.791	3.68jk	3.72i
Wood ash + pig dung	3.20p	3.75n	3.81lm	3.90l	3.85j
Wood ash + poultry dung	3.31q	3.82o	3.88n	3.911	3.99j
Cocoa husk (sole)	2.67h	2.73f	2.82f	2.98hi	2.94e
Cocoa husk + goat dung	3.38s	3.46l	3.64k	3.71k	3.75i
Cocoa husk + pig dung	3.32r	3.44kl	3.87m	3.93m	3.98j
Cocoa husk + poultry manure	3.59t	3.67m	3.801	3.97n	4.06k
Rice bran (sole)	2.24d	2.33d	2.35cd	2.40d	2.45cd
Rice bran + goat dung	2.63g	2.68ef	2.70de	2.76ef	2.80de
Rice bran + pig dung	2.70i	2.76f	2.87fg	2.90g	3.02f
Rice bran + poultry manure	2.73j	2.80fg	2.99h	3.04i	3.17g
Spent grain (sole)	2.36e	2.43de	2.60d	2.63e	2.72d
Spent grain + goat dung	2.971	3.00h	3.13j	3.20j	3.25h
Spent grain + pig dung	3.03m	3.10i	3.21j	3.15	3.18g
Spent grain + poultry manure	3.07n	3.20j	3.30jk	3.22j	3.29hi
Saw dust (sole)	2.19c	2.22c	2.29c	2.34c	2.40c
Saw dust + goat dung	2.53f	2.67e	2.70de	2.73ef	2.78d
Saw dust + pig dung	2.53f	2.66e	2.76e	2.80f	2.80de
Saw dust + poultry dung	2.70i	2.78f	2.99h	3.03i	2.97ef
Control (no fertilizer)	0.27a	0.28a	0.28a	0.27a	0.28a
NPK 15-15-15	0.43b	0.41b	0.40b	0.42b	0.39b

Treatment means within each group of column followed by the same letters are not significantly different from each other using DMRT at 5% level.

The responses of okra to the application of plant residues, which ranged from 2 t ha^{-1} to 6 t ha^{-1} could be attributed to the initial soil fertility. At 8 and 10 t ha^{-1} residues or residues plus manure application okra pod formation was retarded and flowering delayed. This is also consistent with the R^2 values in Table 13 where the highest R^2 values (0.907) were obtained at 6 t ha^{-1}. This implied that 90.7% of the okra pod weight variation was adduced to the soil nutrients and okra could respond positively to soil fertility. The above observation corroborates that of Rehim *et al.* (1981) who noted that the P, K, Ca, Mg, Na concentrations in crops decreased with the amount of applied organic fertilizers. The reduction was of plant nutrients dilution resulting from increased starch formation especially at 8 and 10 t ha^{-1} residues application. Above the 6 t ha^{-1} of treatment application and the associated soil nutrient levels of N, P, K, Ca, Mg, Na, soil OM and pH, the principle guiding the law of diminishing returns in fertilizer use was applied (Yayock, 1986). He reported that further addition to the soil above the soil critical level, would lead to little or no addition in crop yield and it should be discontinued.

The implication is that at 6 t ha^{-1} of residues or residue + manure application, the optimum yield of okra has been attained and soil nutrient status (N, P, K, Ca, Mg, Na, OM) had been built up to support the growth of the crop. Therefore by discontinuing application of these treatments to the soil, they will also release sufficient nutrients for crops such as yam, maize, cassava and others to be grown in rotation with okra in future (residual effect).

This is the philosophy of soil fertilization (i.e. adding fertilizers to soils before planting) that supports multiple cropping systems of peasant farmers in the tropics. The observation was supported by the work of Folorunso *et al.* (2000), which emphasized the use of three fertilizer models to evaluate the residual effects of P and K fertilizers for maize growth. They noted that soil exchange sites still released nutrients to crops after application of fertilizers to soil was stopped.

Excessive application of fertilizers to the soil especially at 8 and 10 t ha^{-1} would lead to nutrient imbalance and subsequently poor uptake of essential nutrients for crop growth.

The soil critical levels for N, P, K, Ca, Mg, Na and OM. differed slightly from the earlier ones developed by Agboola and Corey (1973) and Adeoye (1980) signifying the importance of updating research on soil calibration for these nutrients. Besides, the study helps to provide a framework for soil critical levels using applied plant residues or residues + manure which are presently adopted by farmers.

CONCLUSIONS

Plant residues (sole and amended forms) were effective as fertilizer and sources of nutrients for okra crop. Their application enhanced crop growth and pod weight of okra. Amendment of residues with pig, goat and poultry manures improved their effects on the pod weight of okra. The research showed that soil nutrient or critical levels and okra pod weight at 6 t ha^{-1} residue or residues + manure gave the best yield response and above which at 8 and 10 t ha^{-1} crop responses decreased.

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Ultrastructural Studies of Soybean Seed-borne Infection by *Diaporthe phaseolorum* var. *sojae* and Screening of Antagonistic Potentiality by Selected Biocontrol Agents *in vitro*

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ABSTRACT

The association of *Diaporthe phaseolorum* var. sojae with soybean seed decay was examined by ultrastructural studies using light microscopy and scanning electron microscopy. The fungus was detected over the seed surface as whitish-grey mycelial growth and scattered black pycnidia. Hyphae and pycnidia were also observed in palisade, hourglass and parenchyma cell layers of the seed coat. Mycelial growth of *D. phaseolorum* var. *sojae* was abundant in the hourglass cell layer compared with other layers of the seed coat. Neither mycelium nor pycnidium was found in any tissues of the cotyledon and embryo of the infected seed. Asymptomatic seeds were free from infections. Artificial seed inoculation with *D. phaseolorum* var. *sojae* significantly reduced seed germination over control by 21.2% and increased seed rot by 120% in sterilized soil under glass house conditions. Six isolates of Trichoderma and three isolates of bacteria were tested in vitro against *D. phaseolorum* var. sojae in dual culture test. Among these organisms, *T. harzianum* isolate UPM40 exhibited the most antagonistic potential based on Percent Inhibition Radial Growth (PIRG) of 92.9% and shortest time needed (7 days) to overgrow the *D. phaseolorum* var. sojae colony as compared to other tested isolates.

Keywords: *Diaporthe phaseolorum*, screening, soybean, biocontrol agents, ultrastructure

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) seed decay caused by *Diaporthe/Phomopsis* complex is one of the major fungal diseases of soybeans in most soybean-producing areas of the world resulting in reduced seed germination and quality (Sinclair, 1988; Sinclair and Backman, 1989; Zorrilla *et al.*, 1994; Fabrega *et al.*, 2000). The most frequently recovered fungus, *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. var. *sojae* (Leh.) Wehm. (anamorph *Phomopsis sojae* Leh.), is the most important causal organism of *Phomopsis* seed decay (Minor *et al.,* 1995). This fungus is seed-borne and responsible for pod and stem blight of soybean. It colonizes both the immature and mature seed tissues of soybean with or without symptoms (Roy and Ratnayake, 1997). Infected seed appears as moldy, smaller in size and fissured which reduces seed quality under warm and humid conditions (Wrather and Sweets 1998; Jackson *et al.*, 2005). *Diaporthe phaseolorum* var. *sojae* is more variable in cultural characteristics and produce typical pycnidia with both α and β conidia (Zhang *et al.*, 1997). A number of practices have been recommended for the control of *Phomopsis* seed decay. Several cultural practices can reduce the level of infection, but not effective enough (Ploper and Backman, 1992). Fungicide has been used extensively but, it is not a widely accepted practice in the tropics, mainly because of its negative effect on the environment and pathogenic resistance (Hartman and Sinclair, 1992). However, control of *Phomopsis* seed decay by genetic resistance or commercial cultivars has not been well explored (Minor *et al.*, 1995; Jackson *et al.*, 2005).

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Research on the use of microbial antagonists in biocontrol programs has gained considerable attention for its potential to augment or replace fungicides (Wilson and Wisniewski, 1994). Biological control has been used in many crops to control disease, but the case of soybean is at an embryonic stage (Backman and Jacobsen, 1992). Efforts have been made to use biological control agents to protect seeds from pathogen of soybean. The biocontrol agent *Trichoderma* is well known for having a broad antagonism against different pathogenic fungi in a wide variety of economically important crops (Kloepper *et al.*, 1992; Tronsmo and Hjeljord, 1998; Gardener and Fravel, 2002). Moreover, *Trichoderma* spp. can enhance germination and vigour of poor quality seeds (Harman, 2007). Rhizosphere-colonizing bacteria, such as *Pseudomonas* spp., *Burkholderia* spp., *Bacillus* spp., and *Serratia* spp., are commonly associated with plants in a non-pathogenic manner and are antagonistic against soil-borne plant pathogens (Compant *et al.*, 2005). However, there is no information regarding potential isolates of either *Trichoderma* or rhizobacteria against *D. phaseolorum* var. *sojae*. The aim of this research was to establish the site of infection by *D. phaseolorum* var. *sojae* and its effect on seed germination and seedling survival. Attempts were also made to search for any antagonistic potential of different isolates of *Trichoderma* and rhizobacteria against *D. phaseolorum* var. *sojae in vitro*.

MATERIALS AND METHODS

Microscopic Observations

Ultrastructural studies to determine the site of infection in naturally infected soybean seeds var. Palmetto were conducted in the Plant Pathology Laboratory, Faculty of Agriculture, Universiti Putra Malaysia (UPM) in Serdang, Selangor using light microscopy (LM) and scanning electron microscopy (SEM) through 5 repetitions. Twenty seed samples were randomly selected and plated on moist blotter paper and incubated at alternating cycles of 12 h near-ultra violet (NUV) light and darkness. *Diaporthe phaseolorum* var. *sojae* was identified by cultural and morphological characteristics (Pioli *et al.*, 2003). After four days of incubation, seeds producing fruiting structures of *D. phaseolorum* var. *sojae* as well as asymptomatic seeds were selected. Different seed components viz. seed coat, cotyledon and embryo were separated carefully and cut transversely into 2-3 mm^2 pieces.

Light Microscopy (LM)

Individual seeds and their components from both symptomatic and asymptomatic seeds were fixed in Bouin's solution (picric acid, formaldehyde 40%, glacial acetic acid 15:5:1 v/v/v) and vacuum extracted overnight. Samples were then dehydrated in eight series of concentrations of ethanol (30, 40, 50, 60, 70, 80, 90 and 100%) and washed twice in 98% methyl benzoate plus 2% celloidine for 96 h. Samples were then infiltrated in the oven at 60°C overnight in each of the mixture of xylene and paraplast in proportions of 75:25; 50:50; 25:75; and 100%, respectively. Finally, the samples were embedded in paraffin wax and mounted in small paper boxes. Serial paraffin sections 10-12 μm thick were cut using an ordinary rotary microtome (Model 820 Spencer). Fine sections were placed on glass slides using albumin-glycerine solution. Sections were deparafinized with xylene and stained with 0.05% toluidine blue. All stained sections were mounted in DPX mounting media and viewed under light microscope (Model Nikon FX-35DX) (Johansen, 1940).

Scanning Electron Microscopy (SEM)

Seed samples from symptomatic and asymptomatic seeds were fixed separately in 2.5% buffered glutaraldehyde for 24 h at 4°C. Samples were washed with 0.1 M sodium cacodylate buffer (pH 7.7) and post-fixed in 1 % osmium tetraoxide for 2 h at 4°C and then washed again with 0.1 M sodium cacodylate buffer three times for 10 min each. A series of dehydration was performed in seven different concentrations of ethanol (30, 40, 50, 60, 70, 80 and 90%) three times for 10 min each, and finally for 15 min in 100% ethanol. Samples were dried in Baltec 030 Critical Point Drying (CPD) apparatus for 30 min. Dried samples were stuck on stubs and coated with gold in a Polaron Sputter Coater and viewed under SEM (JOEL JSM 6400) (Benhamou and Chet, 1996).

Fungal Isolation, Seed Inoculation and Disease Assessment

Diaporthe phaseolorum var. *sojae* was isolated from naturally infected soybean seeds by agar plate

method (Begum *et al.*, 2007). The fungus was cultured on PDA for 30 days at room temperature (25±1°C) to induce the formation of pycnidia. The pycnidia were collected and busted with glass rod before washed off with sterilized 1.5% sodium alginate solution. Conidial suspension of α and β conidia obtained was adjusted to a concentration of 1×10^7 conidia ml^{-1} by a Neubeaur haemocytometer. Healthy soybean seeds were surface sterilized with 10% Clorox® for 3 min and rinsed thrice with sterilized distilled water, and dried for 1 h in a laminar flow chamber. Seeds were then soaked in spore suspension (1: 2 w/v) of *D. phaseolorum* var. *sojae* for 1 h and surface dried over night. They were subsequently sowed at the depth of 2 cm in plastic trays (39 x 28 x 11 cm) containing sterilized soil mixture (top soil: peat soil: sand = 3: 2: 1). The experiment was done in four replicates containing 25 seeds each. Trays were arranged in a completely randomized design in the glasshouse with each tray considered as a replicate. The glasshouse temperature fluctuated between 31°C (day) and 25°C (night), and 85 ± 5% R.H. After 14 days, the percentages of seed germination and rotten seed were recorded.

Screening of Biocontrol Agents

Six isolates of *Trichoderma* and three isolates of bacteria obtained from the Plant Pathology Laboratory collection, were used in this study (Table 1). The experiment was conducted using a completely randomized design with five replications and repeated four different times.

These isolates were screened for their antagonistic activity against *D. phaseolorum* var. *sojae in vitro* using dual culture tests. The antagonistic activity was determined based on the Percentage Inhibition of Radial Growth (PIRG). A 5 mm diameter mycelial agar disc was cut from the margin of a 7-day-old culture of *D. phaseolorum* var. *sojae* and placed 3 cm from the edge of a 9 cm Petri dish containing PDA medium. Another 5 mm mycelial agar disc from 7-day-old culture of each *Trichoderma* isolate was placed 3 cm away from the former disc on the same plate. The plates were incubated at ambient temperature (25±1°C) for 15 days. Antagonistic activity of *Trichoderma* isolates were assessed after seven days of incubation by measuring the radius of the *D. phaseolorum* var. *sojae* colony using the following formula (Jinantana and Sariah, 1997):

$$\text{PIRG} = \frac{R1 - R2}{R1} \times 100\,\%$$

where R1 indicates the radial growth of the pathogenic fungal colony in the control plates and R2 indicates the radial growth of pathogenic fungal colony in the dual culture plates. During the incubation period, the time needed for full overgrowth on the colony of *D. phaseolorum* var. *sojae* by each of the *Trichoderma* spp. was recorded up to 14 days. Attempts were also made for the recovery of the fungus from the inhibition and overgrowth zone in the dual culture plates. A 5 mm diameter mycelial agar disc of parasitized fungus by each of the *Trichoderma* spp. was transferred from the inhibition and overgrowth zones on the fresh PDA. The regrowth of the *D. phaseolorum* var. *sojae* was observed after 7 days of incubation.

TABLE 1
Isolates of different biocontrol agents (BCAs)

Isolates	Species
Fungal BCAs	
UPM40	*Trichoderma harzianum*
UPM29	*Trichoderma harzianum*
TL1	*Trichoderma longibrachiatum*
TK1	*Trichoderma koningii*
TV3	*Trichoderma virens*
TV2	*Trichoderma virens*
Bacterial BCAs	
UPM14 B1	*Burkholderia glumae*
UPM13 B8	*Pseudomonas aeruginosa*
UPM39 B3	*Serratia marcescens*

For testing antagonistic bacteria, a 5 mm diameter of fungal agar disc from a 7-day-old culture was placed in the middle of a 9 cm Petri dish containing nutrient agar (NA). The plates were incubated at ambient temperature (25±1°C) for 24 h. A loopful of bacteria from 48 h NA culture was streaked in a circle at 3 cm away from the fungal agar disc. After incubation at room temperature for 7 days, the inhibitory activity of the bacteria was determined by measuring the PIRG and the zone of mycelial growth inhibition around the bacterial streak. Three ratings were used: - = no inhibition zone and growth of fungus over bacterial streak; + = no inhibition zone, but no growth of fungus on the bacterial streak and ++ = 1-5 mm inhibition zone (Bardin *et al.*, 2004).

Statistical Analysis

Data were analyzed statistically by ANOVA using SAS software (SAS, 1999). Mean separation was carried out using Tukey's Studentized Range (HSD) at $P = 0.05$.

RESULTS

Ultrastructural studies of asymptomatic and symptomatic soybean seeds infected by *D. phaseolorum* var. *sojae* conducted under LM and SEM revealed no fungal propagule externally and internally for asymptomatic seeds (*Figs. 1A* and *B*). Seeds showing symptoms of *D. phaseolorum* var. *sojae* infection indicated a profuse mycelial growth with black pycnidia over the seed surface. The seeds appeared externally white, chalky and shriveled (*Figs. 2A-B, 3A* and *4A*). Pycnidial beak was found in severely infected seeds (*Fig. 2B*). Mycelia and pycnidia of *D. phaseolorum* var. *sojae* were observed in all layers of the seed coat. Fungal hyphae could be distinguished in the seed tissue based on hyphal morphology. Hyphae of this fungus were hyaline, branched and stained light green with toluidine blue (0.1%). The hyphal breadth ranged from 3.5- 7.5 µm (*Figs. 3B* and *4B*). Pycnidia were found in the palisade cell layer, hourglass cell layer and parenchyma cell (*Figs. 3B-D* and *4C*). Mycelial growth was more abundant in the hourglass layer but less in parenchyma and palisade cell layer of the seed coat (*Figs. 3B* and *4B*). Hyphae and pycnidia were not detected in any tissue of the cotyledons or embryo of infected seeds.

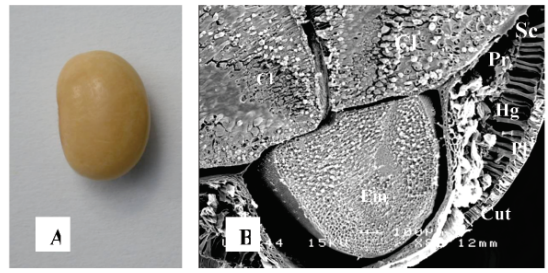

Fig. 1: Photomicrographs showing asymptomatic soybean seeds (A) External view of seed under LM (B); Transverse section of internal seed tissues under SEM (Abbreviations: Sc, Seed coat; Cl, Cotyledon; Em, Embryo; Cut, Cuticle; Pl, Palisade cell; Hg, Hourglass cell; Pr, Parenchyma cell)

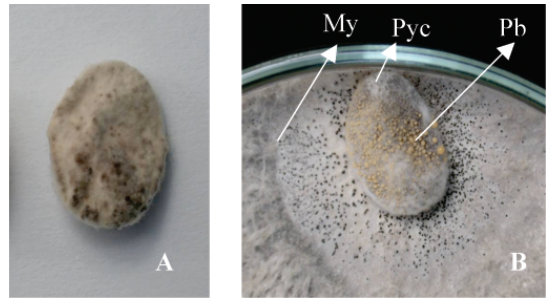

Fig. 2: Light microscopic photomicrographs showing naturally infected soybean seeds by D. phaseolorum *var.* sojae *with grayish-white moldy and scattered black pycnidia over seed surface (A) on moist blotter paper; (B) on PDA (My, Mycelia; Pyc, Pycnidia and Pb, Pycnidial beak)*

The effect of seed inoculation with *D. phaseolorum* var. *sojae* was evaluated on seed germination, and rot of soybeans under glass house conditions (Table 2). Inoculated seeds resulted in lower seed germination at 67.0%, whereas 85.0% was recorded in uninoculated seeds. The results obtained clearly indicated that the fungus reduced seed germination significantly (P=0.05) by 21.2% in comparison with uninoculated seeds (control). A higher percentage (33.0%) of seed rot was also recorded in inoculated seeds than that of uninoculated seeds (15.0%), which corresponded to an increasing seed rot by as much as 120%.

All *Trichoderma* isolates were found to inhibit the radial growth of *D. phaseolorum* var. *sojae* at different degrees of inhibition (Table 3 and

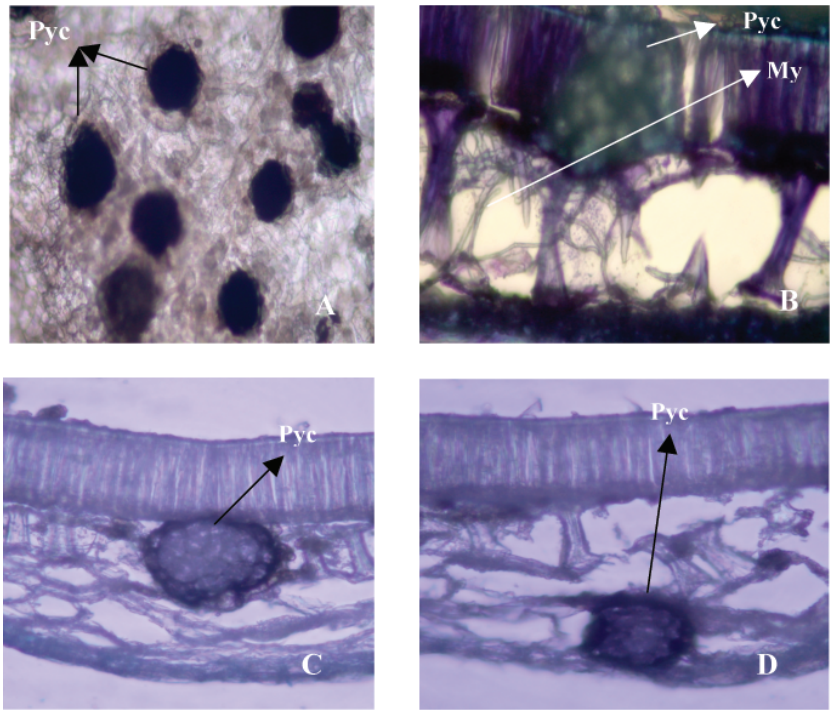

Fig. 3: Light microscopic photomicrographs showing mycelia and pycnidia of D. phaseolorum *var.* sojae *on and in the seed coat of soybean seeds (A); Black pycnidia and mycelia on the seed coat (B); Pycnidia in palisade layer and mycelial growth in the internal layer of the seed coat; (C) Pycnidia in hourglass cell of the seed coat (D) Pycnidia in parenchyma cell of the seed coat (Abbreviations: Pyc, Pycnidia; My, Mycelia)*

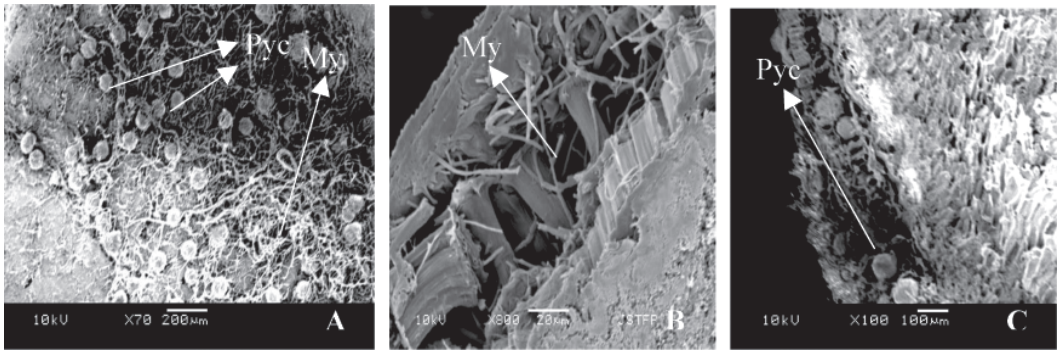

Fig. 4: Scanning electron micrographs (SEM) showing mycelia and pycnidia of D. phaseolorum *var.* sojae *on and in the seed coat in naturally infected soybean seeds (A) Black pycnidia and mycelia on the seed coat (B) Profuse mycelial growth in the internal layer of the seed coat (C) Pycnidia in all layer of the seed coat (Abbreviations: Pyc, Pycnidia; My, Mycelia)*

Fig. 5). The highest PIRG value recorded was UPM40 (92.9%) followed by TV2 (74.5%), TL1 (73.9%), TV3 (71.3%), TK1 (59.9%) and UPM29 (51.9%). The time needed for overgrowth on the pathogenic fungal colony by UPM40 was shortest as compared to other *Trichoderma* isolates. UPM40 completely grew over the fungal pathogen colony 7 days after incubation. The fungal pathogen failed to re-grow when colonized by all *Trichoderma* spp. on fresh PDA (*Fig. 6*). No

TABLE 2
Effect of seed inoculation by *D. phaseolorum* var. *sojae* on seed germination and seed rot of soybean

Treatment	Seed germination (%)	Reduction of germination over control (%)	Seed rot (%)	Increased seed rot over control (%)
Inoculated seed	67.00 b	21.2	33.00 c	120
Uninoculated seed (Control)	85.00 a	0.0	15.00 d	0.0

Means within the same column followed by the same letter are not significantly different at P=0.05 according to Tukey's Studentized Range (HSD) performed on arcsine transformed data.

TABLE 3
Antagonistic effect of *Trichoderma* isolates against *D. phaseolorum* var. *sojae* in the dual culture test

Isolates code no.	Genus/Species name	Antagonism (PIRG)*	Time of overgrowth
UPM40	*Trichoderma harzianum*	92.9 a	7-days
UPM29	*T. harzianum*	51.9 c	-
TL1	*T. longibrachiatum*	73.9 b	9-days
TK1	*T. koningii*	59.9 bc	-
TV3	*T. virens*	71.3 b	11-days
TV2	*T. virens*	74.5 b	9- days

- indicates no overgrowth up to 14 days
* indicates percent inhibition of radial growth (PIRG) at 7 days after incubation
Means within the same column followed by the same letter are not significantly different at P=0.05 according to Tukey's Studentized Range (HSD) performed on arcsine transformed data.

bacterial isolates showed potential to control *D. phaseolorum* var. *sojae* in the dual culture test (Table 4 and *Fig. 7*). They exhibited slight inhibition (+), which was less then 50% after 7 days of incubation. There was no inhibition zone formed between bacterial isolates and *D. Phaseolorum* var. *sojae*.

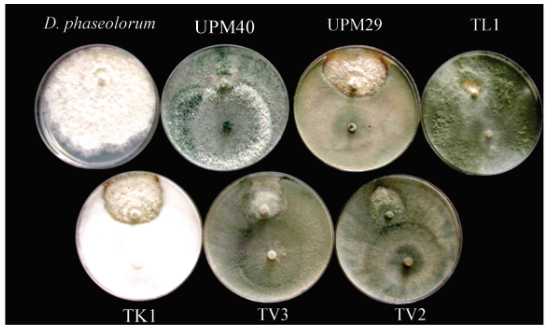

Fig. 5: Radial growth of D. phaseolorum *var.* sojae *in the dual culture test as affected by* Trichoderma *spp, seven days after incubation on PDA*

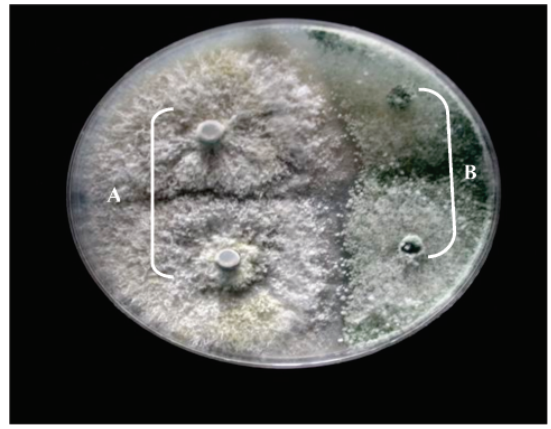

Fig. 6: Regrowth of D. phaseolorum *var.* sojae *in the dual culture test as affected by* Trichoderma *spp, seven 7 days after incubation on PDA. (A)* D. phaseolorum *var. sojae from the control plate; (B) there was no recovery of* D. phaseolorum *var. sojae from the overgrowth zone and interaction zone in the treatment plate*

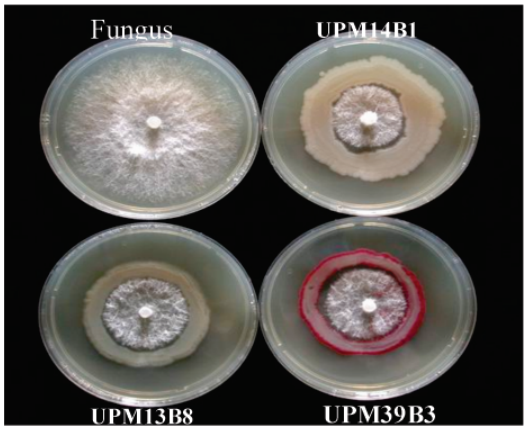

Fig. 7: Radial growth of D. phaseolorum *var.* sojae *in the dual culture test as affected by bacterial isolates, seven days after incubation on NA*

DISCUSSION

Ultrastructure views of symptomatic soybean seeds by *D. phaseolorum* var. *sojae* revealed the colonization of mycelia and pycnidia on and in all layers of the seed coat. The result confirmed that the fungus was an externally and internally seed-borne pathogen and could remain dormant and as latent infection (Sinclair, 1991). Mycelial growth was much more prevalent in the hourglass cell layer than other closely compacted layers of the seed coat. This may be attributed to the large intercellular spaces available that allow for more growth of fungal pathogen in the hourglass cell (Kunwar *et al.*, 1985). Similar trends in the spread of mycelium of *D. phaseolorum* var. *sojae* was observed in the seed coat of infected soybean seeds, but occasionally found in embryonic and cotyledonary tissues (Ilyas *et al.*, 1975). Singh and Sinclair (1986) observed the mycelium of *Phomopsis* sp. on the surface, in all layers of seed coat and the embryo (cotyledons). Kunwar *et al.* (1985) observed that *Phomopsis* spp. colonized seed coat and embryo tissues in mixed infected soybean seeds with *Colletotrichum truncatum*. *Phomopsis* was located in the seed-coat with a relatively low incidence in the embryo tissue (Zorrilla *et al.*, 1994). Spread of the pathogen by fungal propagule in infected seed tissues depended on the severity of infections, seed stage and genotypes (Singh and Mathur, 2004).

Results from the glass house study demonstrated that *D. phaseolorum* var. *sojae* caused seed rot of soybean. The germination of infected seeds was found to be significantly (P=0.05) lower (21.2%) when compared with the uninoculated seeds. *Diaporthe phaseolorum* var. *sojae* was found to be associated with soybean seeds infection both externally and internally of the seed coat which lead to local and systemic infections. Thus, infected seeds reduced germination by progressive rotting of the hypocotyl-radicle axis of soybeans (Vrandecic *et al.*, 2006). Kmetz *et al.* (1978) reported that *D. phaseolorum* var. *sojae* negatively affected seed germination and caused seed rot. Vrandecic *et al.* (2006) found *Phomopsis sojae* to be the most pathogenic to soybean seeds among twelve isolates of *Diaporthe/Phomopsis* species. This fungus caused seed rots after artificial seed inoculation.

In vitro screening is considered the most desirable first step to screen for a large number of potential antagonists in a biocontrol process for possible application in the field (Merriman and Russell, 1990). The preliminary screening of different isolates of *Trichoderma* and bacteria was conducted using dual culture test *in vitro*. *Trichoderma harzianum* (UPM40) showed the highest antagonistic potential to suppress the growth of *D. phaseolorum* var. *sojae* based on higher PIRG value and time needed for complete

TABLE 4
Antagonistic effect of bacterial isolates against *D. phaseolorum* var. *sojae* in the dual culture test

Isolates code no.	Genus/Species name	Antagonism (PIRG)*	Inhibition category*
UPM14 B1	*Burkholderia glumae*	33.9 a	+
UPM13 B8	*Pseudomonas aeruginosa*	33.1 a	+
UPM39 B3	*Serratia marcescens*	34.1 a	+

* indicates percent inhibition of radial growth (PIRG) at 7 days of incubation

Means within the same column followed by the same letter are not significantly different at *P*=0.05 according to Tukey's Studentized Range (HSD) performed on arcsine transformed data.

overgrowth compared to other *Trichoderma* isolates. However, the tested bacterial isolates did not show any prospective inhibitory effect to *D. phaseolorum* var. *sojae*. Although, PIRG values of all bacterial isolates indicated positive results, the values were less than 75%. A PIRG value of more than 75% is required to be considered as a potential antagonist (Narayanasamy, 2006). These bacterial isolates were isolated from oil palm roots and tested for their antagonistic activity against *Ganoderma boninense*, a soilborne pathogen (Zaiton, 2006). Specificity of the bacterial isolates could lead to the low *in-vitro* effectiveness against *D. phaseolorum*. The time needed for overgrowth on the pathogen colony is an important parameter in the assessment of the antagonistic ability to compete against the pathogen for limited nutrient resources and space (Ibrahim, 2005). *Trichoderma harzianum* was able to completely overgrow the fungal pathogen colony within 7 days. There was no recovery of *D. phaseolorum* var. *sojae* from the parasitized mycelia by *Trichoderma* spp. Fernandez (1992) reported that application of *T. harzianum* to soybean residues resulted in a significant decrease in the incidence of soybean pathogens. In field plots, *T. harzianum* increased soybean plant survival by 40% (Menendez and Godeas, 1998). The inhibition of radial growth of fungal mycelia in this study was considered to be either competition or mycoparasitism or antibiosis which inhibited fungal pathogen activity and caused their lyses (Wilson and Wisniewski, 1994; Harman, 2005). The activity of *T. harzianum* in this study suggested that it can be used as a potential antagonist to suppress *D. phaseolorum* var. *sojae* of soybean in the field. Further research is needed to develop formulation and the most suitable technique for field application of biocontrol agents in comparison to recommended fungicides.

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Protein Expression of Late Elongated Hypocotyl (LHY) Homolog Genes of Teak in *Escherichia coli*

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ABSTRACT

Expression of an isolated gene in a system that directly translates it into a protein is an important step to study the protein encoded by the gene. The isolated gene can be expressed in vivo by a heterologous system. In this study, a bacteria system was used to translate the *Tectona grandis* Late Elongated Hypocotyl (Tg-LHY) gene, which was isolated from flowering tissues of teak (*Tectona grandis*). The gene was cloned into the pET 14b vector (Novagen) and transformed into BL 21(DE3)/pLysS and Rosetta 2 expression host cells (Novagen). Rosetta 2 host cell has been found to be a good candidate to express the Tg-LHY protein from plant origin, as it recognizes the codon that was found in plant but rarely used in bacteria. The expressed protein was about an expected size, which was 90 kD. Western blot analysis using antibody against His-tag, which was fused to the Tg-LHY protein, proved that the expressed protein was Tg-LHY protein.

Keywords: Heterologous protein expression, *Tectona grandis*, LHY homolog genes

INTRODUCTION

The Late Elongated Hypocotyl (LHY) homolog gene has been isolated from flowering tissues of teak (*Tectona grandis*) using subtractive hybridization technique, and was named *Tectona grandis* Late Elongated Hypocotyl (Tg-LHY) (Norlia *et al.*, 2006). This gene has similarities to LHY genes from a few plant species. It is 57% identical to LHY of *Castanea sativa*, 52% identical to *Phaseolus vulgaris* and 43% identical to *Arabidopsis thaliana* (Ramos *et al.*, 2005; Kaldis *et al.*, 2003; Schaffer *et al.*, 1998). LHY gene has been reported as one of the important genes in plant circadian clock system (Schaffer *et al.*, 1998). Other genes involved in plant circadian clock oscillation are Circadian Clock Associate 1 (CCA1) and Timing of CAB Expression 1 (TOC1) (Carre, 2002). In *Arabidopsis*, a reciprocal regulation between TOC1 and LHY/CCA1 led to oscillation of circadian clock system (Alabadi, 2001). Circadian clock systems are complex signalling networks that allow organisms to adjust cellular and physiological activities in anticipation of periodic changes in the environment. Circadian clock governs many plant processes including movement of organs like leaves and petals, hypocotyl elongation, stomata opening, expression of several genes and flowering time (Jarillo *et al.*, 2004).

It has been suggested that circadian rhythmicity depends on clock protein. In *Arabidopsis*, the most circadian clock plant studied, light was shown to modulate expression of LHY at the translation level which coincides with expression of LHY mRNA at dawn (Kim *et al.*, 2003). This simultaneous translation induction and transcription repression of LHY expression are thought to play a role in narrowing the peak of LHY protein synthesis at dawn and increasing the robustness and accuracy of circadian oscillator.

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The expression of LHY gene in flowering tissue of teak suggested the involvement of the circadian clock system in flower development of teak. In *Arabidopsis*, which is a long day plant, the long photoperiod detected by the circadian clock led to early flower development (Blazque, 2000). Teak, which is planted in neutral day places like Malaysia, photoperiod might not be the environmental factor detected by its circadian clock system towards flower development. Comparison between five years temperature data and the reproductive cycle of teak at Mata Ayer, Perlis suggest that temperature might be the environmental factor detected by the circadian clock system of teak (Norlia, 2007). To further investigate the assumption, Tg-LHY protein in correlation with the temperature would be an indicator for the environmental factor that was detected by circadian clock system of teak.

The objective of this study was to isolate the Tg-LHY protein, which will be useful in preparing the Tg-LHY specific antibodies for further Tg-LHY protein analysis in flowering tissues of teak. In this paper we report the cloning and expressing of Tg-LHY protein in a bacteria system.

MATERIALS AND METHODS

Primer Design and Cloning

The pET 14b plasmid vector (Novagen, USA) used in this study is a translation vector, which carries a His-Tag sequence and strong bacteriophage T7 translation signal at the 5' end of the multiple cloning site (*Fig. 1*). Cloning sites of this vector only consists of 3 restriction enzyme sites, which are *Bam H*1, *Xho* 1 and *Nde* 1 (*Fig. 1*). Therefore, primers were designed as such to include either one of these restriction enzyme sites, however the selected restriction enzyme site should not exist within the cDNA fragment. The primers designed were LHYXhof (5'-GCG CTC GAG **ATG** GAC CCT TAT TCA TCT-3') and LHYBamr (5'-GCG GGA TCC **TTA** AGT AGA AGC CTC TCC-3'), which contained restriction enzyme sites of *Xho* I and *Bam H*I, respectively (underlined bases). In designing the primer, the start codon of the gene was placed immediately after the restriction site for the 5'-primer and the stop codon was placed immediately before the restriction site for the 3'-primer (bolded bases).

PCR to amplify the full-length of Tg-LHY cDNA fragment was carried out using PE GenAmp System 9600, The program used was denaturing at 94°C for 3 min, followed by 30 cycles of denaturing at 94°C for 45 sec, annealing at 60°C for 1.3 min and extension at 70°C for 10 min. The reaction was carried out by adding 2.5 µl 10X Pfx Amplification buffer (Invitrogen, USA), 1.5 µl 10 mM dNTP mixture (Fermentas, USA), 10 µM of each primer, 150 ng recombinant plasmid containing full length of Tg-LHY, 1 U Platinum Pfx DNA Polymerase and distilled water to the total volume of 25 µl. The amplified Tg-LHY fragment was then eluted and ligated into PCR 2.1 plasmid vector (Invitrogen, USA) and transformed into One Shot competent cell (Invitrogen, USA). The plasmid was digested with both restriction enzymes and the insert was sub-cloned into an expression vector, pET 14b. Recombinant plasmid was then transformed into competent DH5µ cell. The recombinant plasmids were then extracted and sequenced. The recombinant plasmids, which showed a right reading frame after sequencing, were transformed into an expression host cell, either BL 21(DE3)/pLysS or Rosetta 2 (Novagen). Both strains carry a chromosomal copy of the T7 RNA polymerase gene under the control of *lacUV5* promoter, which

Fig. 1: Multiple cloning site of vector pET 14b (reproduced from pET 14b map, Novagen, USA)

is suitable for the production of protein from target genes cloned in pET vector. Strain Rosetta 2 has another feature that enhances the expression of eukaryotic proteins encoded by codons rarely used in *E. coli* such as AUA, AGG, AGA, CUA, CCC and GGA (Del Tito *et al.*, 1995).

Protein Induction and Denaturation

Transformed cells were incubated at 37°C with shaking at 250 rpm in Luria Bertani medium containing 50 l/ml ampicillin and 34 g/ml chloramphenicol until the OD_{600} reached 0.6. One ml of the medium containing growth cell was removed for the un-induced control. IPTG was added to the remaining cells to the final concentration of 0.4 mM. The incubation was continued until 5 hr; every one hour, 1 ml of sample was removed for time course expression analysis. The five samples collected at different time periods together with the un-induced control were centrifuged at 10,000 g for 1 min and each pellet was dissolved in 100 l of 1 X Phosphate Buffer Saline (PBS). Two hundred microlitres of 2X sample buffer (125 mM tris-HCl [pH 6.8], 4% [w/v] SDS, 5% [v/v] 2-Mercaptoethanol, 20% [v/v] glycerol and 0.1% [w/v] bromophenol blue) were added and passed through a 27-gauge needle five times to reduce the viscosity. Each sample was denatured by heating at 85°C for 5 min and stored at -20°C until further analysis.

SDS-PAGE

The expressed proteins were analysed using 12%(w/v) SDS-PAGE, which was prepared according to the Protein Electrophoresis Technical Manual of Amersham Pharmacia Biotech. The gel was stained with coomassie blue and dried in between two sheets of porous cellophane and locked into the drying frame. The framed gel was allowed to dry for two days.

Western Blot Analysis

Western blotting was performed in order to identify the protein fragments of expressed inserted genes using antibodies. His-Tag AP Western reagent kit and His-Tag monoclonal antibody (Novagen, USA) were used to detect the heterologous protein. Separated proteins on 12% (w/v) SDS-PAGE were electrophoretically transferred to a PVDF Western Blotting Membrane (Roche) using a semi-dry electrophoretic transfer (Biorad). Colorimetric detection of His-tag protein, encoded by pET 14b, which was fused to N-terminal of the expressed protein, was carried out according to the User Protocol of His-Tag Monoclonal Antibody supplied by the manufacturer (Novagen).

RESULTS AND DISCUSSION

Tg-LHY cDNA amplified using LHYfXho and LHYrBam primer pair, were about 2.4 kb (*Fig. 2*). The PCR fragments were ligated into the PCR 2.1 vector before digestion with *Xho* I and *Bam HI* enzyme. This was carried out to ensure complete digestion of the fragment. The digested fragments (*Fig. 3*) were then ligated into the digested pET 14b vector with similar enzymes and transformed into DH5 for recombinant clone analysis. PCR method using LHYfXho and LHYrBam primers was carried out to screen for

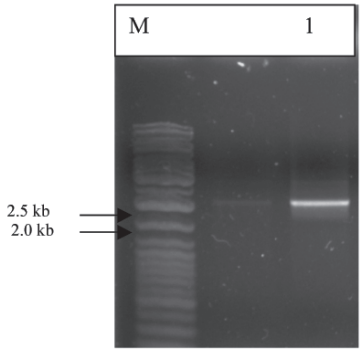

Fig. 2: PCR product of Tg-LHY cDNA using LHYfXho and LHYrBam primer pair. The amplified fragment is about 2.4 kb (1). M is DNA marker of GeneRuler Ladder Mix (Fermentas)

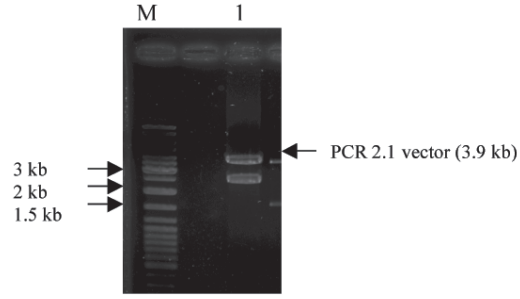

Fig. 3: Complete digestion of pET14b-TgLHY construct. (1) Tg-LHY containing Bam HI and Eco RI cutting site on its ends was digested with respective enzymes. M is DNA marker of GeneRuler Ladder Mix (Fermentas)

the presence of Tg-LHY cDNA in the recombinant plasmid (*Fig. 4*).

The positive recombinant plasmid with compatible reading frame was transformed into an expression host cell. Earlier in this experiment, BL 21(DE3)/pLysS (Novagen) host cell was used. Time course gene expression analysis showed an increase in protein synthesis at an expected size (as pointed by slunt arrow in *Fig. 5a*). The size expectation was calculated based on the assumption that the mean molecular weight of the amino acid is 110 Da, therefore the expressed protein was estimated to be about 90 kD (84 kD of Tg-LHY and 4.4 kD of His-tag protein). The size of Tg-LHY protein expressed was similar to the size of Arabidopisi LHY protein, which was reported to be about 88 kDa (Kim *et al.*, 2003). However, few other bands of expressed protein were also observed (as pointed by straight arrows in *Fig. 5a*). Based on Western analysis, the smaller protein synthesized was found to contain the His-tag protein (*Fig. 5b*). Therefore the proteins were believed to be due to premature translation termination. The phenomenon occurs as a result of differences in codon usage between the inserted gene origin and the host cell, *E. coli.* In *E. coli,* codon such as AGG, AGA, ATA, CTA, CCC, GGA and CGG are rarely used. However in Tg-LHY cDNA, about 7% of its total codons were the rare codons of *E. coli.*

Recombinant plasmid was then transformed into Rosetta 2 host cell in order to enhance the expression of eukaryotic protein that contains codons rarely used in *E. coli.* Rosetta 2 host cell is a BL21 derivative, which was engineered to contain tRNAs and able to translate a rare codon of *E. coli.* Time course protein synthesized analysis showed that after 2 hours induction with IPTG, a protein band of about 90 kD was synthesized (*Fig. 6a*). Western blot analysis against His-tag, which was fused to the N-terminal of the synthesized protein, confirmed that the bands correspond to the protein translated from the inserted genes (*Fig. 6b*).

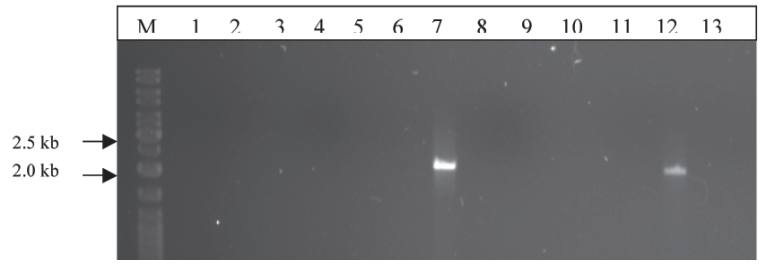

Fig. 4: PCR screening of recombinant clones containing pET14b-TgLHY. LHYfXho and LHYrBam primers were used to amplify Tg-LHY, and out of 13 clones (1 –13) screened, 2 clones (7 and 12) amplified the fragment. M is DNA marker, GeneRuler Ladder Mix (Fermentas, USA)

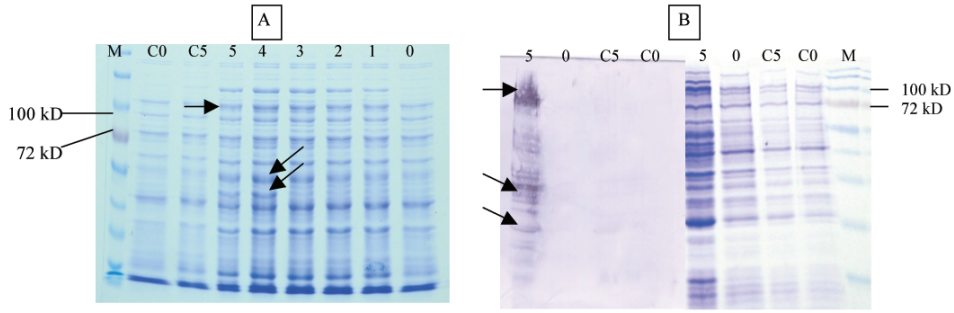

Fig. 5: SDS-PAGE analysis (A) and Western blot analysis (B) of Tg-LHY transformed into BL 21 host cell. Tg-LHY expressed protein at an expected size was marked by straight arrows and truncated expressed proteins were marked by slunt arrows. Protein loaded in the lane of SDS-PAGE were harvested before IPTG was added (0), and 1 hr (1), 2 hr (2), 3 hr (3), 4 hr (4) and 5 hr (5) after IPTG induction. The vector cell cultured (pET 14b in BL 21 host cell) was used as a control and was harvested before induction (C0) and after 5 hours induction (C5). M in both analysis is protein marker Protein prestained ladder (Crystalgen)

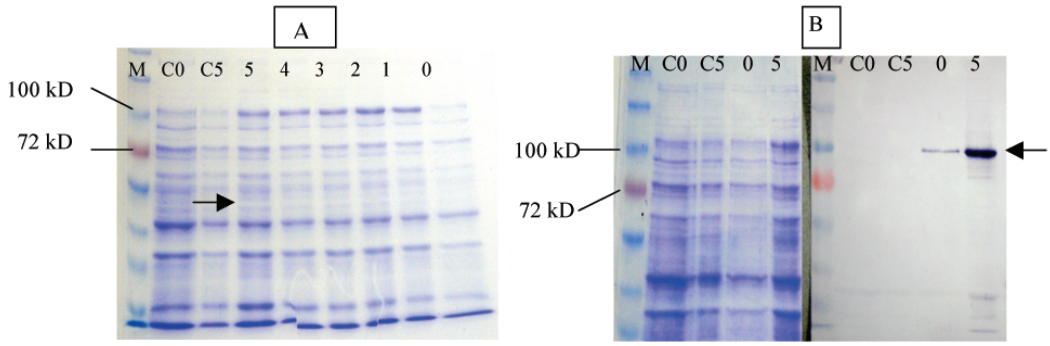

Fig. 6: SDS-PAGE analysis (A) and Western blot analysis (B) of Tg-LHY transformed into Rosetta 2 host cell. Tg-LHY expressed protein at an expected size was marked by straight arrows. Protein loaded in the lane of SDS-PAGE were harvested before IPTG was added (0), and 1 hr (1), 2 hr (2), 3 hr (3), 4 hr (4) and 5 hr (5) after IPTG induction. The vector cell cultured (pET 14b in respective host cell) was used as a control and was harvested before induction (C0) and after 5 hours induction (C5). M in both analysis is protein marker Protein prestained ladder (Crystalgen)

CONCLUSIONS

The protein of Tg-LHY gene isolated from teak was expressed in a bacteria system. However, due to the different codon usage between bacteria and plant, expression host cell that was able to recognize the rare codon should be used. In this study, Rosetta 2 has been found to be a better host cell for the expression of Tg-LHY protein, which originated from plant.

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Molecular Systematics of Mahseers (Cyprinidae) in Malaysia Inferred from Sequencing of a Mitochondrial Cytochrome C Oxidase I (COI) Gene

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ABSTRACT

This study examined the molecular systematics among three Mahseers (*Tor douronensis, Tor tambroides* and *Neolissochilus stracheyi*) using partial sequencing of a Cytochrome C Oxidase I (COI) mitochondrial DNA segment (466bp). The phylogenetic results using the Neighbour-Joining (NJ) method supported the monophyletic status (hence the taxonomic status) among the three putative Mahseer species. The close genetic relationships (0.1-0.4%) found between *T. tambroides* samples from Peninsular Malaysia (kelah fish) and those from Sarawak (empurau fish) also supported their classification as belonging to the same species. The phylogenetic analysis also showed that the *T. douronensis* mtDNA consisted of three highly distinct lineages supported by high bootstrap values, with the Sabah samples forming its own cluster. Thus, this phylogenetic study, although based on a limited number of samples and only a single mtDNA gene managed to provide useful insights into the systematic status of the Mahseers found in Malaysia.

Keywords: Freshwater fish, Mahseers, COI, molecular systematics

INTRODUCTION

Freshwater fishes of the genus *Tor* Gray, commonly known as the Mahseers, belong to the family Cyprinidae (subfamily Cyprininae) (Inger and Chin, 1962; Mohsin and Ambak, 1983; Roberts, 1989; Kottelat *et al.,* 1993). There are currently 17 described species under the genus *Tor* from all across Asia (Ng, 2004) but only three species were reported in Malaysia: *Tor tambroides* Bleeker, *Tor tambra* Valenciennes, and *Tor douronensis* Valenciennes (Kottelat *et al.,* 1993; Kottelat and Whitten, 1996; Ng, 2004). The taxonomic status of *Tor soro* Valenciennes had been revised and it is currently re-classified as *Neolissochilus stracheyi* (Rainboth, 1996). Mahseers are important as food fish as well as ornamental and recreational fishes. However, recently the population sizes of their natural stocks are decreasing rapidly due to environmental degradation and increased fishing pressure (Ng, 2004).

So far, very little taxonomic work to systematically sort out Malaysian Mahseer has been documented. The most cited work was by Mohsin and Ambak (1983) who described *Tor tambroides* and *Tor soro* as two valid Mahseers found in Peninsular Malaysia while a more recent view by Ng (2004) suggested the occurrence of three species; *T. tambroides*, *T. tambra* and *T. douronensis*. Other taxonomic works recognized *T. douronensis* and *T. tambroides* as two valid species (Roberts, 1989; Kottelat *et al.*, 1993; Rainboth, 1996; Zhou and Chu, 1996), although Roberts (1999) classified them to be a single species, and a junior synonym to *T. tambra*. The presence of the median lobe has been characterized as a

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diagnostic morphological character distinguishing the genus *Tor* from the genus *Neolissochillus* (Rainboth, 1996; Ng, 2004), though it cannot be used consistently as a marker to discriminate between fishes of the genus *Tor*. Thus, the application of molecular techniques (such as DNA sequencing) can provide new and better insights into the unresolved taxonomy and phylogenetic relationships of all the putative Mahseers in Malaysia (Nguyen *et al.*, 2006).

Nguyen *et al.* (2006) recently produced the first molecular work on Mahseers in Malaysia by examining the genetic diversity and phylogenetic relationships of broodstocks of *T. douronensis* and *T. tambroides* cultured in Sarawak (Borneo) through sequencing analysis of the mitochondrial DNA (mtDNA) 16S rRNA gene region. Thus, the present study also aimed to clarify the phylogenetic relationships among Mahseer fishes in Malaysia but with a few different approaches. First, we utilized direct sequencing of the *Cytochrome C Oxidase* I (COI) mtDNA gene region, a gene with a faster evolutionary rate compared to the 16S rRNA (Simon *et al.*, 1994), and thus capable of providing a better resolution at the interspecific level. Secondly, we included additional *Tor* samples from Peninsular Malaysia (kelah fish) and Sabah (pelian fish), to compare with the *T. douronensis* (semah fish) and *T. tambroides* (empurau fish) of Sarawak. Thirdly, *N. stracheyi* representing the genus *Neolissochilus* were included in the phylogenetic study to quantify the genetic differences between the two genera.

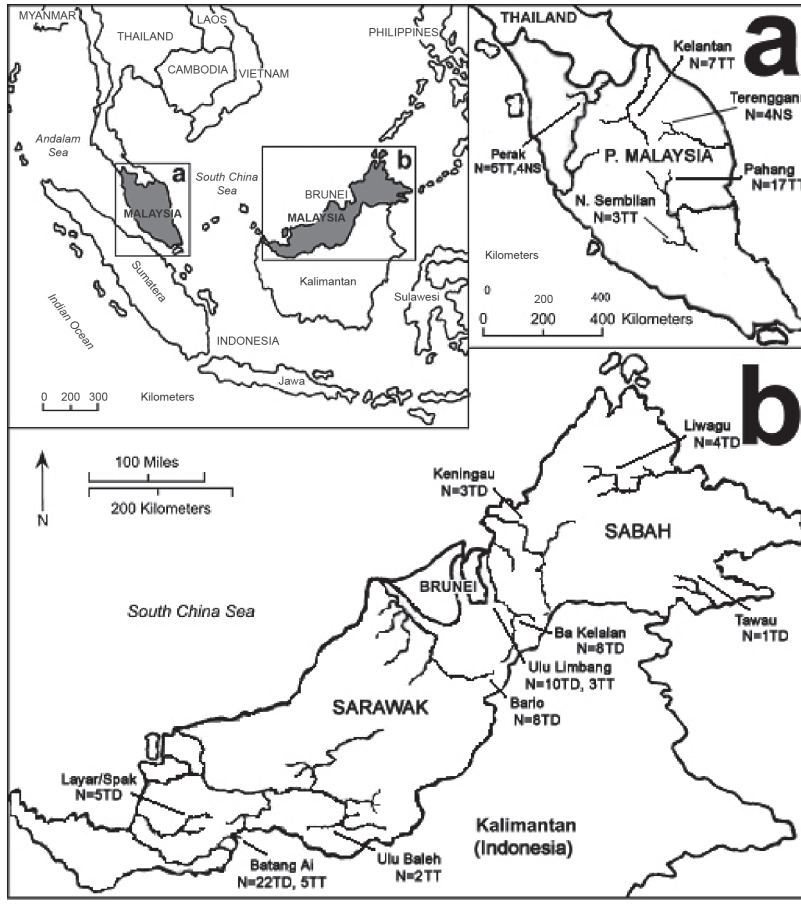

Fig. 1: Map showing sampling locations and sample size (N) in Peninsular Malaysia, Sarawak and Sabah. TD= T. douronensis, *TT*= T. tambroides, *NS*= N. stracheyi

MATERIALS AND METHODS

A total of 111 individuals of the three putative mahseers (*T. douronensis*, *T. tambroides* and *N. stracheyi*) were collected from several locations in Peninsular Malaysia, Sarawak and Sabah (*Fig. 1*). Total DNA was extracted using a modified CTAB method (Grewe *et al.*, 1993) in the presence of Proteinase K. The isolated genomic DNA was used for the mtDNA analysis.

A 500 bp segment of the COI gene was amplified with the oligonucleotide primers COIf (5' CCTGCAGGAGGAGGAGAYCC 3', forward) and COIe (5' CCAGAGATTAGA GGGAATC AG TG 3', reverse) (Palumbi *et al.* 1991). Approximately, 50-100 ng of the template DNA was amplified in a 25 ml reaction mixture containing 50 mM 10X buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP (Promega), 0.1 mM of each primer, and 0.5 units of *Taq* DNA Polymerase (Promega). The cycle parameters consisted of 35 cycles of denaturation (95°C, 30 seconds), annealing (45°C, 30 seconds), and extension (72°C, 60 seconds). The amplified products were visualized on 1% agarose gel containing ethidium bromide, run for approximately 30 min at 90 V and photographed under UV light. The purified PCR products were directly sequenced using the BigDye® Terminator v3.0 Cycle Sequencing kit (ACGT) on an ABI 377 automated sequencer (PE Applied Biosystem) using only the forward primer (COIf). Sequencing reaction using the reverse primer (COIe) was subsequently carried out on some of the samples (haplotypes) to verify the polymorphism in the DNA sequence initially detected using the forward primer.

The CHROMAS (Version 1.45) program was used to display the fluorescence-based DNA sequencing analysis results. The multiple sequence alignments were done using the CLUSTAL X program version 1.81 (Thompson *et al.*, 1997), and subsequently aligned by eye. The Molecular Evolutionary Genetics Analysis (MEGA) version 3.1 (Kumar *et al.*, 2004) program was used to construct a neighbour-joining (NJ) (Saitou and Nei, 1987) tree using two indigenous cyprinids (*Barbonymus gonionotus* (Genbank accession number: DQ532806) and *Barbonymus schwanenfeldii* (Genbank accession number: DQ532805)) as outgroup species. The phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets. The pairwise genetic distance between populations was calculated using the Tamura-Nei distance (Tamura and Nei, 1993), based on unequal base frequencies and unequal ratios of transition to transversion (Ti:Tv) implemented in MEGA.

RESULTS AND DISCUSSION

The sequence analysis of the partial COI gene (466 base pairs) revealed a total of 24 haplotypes in the nucleotide data set: 14 haplotypes belonging to *T. douronensis*, six haplotypes belonging to *T. tambroides* and four haplotypes belonging to *N. stracheyi* (*Fig. 2*). The sequence of each of the haplotypes was deposited in the GenBank (GeneBank Reference Numbers: DQ532824-DQ532827 and EF192444-192463). In total, 74 (15.9%) variable sites were found, of which 56 (12.0%) were parsimony informative sites, while 392 (84.1%) were monomorphic sites. Transitional changes occurred more frequently than transversional changes as is typical of animal mitochondrial genomes (Briolay *et al.,* 1998).

The phylogenetic results obtained by using the NJ method supported the monophyletic status among the three mahseers (*Fig. 3*), although the bootstrap support between *T. tambroides* and *N. stracheyi* was low (58%). The high genetic divergence separating *T. douronensis* and *T. tambroides* confirmed their status as distinct species (Table 1). Likewise, the high genetic divergence separating the *N. stracheyi* lineage from the *Tor* lineages (7.7-8.7%) also supported its recent reclassification from the genus *Tor* into the genus *Neolissochilus* (Rainboth, 1996).

The close genetic relationships (0.1-0.4%) found between *T. tambroides* samples from Peninsular Malaysia (kelah fish) and those from Sarawak (empurau fish) supported their taxonomic status as belonging to the same species (Table 1). However, the overall very low level of genetic differentiation within and among *T. tambroides* populations may have resulted from the limited number of samples (2-17) analysed for each population, but was consistent with those found by Nguyen *et al.* (2006) in the Sarawak populations.

The phylogenetic analysis also revealed that the *T. douronensis* mtDNA consisted of three highly distinct clusters (Cluster I to III, *Fig. 3*) with the Sabah samples forming its own cluster (Cluster III) with strong bootstrap support. The genetic differentiations between the Sabah

Fig. 2: Summary of nucleotide variations found in the three Mahseers. Only variable sites are shown. Haplotypes are named referring to the species and a number. HS= T. douronensis *haplotype, HKE=* T. tambroides *haplotype, HNS=* N. stracheyi *haplotype. Dots indicate identity with the HS4 haplotype sequence. DQ532805 and DQ532806 are* B. schwanenfeldii *and* B. gonionotus *haplotype sequences, respectively.*

(pelian fish) lineage (Cluster III) and both the Sarawak lineages (Cluster I and II) are relatively high (4.2-4.7%) for a conspecific group, and definitely higher than the two divergent *T. douronensis* lineages from Sarawak (2.0%) found by Nguyen *et al.* (2006) using 16s rRNA (Table 1). However, our phylogenetic analysis did not find any *T. douronensis* lineage genetically more similar to *T. tambroides* (6.8-8.2%) than to each other as was found by Nguyen *et al.* (2006). Thus, we suggest that the *T. douronensis* lineages from Sabah could represent a cryptic species.

Overall, the current study managed to provide insights into the phylogenetic relationships among the three putative species of the important mahseers of Malaysia. Nevertheless, the shortcomings of our results were clearly recognized and the data should be treated with great caution, since it was based on a limited number of samples (especially in *T. tambroides*) and a single maternally inherited gene (COI). Indeed, further studies on their taxonomy, population structures and phylogeography are required based on larger sample sizes per population, samples from other areas of their geographical distributions, a more variable mtDNA region such as the control region (D-Loop) to reveal more variations at the inter and intra population levels, and data from nuclear markers such as single locus microsatellite markers to complement the mtDNA findings.

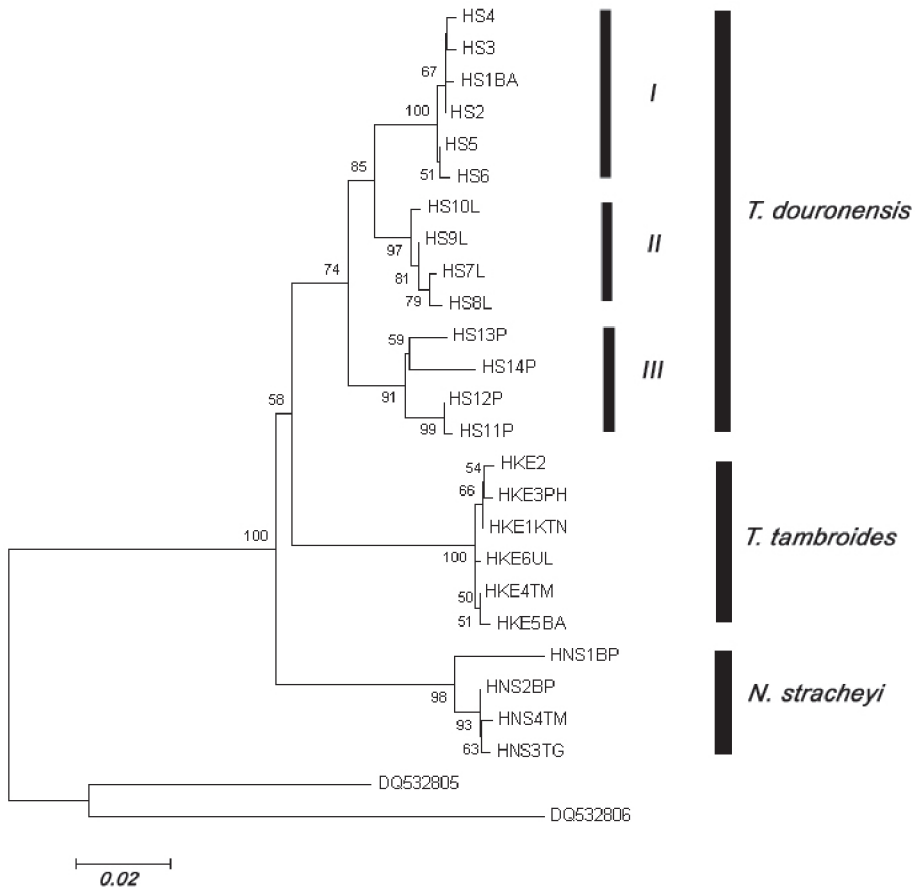

Fig. 3: Neighbour-joining (NJ) phylogram (consensus tree) showing the relationships among COI mtDNA haplotypes of the Mahseers. Haplotypes are named referring to the species and a number. HS= T. douronensis *haplotype, HKE*= T. tambroides *haplotype, HNS*= N. stracheyi *haplotype. Number at each node represents the bootstrap value (%) based on 1000 pseudoreplications for NJ analysis.*

TABLE 1
Pairwise Tamura-Nei genetic distance among the different populations of the three Mahseer species used in this study. Population 1-7 represents *T.tambroides* while population 8-13 represents *T.douromensis*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
T. tambroides	1. Pahang	-													
	2. N. Sembilan	0.000	-												
	3. Kelantan	0.000	0.000	-											
	4. Perak	0.002	0.001	0.001	-										
	5. Batang Ai	0.002	0.002	0.002	0.001	-									
	6. Ulu Limbang	0.004	0.004	0.004	0.002	0.003	-								
	7. Ulu Baleh	0.002	0.002	0.002	0.001	0.001	0.001	-							
T. douronensis	8. Sabah	0.082	0.082	0.082	0.081	0.081	0.081	0.081	-						
	9. Layar/Spak	0.071	0.071	0.071	0.069	0.070	0.068	0.068	0.042	-					
	10. Batang Ai	0.072	0.071	0.071	0.073	0.073	0.075	0.073	0.046	0.024	-				
	11. Ba Kelalan	0.072	0.071	0.071	0.073	0.073	0.076	0.074	0.047	0.027	0.006	-			
	12. Ulu Limbang	0.071	0.071	0.071	0.072	0.073	0.075	0.073	0.046	0.026	0.005	0.001	-		
	13. Bario	0.074	0.073	0.073	0.075	0.075	0.078	0.076	0.047	0.027	0.006	0.005	0.004	-	
	14. *N. stracheyi*	0.083	0.083	0.083	0.081	0.081	0.078	0.080	0.079	0.077	0.085	0.087	0.086	0.086	-

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Genetic Diversity of *Fusarium fujikuroi* Isolated from Bakanae Disease of Rice on the Basis of Vegetative Compatibility

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ABSTRACT

Fusarium fujikuroi which was originally studied in Japan is a pathogen of bakanae disease of rice. The disease was recorded in almost all countries where rice is grown commercially, including Malaysia and Indonesia. A total of 79 strains of *F. fujikuroi* were isolated from rice plants showing typical bakanae symptoms from major granary areas in Malaysia and Indonesia. They were identified using morphological characteristics for species delimitation. The objective of this study was to investigate genetic diversity of the *F. fujikuroi* strains by generating nitrate non-utilizing (*nit*) mutants, followed by phenotyping on diagnostic media, and pairing the mutants on minimal media (MM). About 96.2% of the strains were identified as heterokaryon self-compatible (HSC) based on their ability to form a stable heterokaryon while the remaining 3.8% of the strains were classified as heterokaryon self-incompatible (HSI) based on their inability to form a heterokaryon, even after repeated attempts. Those HSC strains that paired by producing robust growth were classified in the same vegetative compatibility group (VCG). The bakanae strains of *F. fujikuroi* were grouped into 26 VCGs; the largest group was VCG A01 which comprised of 23 strains. Out of 26 VCGs, 12 VCGs contained more than one strain member, and 14 VCGs were represented by a single strain and were not compatible with other strains. The ratio of VCGs to strains of *F. fujikuroi* in these samples was 0.29. The strains of *F. fujikuroi* that caused bakanae disease of rice in Malaysia and Indonesia are genetically diverse based on their multiple VCGs.

Keywords: Bakanae disease, *Fusarium fujikuroi*, heterokaryon, vegetative compatibility group

INTRODUCTION

Vegetative compatibility (VC) also known as heterokaryon compatibility, is another useful tool for identifying fungi and it also reveals the genetic diversity of several fungal genera, including *Fusarium* (Leslie, 1996). VC happens when hyphae of two strains are anastomosed and fused to form a stable heterokaryon and those strains are therefore classified in the same vegetative compatibility group (VCG) (Leslie, 1993), while those that cannot form such heterokaryons are vegetatively incompatible and are therefore grouped in different VCGs. The strains are vegetatively compatible if they have the same allele at each and every incompatible locus. Frequently, the strains in the same VCG have similarity in genetic characteristics; the strains usually share more traits than strains in different VCGs (Leslie, 1993).

Stable heterokaryons in *Fusarium* were generated, first by inducing complementary nitrate non-utilizing (*nit*) mutants, which practically used to force heterokaryosis and subsequently to identify VCGs (Sidhu, 1986; Klittich *et al.*, 1986; Sunder and Satyavir, 1998). Several classes of *nit* mutants e.g. chlorate resistant nitrate utilizing (*crn*), *nit1, nit3* and NitM can be distinguished based on differential growth on phenotyping media containing different nitrogenous compounds as the sole

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source of nitrogen such as hypoxanthine (HX), ammonium tartrate (NH_4), natrium nitrate ($NaNO_3$) and natrium nitrite ($NaNO_2$) (Correll *et al.*, 1987). Heterokayon formation is a complex process that depends on more than just the capability of the strains to complement each other physiologically. Strains that are unable to form heterokaryon, including even between mutants derived from the same strain have been identified as heterokaryon self-incompatible (HSI), but if stable heterokaryons are formed, the strains are determined as heterokaryon self-compatible (HSC). The process is accomplished by pairing distinct *nit* mutants from different strains in order to classify the strains into VCG.

Several researchers have used VC test to determine the genetic diversity of *F. graminearum* (McCallum *et al.*, 2004), *F. moniliforme* (Puhalla and Spieth, 1985,; Klittich *et al.*, 1986; Sunder and Satyavir, 1998), *F. proliferatum* (Elmer, 1991; Elmer *et al.*, 1999), *F. subglutinans* (Zheng and Ploetz, 2002) and *F. verticillioides* (Chulze *et al.*, 2000). The objectives of this study were to investigate the genetic diversity of *F. fujikuroi* strains that were isolated from bakanae disease of rice and to classify the strains into VCGs.

MATERIALS AND METHODS

Fusarium Strains

A total of 79 strains of *F. fujikuroi* were isolated from bakanae-infected rice from major granary areas in Malaysia and Indonesia. All strains were purified through sub-culturing of single conidia and identified by using morphological characteristics following Burgess *et al.* (1994) before starting the *nit* mutant's generation.

Generation of Chlorate Resistant Sectors (CRSs) and Nit Mutants

Pure cultures of the strains were placed on a complete medium (CM; Correll *et al.*, 1987) for generation of actively growing colonies with a dense fungal growth. Preliminary studies on concentrations of chlorate in media i.e. minimal medium (MM) and potatoes dextrose agar (PDA) amended with 1.5%, 2.0%, 2.5% and 3.0% of $KClO_3$, hence designated as MMC and PDC respectively, were done earlier. The results indicated that 2.5% of $KClO_3$ concentration was selected as the optimum concentration for generation of CRSs. Plates of MMC and PDC containing 2.5% of $KClO_3$ were inoculated with 2 mm^2 mycelial discs taken from an actively growing colony and incubated under standard growth conditions (Salleh and Sulaiman, 1984). The colonies began to produce CRSs that appeared like sectors or fans after 7 days. The individual sectors from each colony was transferred to slant agar of MM containing $NaNO_3$ as the sole of nitrogen and incubated as above (Puhalla, 1985). Sectors that produced thin growth on MM were selected and considered to be *nit* mutants as they could not reduce nitrate present in the medium and subsequently kept at 4°C for phenotyping. Those that grew densely as wild type or reverted cultures were discarded.

Phenotyping of Nit Mutants

The physiological phenotyping of *nit* mutants were interpreted by their growth on MM that was modified by replacing $NaNO_3$ with $NaNO_2$ (0.5 gL^{-1}), hypoxanthine (0.2 gL^{-1}) or ammonium tartrate (1.0 gL^{-1}). The plates were incubated in complete darkness and colony growth was scored after 4 days of incubation. The colony growth of *nit* mutants on these nitrogen sources was recorded for identification of mutants as *nit1, nit3* or NitM following Klittich and Leslie (1988) (*Fig. 1*; Table 1). The mutants that grew vigorously on MM were either reverted, wild-type, mixed or *crn* and these cultures were discarded.

Pairing of Complementary Nit Mutants

Pairings of complementary *nit* mutants were made on MM following a procedure described by Leslie (1993). All inoculated plates were incubated at room temperature in complete darkness and the pairing results were recorded after 7 - 21 days. The strain was classified either HSC that formed heterokaryon (robust growth) or HSI when the *nit* mutants were unable to form heterokaryon (only thin growth at intersection of the colonies) at the line of contact between the mutants. Only *nit* mutants of HSC strains obtained from different parents were paired between each other. Two complementing strains were termed vegetatively compatible and designed as members of the same VCGs, whereas, strains that did not complement each other were termed vegetatively incompatible and assigned in different VCGs. VCGs of all *F. fujikuroi* strains were identified by pairing all possible combinations of *nit* mutants from each strain.

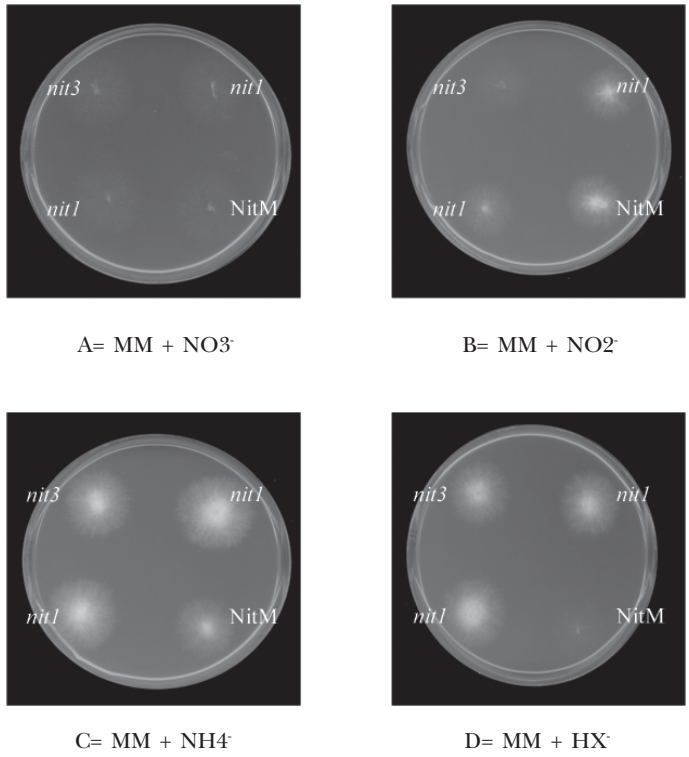


Fig. 1: Growth of three nitrate non-utilizing (nit) mutant phenotypes of F. fujikuroi *strain B3132P on media with one of four different nitrogen sources*

TABLE 1
Phenotyping of *nit* mutants based on colony growth on media with different nitrogen sources

Mutation[a]	Mutant type	Colony growth on MM with different nitrogen sources[b]			
		NO_3^-	NO_2^-	NH_4^-	HX^-
None	*crn*	+	+	+	+
Locus of nitrate reductase	*nit1*	-	+	+	+
Locus of pathway-specific regulator protein	*nit3*	-	-	+	+
Locus of molybdenum co-factor	NitM	-	+	+	-

[a]Carried out by Garrett and Amy (1978) and Marzluf (1981) based on analysis of mutants of *Aspergillus nidulans* and *Neurospora crassa*
[b]Colony growth on BM with different sources of nitrogen
+ = dense, fuzzy growth (wild-type); - = thin, transparent growth without aerial mycelium

RESULTS AND DISCUSSION

Generation of Chlorate-resistant Sectors (CRSs) and Nit Mutants

Spontaneous CRSs were recovered from all wild-type strains of *F. fujikuroi* when cultured on two media i.e. MMC and PDC containing chlorate as a toxic analogue of nitrate. Most of CRSs grew as thin expansive colonies with no aerial mycelium (*nit* mutants) because they were unable to utilize nitrate present in the media. The result revealed that 2.5% $KClO_3$ was the optimum concentration for *nit* mutant's generation. Majority of the *F. fujikuroi* strains and other species tested were insufficiently inhibited when lower or higher

concentrations of $KClO_3$ were applied. All the *nit* mutants had wild-type morphology on media containing an ammonium salt and produced thin sparse growth on nitrate medium. The *nit* mutants that produced wild-type growth on media containing both nitrite and hypoxanthine were classified as *nit1*; whereas, those with wild-type growth on medium with hypoxanthine or nitrite were identified as *nit3* or NitM, respectively.

Chlorate has been very practical for studying nitrate assimilation in *Fusarium* species (Correll *et al.*, 1986, 1987). Therefore, chlorate was used to induce CRSs for compatibility test. The number of CRSs obtained depended on the strains and amount of chlorate in the medium (Liu and Sundheim, 1996). Thus, the concentration of chlorate in the medium is an important variable. Therefore, a preliminary study on the effect of $KClO_3$ concentration on generation of *nit* mutants was conducted. Under normal growth condition, most fungi have the ability to utilize nitrate as a source of nitrogen by the internal reduction of chlorate into the ammonium form via nitrate and nitrite reductase (Garraway and Evan, 1984). However, the strains were unstable when grown on media containing chlorate and the colonies were unable to reduce chlorate to chlorite. This type of unstable growth on chlorate medium was previously observed in *Fusarium* spp. such as *F. oxysporum* (Correll *et al.*, 1987), *F. moniliforme* (Klittich and Leslie, 1988), *F. poae* (Lui and Sundheim, 1996) and *F. proliferatum* (Elmer *et al.*, 1999).

Nit mutants that have been generated are usually unable to reduce chlorate to chloride because of a lesion at one or more loci that control reductase, thus rendering them as a chlorate-resistance (Correll *et al.*, 1986). Previously, some researchers successfully recovered *nit* mutants from some *Fusarium* species associated with those in section Liseola i.e. *F. moniliforme* (Puhalla and Spieth, 1985; Klittich *et al.*, 1986; Sunder and Satyavir, 1998), *F. proliferatum* (Elmer, 1991; Elmer *et al.*, 1999), *F. subglutinans* (Zheng and Ploetz, 2002) and *F. verticillioides* (Chulze *et al.*, 2000). *Nit* mutants have also been recovered from other *Fusarium* species namely *F. poae* (Lui and Sundheim, 1996), *F. solani* (Hawthorne and George, 1996), *F. graminearum* (McCallum *et al.*, 2004) and *F. oxysporum* (Puhalla, 1984; Fernandez *et al.*, 1994; Vakalounakis and Fragkiadakis, 1999; Katan, 1999; Katan and Katan, 1999; Mes *et al.*, 1999) and other fungus, including *Colletotrichum acutatum* (Freeman *et al.*, 2000), *Neurospora crassa* (Marzluf, 1981), *Aspergillus flavus* (Papa, 1986) and *Verticillium albo-atrum* (Gordon *et al.,* 1986). In this study, complementary *nit* mutants recovered from each strain were categorized into one of the several phenotypic classes by their relative growth on phenotyping media containing different nitrogen sources. These classes of *nit* mutants presumably reflect mutations at a nitrate reductase structural locus (*nit1*); a nitrate assimilation pathway-specific regulatory locus (*nit3*), and the loci (at least five) that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity (NitM) (Klittich *et al.*, 1986). Some chlorate-resistant mutants, however, reverted to wild-type growth (dense growth); these mutants were classified as *crn* mutants.

Complementation Test

The complementation occurred more rapidly and subsequent growth of the resulting heterokaryon was more robust in the pairings between NitM and *nit1* or NitM and *nit3* mutants than those between *nit1* and *nit3* mutants, which usually formed weak heterokaryons. Results from this study showed that seventy-six strains (96.2%) were classified as HSC (*Fig. 2A*; Table 2). Majority of the strains from the same location were classified in the same group of VCG; e.g. most strains from Kuala Selangor were grouped in VCG A01, all strains from Melaka were in VCG A07 and all Indonesian strains were in VCG A03 as well as VCG A05.

Complementation did not occur between phenotypically distinct *nit* mutants from three strains i.e. A3059P, B3111P and D0673P, where no heterokaryon was formed as an indicator. The lack of complementation between phenotypically distinct *nit* mutants recovered from those strains, even after repeated attempts lead to the designation of these strains as HSI *(Fig. 2B)*. This lack of complementation observed due to a different inability to anastomose among the strains. HSI have been observed in several strains of *Fusarium* species such as *F. moniliforme* (Sidhu, 1986; Correll *et al.*, 1987, 1989), *F. oxysporum* (Jacobson and Gordon, 1988; Vakalounakis and Fragkiadakis, 1999), *F. solani* (Hawthorne and George, 1996), *F. proliferatum* (Al-Amodi, 2006) and *F. graminearum* (McCallum *et al.*, 2004). HSI has also been observed in other

filamentous Ascomycetes including *Neurospora crassa* and *Podospora anserine* (Saupe, 2000) and *Aspergillus sp.* (Papa, 1986). Correll *et al.* (1987) suggested that HSI might be due to anomaly of the *nit* mutants themselves, in the lack of anastomosis. In addition, the lack of complementation has also been caused by a double mutation in some of the *nit* mutants (Papa, 1986). On the other hand, Correll *et al.* (1989) reported that low frequency of hyphal fusions per mm^2 on MM after they were paired may also caused HSI. HSI strains averaged 0.2 and 1.1 hyphal fusions per mm^2 compared with 6.9 and 8.1 fusions per mm^2 for HSC, which formed heterokaryon normally. Correll *et al.* (1989) also reported that when HSI strains branched less frequently, then there would be fewer anastomosed cell formation.

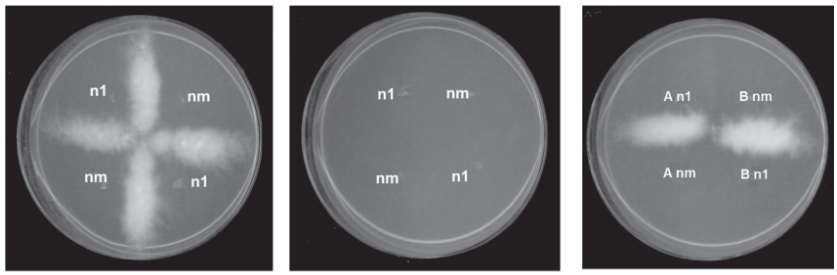
From the results, twenty-six distinct VCGs were identified among 76 strains of *F. fujikuroi* (Table 2), the ratio of VCGs to strains was 0.29. In comparison, Hsieh *et al.* (1977) reported 58 strains of *F. moniliforme,* belong to MP-C recovered from bakanae-infected rice were assigned to 22 VCGs (ratio; 0.37). In addition, Puhalla and Spieth (1985) recorded 12 VCGs of MP-C strains from China and Taiwan, whereas, Sunder and Satyavir (1998) recorded 10 VCGs for 28 strains with a ratio of 0.36. These phenomena took place, probably due to effects of the small numbers and sources of *Fusarium* strains. The HSC strains were classified in different VCGs when the mutants of the strains were unable to form heterokaryons during the compatibility test (*Fig. 2C*). The multiple VCGs of *F. fujikuroi* associated with bakanae disease of rice in Malaysia and Indonesia indicated the existence of substantial genetic diversity.

TABLE 2
Geographical location and VCGs of the 76 strains of *F. fujikuroi* isolated from rice in Malaysia and Indonesia, classified as HSC

VCGs	No.	Strain	Geographic locations	*Nit* mutants[1]		
				nit1	*nit3*	NitM
VCG A01	1	B2449P	Tanjong Karang, Selangor, Malaysia	+	-	+
	2	B2453P	Tanjong Karang, Selangor, Malaysia	+	-	+
	3	B3092P	Kuala Selangor, Selangor, Malaysia	+	-	+
	4	B3099P	Kuala Selangor, Selangor, Malaysia	+	+	+
	5	B3101P	Kuala Selangor, Selangor, Malaysia	+	+	-
	6	B3104P	Kuala Selangor, Selangor, Malaysia	+	+	-
	7	B3106P	Kuala Selangor, Selangor, Malaysia	+	+	+
	8	B3114P	Kuala Selangor, Selangor, Malaysia	+	+	-
	9	B3115P	Kuala Selangor, Selangor, Malaysia	+	+	+
	10	B3116P	Kuala Selangor, Selangor, Malaysia	+	+	-
	11	B3117P	Kuala Selangor, Selangor, Malaysia	+	+	-
	12	B3119P	Kuala Selangor, Selangor, Malaysia	+	+	+
	13	B3120P	Kuala Selangor, Selangor, Malaysia	+	-	+
	14	B3121P	Kuala Selangor, Selangor, Malaysia	+	+	+
	15	B3122P	Kuala Selangor, Selangor, Malaysia	+	-	+
	16	B3123P	Kuala Selangor, Selangor, Malaysia	+	-	+
	17	B3129P	Sungai Besar, Selangor, Malaysia	+	+	+
	18	B3131P	Sungai Besar, Selangor, Malaysia	+	-	+
	19	B3132P	Sungai Besar, Selangor, Malaysia	+	+	+
	20	B3133P	Sungai Besar, Selangor, Malaysia	+	+	-
	21	B3136P	Sungai Besar, Selangor, Malaysia	+	+	+
	22	B3139P	Sungai Besar, Selangor, Malaysia	+	+	-
	23	B3143P	Sungai Besar, Selangor, Malaysia	+	+	+
VCG A02	24	A3066P	Seberang Perak, Perak, Malaysia	+	+	+
	25	B3093P	Kuala Selangor, Selangor, Malaysia	+	+	+
	26	B3094P	Kuala Selangor, Selangor, Malaysia	+	-	+
	27	B3097P	Kuala Selangor, Selangor, Malaysia	+	-	+

Table 2 Cont.

	28	B3098P	Kuala Selangor, Selangor, Malaysia	+	-	+
	29	B3109P	Kuala Selangor, Selangor, Malaysia	+	+	+
	30	B3110P	Kuala Selangor, Selangor, Malaysia	+	-	+
	31	D3076P	Tumpat, Kelantan, Malaysia	+	+	+
VCG A03	32	I3206P	Padang, Sumatra, Indonesia	+	+	+
	33	I3209P	Padang, Sumatra, Indonesia	+	-	+
	34	I3211P	Padang, Sumatra, Indonesia	+	-	+
	35	I3213P	Padang, Sumatra, Indonesia	+	+	+
	36	I3215P	Padang, Sumatra, Indonesia	+	+	-
VCG A04	37	B3138P	Sungai Besar, Selangor, Malaysia	+	+	-
	38	B3140P	Sungai Besar, Selangor, Malaysia	+	+	+
	39	B3141P	Sungai Besar, Selangor, Malaysia	+	+	+
	40	B3142P	Sungai Besar, Selangor, Malaysia	+	-	+
VCG A05	41	I3207P	Padang, Sumatra, Indonesia	+	+	+
	42	I3208P	Padang, Sumatra, Indonesia	+	+	+
	43	I3214P	Padang, Sumatra, Indonesia	+	+	-
	44	I3216P	Padang, Sumatra, Indonesia	+	+	-
VCG A06	45	K0661P	Kampung Paya, Kedah, Malaysia	+	+	+
	45	K0686P	Kampung Paya, Kedah, Malaysia	+	+	+
	47	K3219P	Pendang, Kedah, Malaysia	+	+	+
	48	K3220P	Pendang, Kedah, Malaysia	+	+	-
VCG A07	49	M3234P	Merlimau, Melaka, Malaysia	+	-	+
	50	M3235P	Merlimau, Melaka, Malaysia	+	+	+
	51	M3236P	Merlimau, Melaka, Malaysia	+	+	+
	52	M3237P	Merlimau, Melaka, Malaysia	+	+	+
VCG A08	53	B3102P	Kuala Selangor, Selangor, Malaysia	+	+	+
	54	B3105P	Kuala Selangor, Selangor, Malaysia	+	-	+
VCG A09	55	B3127P	Sungai Besar, Selangor, Malaysia	+	+	+
	56	B3128P	Sungai Besar, Selangor, Malaysia	+	+	+
VCG A10	57	C3090P	Rompin, Pahang, Malaysia	+	+	+
	58	C3091P	Rompin, Pahang, Malaysia	+	+	+
VCG A11	59	P0654P	Seberang Perai, Penang, Malaysia	+	+	+
	60	P0655P	Seberang Perai, Penang, Malaysia	+	+	+
VCG A12	61	T3067P	Jabi, Terengganu, Malaysia	+	+	+
	62	T3068P	Jabi, Terengganu, Malaysia	+	+	+
VCG A13	63	A3052P	Seberang Perak, Perak, Malaysia	+	+	-
VCG A14	64	A3060P	Seberang Perak, Perak, Malaysia	+	-	+
VCG A15	65	B3100P	Kuala Selangor, Selangor, Malaysia	+	+	+
VCG A16	66	B3103P	Kuala Selangor, Selangor, Malaysia	+	+	+
VCG A17	67	B3107P	Kuala Selangor, Selangor, Malaysia	+	+	+
VCG A18	68	B3112P	Kuala Selangor, Selangor, Malaysia	+	-	+
VCG A19	69	B3113P	Kuala Selangor, Selangor, Malaysia	+	+	+
VCG A20	70	B3118P	Kuala Selangor, Selangor, Malaysia	+	+	+
VCG A21	71	B3130P	Sungai Besar, Selangor, Malaysia	+	+	+
VCG A22	72	B3134P	Sungai Besar, Selangor, Malaysia	+	+	-
VCG A23	73	D0674P	Peringat, Kelantan, Malaysia	-	+	+
VCG A24	74	I3422P	Samarinda, Kalimantan, Indonesia	+	-	+
VCG A25	75	K0688P	Kampung Paya, Kedah, Malaysia	+	-	+
VCG A26	76	R0621P	Cuping, Perlis, Malaysia	+	-	+



A B C

Fig. 2: Compatible interactions. A) HSC strain B) HSI strain; C) Two incompatible strains (A and B) were grouped in the different VCGs. n1 = nit1, nm = NitM

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Distribution, Morphological Characterization and Pathogenicity of *Fusarium sacchari* Associated with Pokkah Boeng Disease of Sugarcane in Peninsular Malaysia

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ABSTRACT

Pokkah boeng disease on sugarcane has been recorded in almost all countries where sugarcane is grown commercially. The objectives of this study were to survey the distribution of *Fusarium sacchari* associated with pokkah boeng disease throughout Peninsular Malaysia, to isolate and identify the causal organisms by using morphological characteristics, and to ascertain the pathogenicity of *F. sacchari* based on Koch's postulates. A total of 58 strains of *F. sacchari* were obtained throughout sugarcane plantations, small holders and household compounds within seven states i.e. Kedah, Perlis, Penang, Kelantan, Terengganu, Pahang and Johor in Peninsular Malaysia. The highest number of *F. sacchari* strains was obtained from Kedah (48%), followed by Perlis (25%), Penang (3%), Pahang (3%), Kelantan (5%), Terengganu (8%) and Johor (8%). For identification of *F. sacchari*, carnation leaves agar (CLA) and potato dextrose agar (PDA) media were used with emphasis for characterizations of colony features, growth rates, shapes and sizes of macroconidia and microconidia, conidiogeneous cells and chlamydospores. In plant house pathogenicity tests, healthy seedlings of sugarcane cultivar PS-81-362 were inoculated by injection and soaking techniques with conidial suspension ($2x10^6$ conidia/ml) of selected strains of *F. sacchari, F. proliferatum and F. subglutinans*. All strains of F. sacchari tested were pathogenic to sugarcane plants with disease severity index (DSI) varying from 0.3 to 5.0 (0 for no visible symptoms and 5 for plants with symptoms of twisted, wrinkled and shortened leaves or death). There were no significant ($p>0.05$) difference in DSI caused by strains of *F. sacchari* on variety PS-81-362 for both inoculation techniques, although they were significantly different compared with the control. This knowledge would be invaluable in developing our understanding on the interaction between *F. sacchari* with the host plants.

Keywords: Sugarcane, pokkah boeng, *Fusarium sacchari*

INTRODUCTION

In Peninsular Malaysia, the largest sugarcane plantations are situated in Felda Chuping, Perlis and Gula Padang Terap, Kedah for local consumption. The increased importation of sugarcane and other sugar crops from 5,304.48 tonnes in 1990 to 10,491.73 tonnes in 2004 based on the Food and Agriculture Organization of the United Nations (FAO) statistics indicates the demand for sugar in Malaysia. One of the limiting factors in sugarcane production is diseases caused by fungi, bacteria, viruses and

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nematodes that affect the quantity and/or quality of harvested crops (Edgerton 1955; Martin *et al.,* 1961; Sharma, 2006). Moreover, one or more diseases can occur on virtually every sugarcane plant in the field (Barnes, 1974; Hideo, 1988). Pokkah boeng (a Javanese word) is one of the most important diseases of sugarcane in Southeast Asia (Benigno and Quebral, 1977; Giatgong, 1980; Salleh, 1994, 2007; Semangun, 1988; Singh, 1980) and the world over (Agrios, 2005). Provided the environment is conducive, the disease can cause significant quality reduction in varieties with high sugar yields (Dohare *et al.,* 2003; Duttamajumder *et al.*, 2004). Approximately 40.8 - 64.5% sugars can be reduced from sugarcanes infected with pokkah boeng disease, depending on the cultivars (Dohare *et al.*, 2003).

Fusarium sacchari in the Section Liseola is generally responsible for causing this disease (Egan *et al.*, 1997; Nirenbergh and O'Donnell, 1998; Leslie and Summerell, 2006). The pathogen is transmitted by air currents (Martin *et al.*, 1961; Raid and Lentini, 1991) and airborne spores will colonize the leaves, flowers and stems of the plant (Burgess, 1981). The curve structure of macroconidia of *Fusarium* species can easily be dispersed by rain splash (Deacon, 2006).

The objectives of this study were to survey the distribution of *F. sacchari* associated with pokkah boeng disease throughout Peninsular Malaysia, to isolate and identify the causal organisms based on morphological characteristics, and to ascertain the pathogenicity of *F. sacchari* based on Koch's postulates. Data from the distribution, morphological characteristics of causal organism, and the pathogenicity test on healthy sugarcane will provide integrated information in formulating feasible control measures against the disease in Malaysia.

MATERIALS AND METHODS

Morphological Characteristics and Identification

Samples of sugarcane plants with pokkah boeng symptoms were observed and collected from sugarcane plantations, small holders and household compounds in seven states Kedah, Perlis, Penang, Kelantan, Terengganu, Pahang and Johor in Peninsular Malaysia between November 2004 and March 2005 (Table 1).

Fifty-eight single-spored cultural strains of fungi grown on PDA (potato dextrose agar) and CLA (carnation leaves agar) plates were used for identification based on morphological characteristics. PDA was used to measure fungal growth rates and pigmentation, while CLA was used to determine the shapes and sizes of macroconidia and microconidia, conidiogenous cells and chlamydospores formation. Morphological characteristics on CLA were observed after 7 - 10 days of incubation under standard growth conditions (Salleh and Sulaiman, 1984). The identification of *F. sacchari* was done based on the taxonomic guidelines by Nirenberg and O'Donnell (1998) and Leslie and Summerell (2006).

Pathogenicity Tests

The experiment was conducted on PS-81-362 sugarcane seedlings variety (provided by Gula Padang Terap Plantation in Kedah) between September 2005 and March 2006 in the plant house at the School of Biological Sciences, Universiti Sains Malaysia (USM). Each strain of *F. sacchari, F. proliferatum and F. subglutinans* isolates were grown on PDA plates as described by Salleh and Sulaiman (1984). Conidial suspension of each strain was prepared by pouring sterile distilled water onto 7 day-old cultures, shaken thoroughly, pooled, and the concentration was adjusted to $2x10^6$ conidia/ml by using a haemocytometer. Apparently healthy stalks were cut into a single-bud with one node. The stalks were surface-disinfected by dipping in 0.26% NaOCl solution for 30 min before inoculation with the inoculum.

Inoculation was carried out first by injecting the suspension (5 ml/plant) into young spindle leaves with a sterile syringe needle. In the second technique, single-bud stalks (one-node) were soaked overnight at room temperature in 500 ml of the inoculum suspension in a plastic container. The single-bud stalk pieces were then planted in sterile garden soil (autoclaved for 20 min at 1.05 $kgcm^{-2}$). All control plants were inoculated with sterile water. Every treatment was replicated three times. After 15 days of inoculation (dai), the plants were checked twice a week for six months in the plant house. The day temperature was 30.3 - 35.1°C and the night temperature was 23.3 - 30.6°C. All tissues of sugarcane plants inoculated with *F. sacchari* strains that showed pokkah boeng symptoms and controls were re-isolated for *F. sacchari* and re-identified as described above.

TABLE 1
Source of selected strains of *F. sacchari* used in pathogenicity tests

Species	Strain no.	Locality
F. sacchari	K3257U	Gula Padang Terap, Kedah
	K3268U	Gula Padang Terap, Kedah
	K3271U	Felda Chuping, Perlis
	R3287U	Felda Chuping, Perlis
	K3305U	Gula Padang Terap, Kedah
	D3325U	Rantau Panjang, Kelantan
	K3352U	Baling, Kedah
	T3334U	Setiu, Terengganu
F. proliferatum	K3240U	Gula Padang Terap, Kedah
	K3241U	Gula Padang Terap, Kedah
	K3245U	Gula Padang Terap, Kedah
	C3336U	Kuantan, Pahang
	C3340U	Kuantan, Pahang
F. subglutinans	K3267U	Gula Padang Terap, Kedah
	K3270U	Gula Padang Terap, Kedah
	K3308U	Kupang, Kedah
	R3276U	Felda Chuping, Perlis
	R3281U	Felda Chuping, Perlis

Disease Assessment

The symptoms were scored based on disease scale from 0 to 5 by following the scoring system devised by Elmer (2002) (Table 2) with slight modifications for sugarcane at 15, 30, 60, 90, 120, 150 and 180 days after inoculation (dai).

Disease Severity Index (DSI) was calculated by using the disease scale as follows:

$$DSI = \frac{\sum(A \times n)}{\sum B} \times \frac{100}{6}$$

A = disease scale (0, 1, 2, 3, 4 or 5)
n = number of stalks for each disease scale
B = total number of stalks

Statistical Analysis

DSI was analysed using non-parametric statistics; Friedman test and Mann-Whitney tests ($P<0.05$) were used to compare the inoculation techniques (soaking and injection) using SPSS programme version 11.5.

RESULTS AND DISCUSSION

The general observable symptoms of pokkah boeng in the field are yellowing or chlorosis of young leaves with some showing red specks at early stages of infection, followed by crumpled and twisted leaves. These symptoms were mostly found in Padang Terap, Kedah and Chuping, Perlis, the two biggest sugarcane plantations in Peninsular Malaysia. Throughout the samplings, the sugarcane leaves were the main parts that showed noticeable symptoms of pokkah boeng. This occurred due to the pathogen involved being reported to be an air borne fungi (Martin *et al.*, 1961; Raid and Lentini, 1991). The highest numbers of strains (48%) were obtained from Kedah, followed by Perlis (25%), Terengganu (8%), Johor (8%), Kelantan (5%), Penang (3%) and Pahang (3%) from 58 strains (See *Fig. 1*).

TABLE 2
Disease severity index (DSI) used in disease assessment following Elmer (2002) with slight modifications for sugarcane

Disease scale	Symptom
0	no visible symptoms
1	slight chlorosis on young leaves
2	<10% of the leave areas showing chlorosis and/or 10% of the plant with twisted leaves symptoms
3	11% to 25% of the plant with twisted leaves symptoms
4	26 to 50% of the plant with twisted leaves symptoms and/or reddish specks develop within chlorotic parts
5	51 to 100% of the plant with twisted, wrinkled and shortened leaves symptoms or plant death

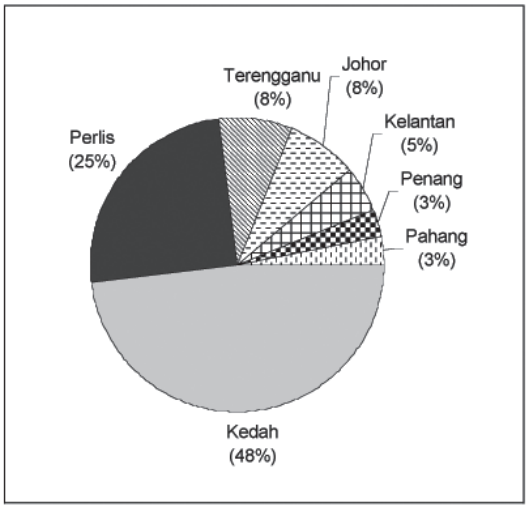

Fig. 1: Distribution of F. sacchari *according to states surveyed in Peninsular Malaysia*

All strains were identified as *F. sacchari* based on their morphological characteristics (*Fig. 2*). On PDA, the growth was rapid with abundance of mycelia that were colorless to pale violet and grayish violet. The pigmentations were colorless to grayish orange becoming purple with age. Colonies reached 2.3-3.7 cm in diameter at 25°C and 2.1-3.4 cm at 30°C after 3 days. Conidia produced on PDA were not uniform and not suitable for identification purposes. On CLA plates, *F. sacchari* were characterized by formation of microconidia that were produced abundantly in false-heads (*Fig. 2A*) from monophialides (*Fig. 2B*) and also polyphialides (*Fig. 2C*). The conidiophores were mostly branched at one level (*Fig. 2D*). Macroconidia (*Fig. 2E*) produced on CLA were sparse, slender, slightly falcate and thin-walled, 3 - 4 septate and the size ranges from 19.0 - 46.2 x 2.7 - 3.3 µm. The microconidia (*Fig. 2F*) were oval and slender 0 - 1 septate with sizes ranging from 5.4 - 15.0 x 1.9 - 4.1 µm. CLA is a natural substrate medium (Fisher *et al.*, 1982) that promotes sporulation rather than mycelial growth and at the same time false-heads can be seen directly in situ. On CLA plates the conidia were more abundant and uniform. The chlamydospores and microconidial chains were absent and these criteria are important in differentiating other Fusarium species in the Section Liseola (Nelson *et al.*, 1983).

Within six months of observation, the symptoms of pokkah boeng on young sugarcane plants variety PS-81-362 inoculated with different strains of *F. sacchari* varied. On day 15 until day 60 after inoculation, most of the inoculated sugarcane plants showed typical symptoms of pokkah boeng with chlorosis on the young leaves and some with twisted leaves. On day 90 until day 180 after inoculation, more twisted leaves appeared and reddish specks developed on the chlorotic parts. Some plants died and mycelium can be seen clearly at the nodes. Other young sugarcane plants which were inoculated with *F. proliferatum, F. subglutinans* and control plants remained healthy. There were no visible symptoms of pokkah boeng on sugarcane inoculated with *F. subglutinans* and *F. proliferatum* for both injection and soaking techniques. *F. sacchari* that were re-isolated from inoculated plants with pokkah boeng symptoms were identical to the original organisms isolated.

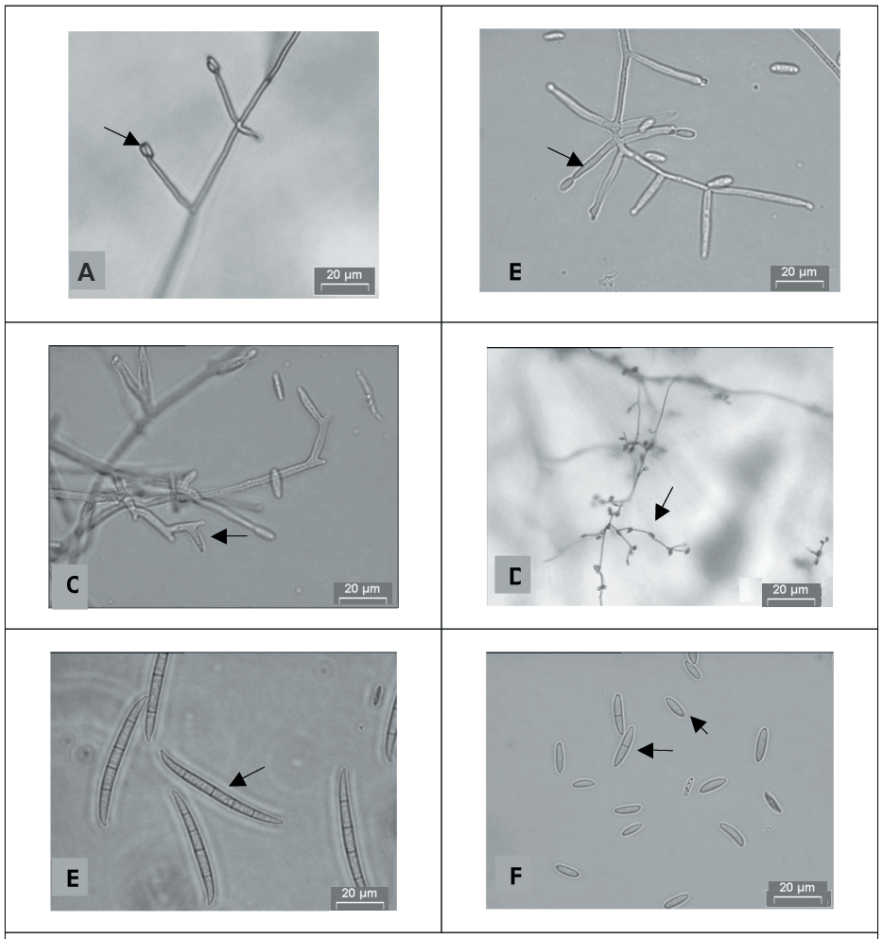

Fig. 2: Morphological characteristics of F. sacchari *from CLA; (A) Microconidia in false heads, (B) Simple monophialidic conidiophores, (C) Simple polyphialidic conidiophores, (D) Aerial mycelium with simple and prostrate conidiophores, (E) macroconidia with 4 septate, (F) 0 - 1 septate microconidia*

The DSI on variety PS-81-362 that was inoculated with strains of *F. sacchari* using injection (*Fig. 3*) and soaking (*Fig. 4)* techniques showed a steady increased in disease severity from 15 to 180 dai. Isolate R3287U showed the highest DSI for both techniques i.e. 4.3 for injection (*Fig. 3*) and 5.0 for soaking (*Fig. 4*) techniques. All strains of *F. sacchari* tested were pathogenic to sugarcane plants, as indicated by a mean DSI that was significantly different ($p<0.05$) from 15 to 180 dai, compared to that of control plants. There was no significant ($p>0.05$) difference in DSI between inoculation techniques by soaking and injection.

The results of inoculation tests in the plant house confirmed that pokkah boeng disease of sugarcane was caused by *F. sacchari*. The symptoms on inoculated plants were similar to those observed in the fields. The development of disease symptoms in the plant house was completed in six months when the syndromes of the typical pokkah boeng disease were observed. Sugarcane plants at actively growing stages are more susceptible to infection than the older canes (Martin *et al.*, 1961; Raid and Lentini, 1991). Therefore, susceptible sugarcane plants are easily infected with pokkah boeng disease during the early growth stages. The DSI for all sugarcane plants inoculated with different strains *F. sacchari* by both techniques steadily increased from 90-180 dai.

Both inoculation techniques were successful in inducing pokkah boeng symptoms on variety PS-81-362. Therefore, inocula at the concentration

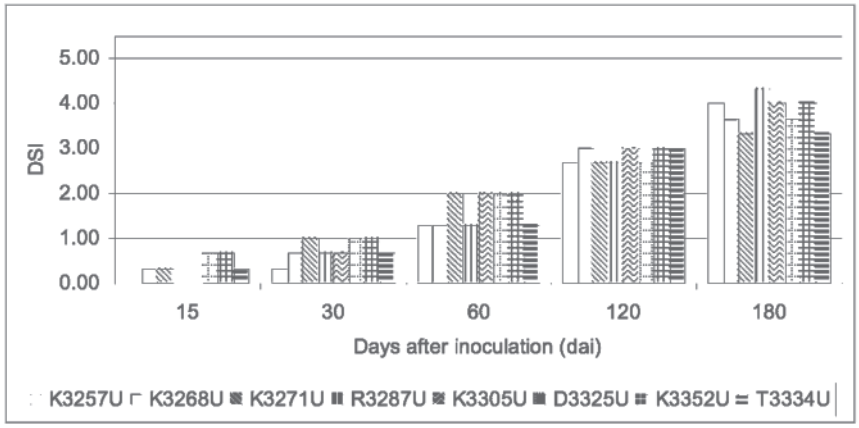

Fig. 3: Disease severity index (DSI) of young sugarcane plants var. PS-81-362 at different days after inoculation using injection technique with selected strains of F. sacchari

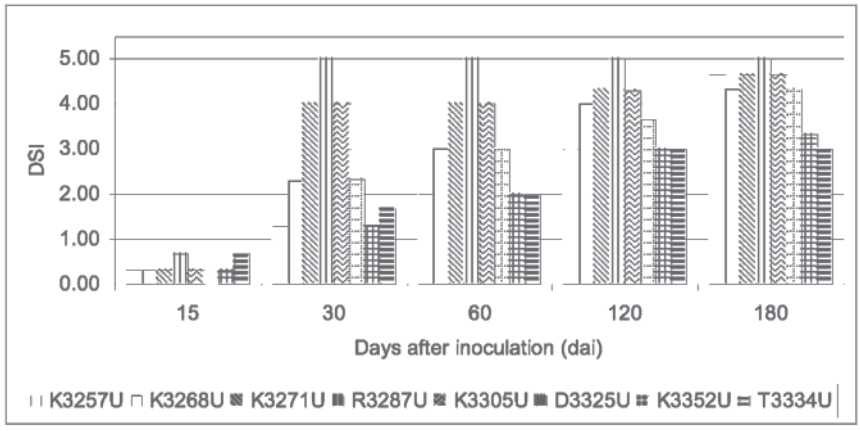

Fig. 4: Disease severity index (DSI) of young sugarcane plants var. PS-81-362 at different days after inoculation using soaking technique with selected strains of F. sacchari

of 2 x 10^6 conidia/ml injected into healthy plants appeared to be successfully transferred to the tender and fragile spindle leaves that facilitate pathogen penetration. Natural openings such as stomata and hydathodes on the leaves could also facilitate spore germination and penetration host of the tissues (Dickinson and Lucas, 1982; Agrios, 2005). For the soaking technique, dipping the plant into the inoculum suspension overnight provided direct exposure of the pathogen to the host making the penetration process easier. The duration of stalk immersion in the inoculum suspension influenced disease severity with longer immersion resulting in more severe symptoms (Aragaki, 1975).

Previous studies indicated that pokkah boeng disease on sugarcane was caused by *F. moniliforme var. subglutinans* in Peninsular Malaysia (Geh, 1973) but recent research reported that *F. sacchari* is the pathogen (Egan *et al.*, 1997; Nirenbergh and O'Donnell, 1998; Leslie and Summerell, 2006). *F. sacchar*i was successfully re-isolated from all symptomatic leaves that were inoculated in the plant house.

CONCLUSIONS

The study showed that pokkah boeng disease was widely distributed in growing sugarcane areas of in seven states i.e. Kedah, Perlis, Penang, Kelantan, Terengganu, Pahang and Johor, in

Peninsular Malaysia. Pathogenicity test results confirmed that the disease was caused by *F. sacchari*. The morphological characteristics that were described and derived from this research will assist in correct and quick identification of *F. sacchari* and other closely related *Fusarium* species in Section Liseola. The data from the distribution, morphological characteristics and the pathogenicity test on healthy sugarcane will provide integrated information in formulating feasible control measures against this disease in Malaysia.

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Morphometric Analysis as an Application Tool to Differentiate Three Local Pen Shells Species

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ABSTRACT

Pen shells are generally large bivalves, triangular in shape with light yellow-brown to dark brown colour. They live with their pointed end embedded in sediment, attached by abundant fine byssal threads. Pen shells are relatively common at the sandy substrate of the seagrass beds inhabited by *Enhalus acoroides*, *Halophila* spp. and *Cymodocea serulata,* shallow lagoons and coral rubble areas. Specimens of *Pinna bicolor* Gmelin, *Pinna deltodes* Menke and *Pinna atropurpurea* Sowerby were collected from the seagrass beds of Merambong Shoal, Tanjung Adang Shoal and Merambong Island off South Western coast, of Johor, Peninsular Malaysia for morphological studies from August 2005 to June 2006. Naturally, *P. deltodes* is largely found on hard bottom substrate while, *P. bicolor* and *P. atropurpurea* are more associated with soft substrate. Physically, there was no significant external morphological difference between *P. bicolor, P. deltodes* and *P. atropurpurea*. The species was identified on the basis of nine internal and external characteristics of the valves. The three species were found to be morphologically different ($P<0.05$) on the basis of four major characteristics viz width of sulcus (WS), distance between posterior adductor muscle to posterior dorsal nacreous layer (PAMPDNL), dorsal posterior margin length (DPML) and shell width length (WL).

Keywords: Pen shells, Pinnidae, morphometric analysis

INTRODUCTION

Pen shells of the family Pinnidae are widely distributed in the Indo-Pacific from southeastern Africa to Melanesia and New Zealand, north to Japan and to New South Wales and New Zealand (Butler and Keough, 1981; FAO, 1998). Pen shells are also found in Mediterranean and American waters (Rosewater, 1961; Butler, 1987; Zavodnik *et al.,* 1991; Munguia, 2004). *Atrina* and *Pinna* species exist as metapopulations, composed of small groups or patches of individuals. Pen shells are generally large bivalves (30-48 cm long), triangular in shape, thin, shell tapering to a point and light yellow-brown to dark brown in color. Pen shells live with their pointed end embedded in sediments, attached by abundant fine byssal threads (Keen, 1958; FAO, 2002; Tyler-Walters, 2004).

The Pinnids are characterized by a unique, dorsal pallial organ and by the triangular shell shape associated with partial to almost complete burial in the substratum (Beesely *et al.,* 1998). They are extremely proneto breakage from the storm waves and shifting sands, but they have considerable potential of shell repair through utilization of several unique structures that remove debris from the mantel cavity and repositioning of the mantel to form new shell. Repaired shells are seldom shaped like the original, however resulting in great variation in form and sculpture (Rosewater, 1982).

For this reason it is often difficult to identify a specimen without careful examination and even then considerable experience with specific variation may be required to recognize a species with any degree of confidence. A few attempts

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have been made in the literature to present an orderly classification of *Pinnidae* in different areas of the world (Turner and Rosewater, 1958; Rosewater, 1961). The interior nacreous area is divided by an anteroposteriorly directed sulcus into dorsal and ventral lobes, and is a member of the genus *Pinna*. Examination on nacreous areas was distinguished to confirm their identity (Rosewater, 1982; Scheltema, 1983).

A previous report by Winckworth (1929) only described the morphology of pen shell from South India and Ceylon but not in full detail. Rosewater (1961) review provided a key to pen shells in the Indo-Pacific and identified three recent genera *Pinna, Atrina* and *Streptopinna* with seven species in Australia. Subsequently, Scheltema (1983) identified an eighth species.

Pen shells show considerable morphological variation creating taxonomic confusion for many taxonomists (Perry and Larsen, 2004). *Pinna* species are reported to exist with quite similar morphological characteristics when compared to *Atrina* species (Scheltema, 1983). Physically, *Pinna bicolor, P. deltodes and P. atropurpurea* are morphologically similar. Species cannot be differentiated based on shape, colour and others external morphologies. In Malaysia, most of the *Pinna* species live in the same habitat and substratum especially in muddy and sand muddy areas (Idris *et al.*, 2006).

At present, there has been no documentation on the fisheries aspects nor the taxonomic status of Malaysian water Pinnidae. This study started from August 2005 to June 2006. The aim of the study is to generate some information on the taxonomy of the pen shells in the Sungai Pulai seagrass beds. These areas were chosen because of the natural abundance of pen shells that are associated with the seagrasses. In this study, morphometric analysis was used as a tool to differentiate three *Pinna* species. Nine different morphometric characteristics were applied to differentiate three *Pinna* species existing in the study areas.

MATERIALS AND METHODS

Field Sampling of Pen Shell

Specimens of pen shell were collected from Merambong Shoal (N1° 19' 55.62" E103° 35' 57.75"), Tanjung Adang Shoal (N1° 19' 48.03" E103° 33' 59.44") and Merambong Island (N1° 18' 54.83" E103° 36' 33.37") off South Western Johor coast, Malaysia (*Fig. 1*) from August 2005 to June 2006.

By using a hand scope, specimens were removed from the substratum and placed into plastic bags and labeled. All specimens were placed on ice and immediately transported to the laboratory. For morphometric analysis, specimens were stored in a 70% ethanol solution for one week and then placed in a drying oven for 48 hour prior to analysis (Claxton *et al.*, 1997).

Morphometric Characters

Seventy nine individuals were collected from the study areas during low tide and were transferred to the laboratory for identification, labeled specimens were stored and images were taken and recorded. Shells were measured using MITUTOYO digital vernier caliper for total length and other shell morphometric characteristics.

The following parameters were measured :- length of anterior to posterior adductor muscle length (APAML) (1), posterior adductor muscle to posterior shell margin (PAMPSM) (2), dorsal posterior margin length (DPML) (3), dorsal margin length (DML) (4), width length (WL) (5), total length (TL) (6), width of sulcus (WS) (7) posterior adductor muscle to posterior dorsal nacreous layer (PAMPDNL) (8) and dorsal nacreous length (DNL) (9) (*Figs. 2C, 3C* and *4C*). For the identification of the various morphological structures on pen shell species, the methods of Winckworth (1929), Rosewater (1961), Butler and Keough (1981), Scheltema (1983), FAO (1998 and 2002) Ubukata (2002) and Perry and Larsen (2004) were used.

Statistical Analysis

Data was also analysed using SPSS (Statistical Package for Social Science) v13 computer statistical program to compare the means for every morphometric characteristic value between *P. bicolor, P. deltodes* and *P. atropurpurea*. One-way Analysis of variance (ANOVA) was used to compare the morphometric characters of each species. The Tukey HSD post-hoc test was then used to determine which of the characters was significantly different from the others. The morphometric shell measurement data was also used to determine the ratio between the morphometric characteristics of the *Pinna bicolor, P. deltodes* and *P. atropurpurea*.

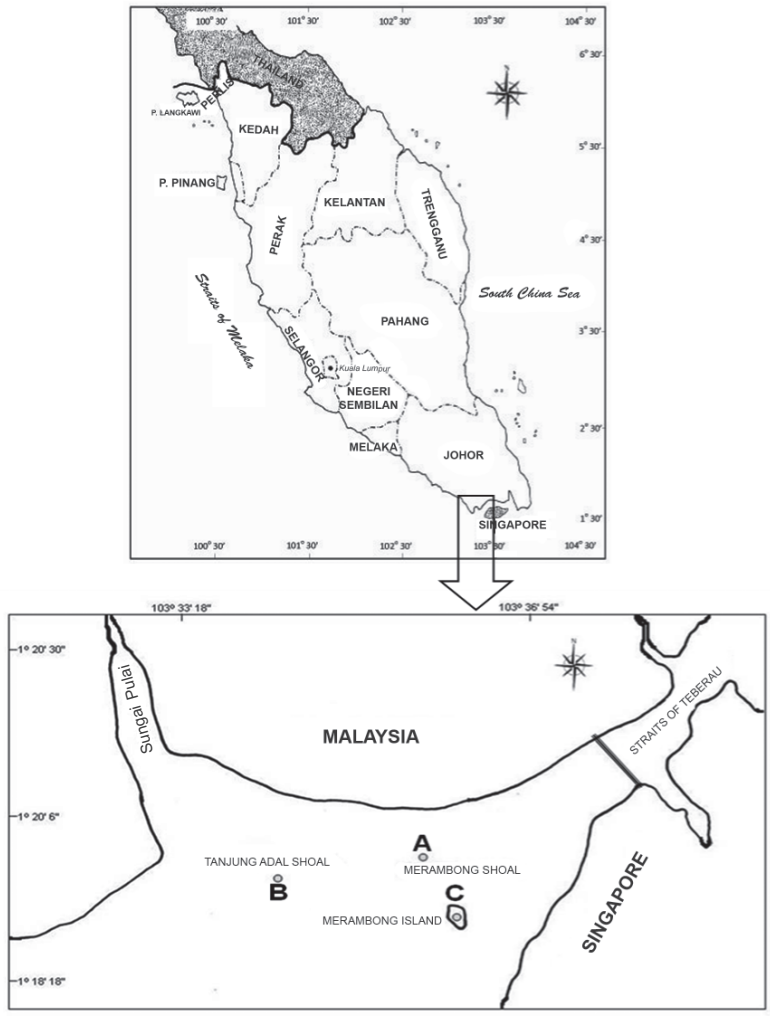

Fig. 1: Map showing the sampling areas. (A)Merambong shoal, (B)Tanjung Adang shoal and (C) Merambong island off South Western Johor coast, Malaysia

Principle component analysis (PCA) was carried out using the correlation matrix of the log-transformed morphometric variables (APAML, PAMPSM, DPML, DML, WL, TL, WS, PAMPDNL and NL) using the program PRIMER v5 (Plymouth Routines In Multivariate Ecological Research) (Clarke and Gorley, 2001). An inspection of the loadings on the principle components was done to determine which represented 'size' and had coefficients of the same sign and which represented 'shape' and had coefficients of mixed signs (Reyment *et al.*, 1984).

RESULTS

Morphometric Characters

A total of 39 specimens of *Pinna bicolor,* 19 specimens of *P. deltodes* and 21 specimens of *P. atropurpurea* were examined for non-overlapping character states. The range and mean values of different morphometric characters of the three local pen shells are shown in Table 1. Differences in the distribution of total width length, dorsal posterior margin length, posterior adductor muscle to posterior dorsal nacreous layer and width of sulcus distinguish *P. bicolor, P. deltodes* and *P. atropurpurea* and correlate with differences

TABLE 1
Analysis of variance (One-way ANOVA) of nine morphometric characteristics showed four characteristics were significantly different (P<0.05) between *P. bicolor*, *P. deltodes* and *P. atropurpurea*

Morphometric Characteristics	Species	N	Range (mm)	Mean (±SD)	F_{value}	Sig.
TL (mm)	*P. bicolor*	39	180.52 - 248.47	225.11(± 22.82)^{a}	2.693	0.74^{ns}
	P. deltodes	19	115.57 - 310.54	225.89 (± 52.95)^{a}		
	P. atropurpurea	21	141.06 - 248.60	206.23 (± 31.09)^{a}		
WL (mm)	*P. bicolor*	39	57.83 - 131.63	93.64 (± 18.23)^{a}	9.05	0.00*
	P. deltodes	19	61.62 - 149.28	116.68 (± 22.16)^{b}		
	P. atropurpurea	21	71.69 - 140.07	103.56 (± 19.19)^{a,b}		
DML (mm)	*P. bicolor*	39	159.75 - 228.80	202.86 (± 21.17)^{a}	2.614	0.80^{ns}
	P. deltodes	19	109.86 - 266.16	200.97 (± 43.86)^{a}		
	P. atropurpurea	21	58.16 - 240.13	182.87 (± 40.71)^{a}		
DPML (mm)	*P. bicolor*	39	25.74 - 74.77	47.43 (± 12.56)^{a}	22.772	0.00*
	P. deltodes	19	33.22 - 92.19	71.86 (± 16.69)^{b}		
	P. atropurpurea	21	34.24 - 64.91	52.54 (± 9.82)^{a}		
PAMPSM (mm)	*P. bicolor*	39	60.10 - 108.17	84.57 (± 11.97)^{a}	0.942	0.39^{ns}
	P. deltodes	19	49.10 - 132.43	89.74 (± 19.79)^{a}		
	P. atropurpurea	21	66.48 - 105.14	84.87 (± 11.19)^{a}		
APAML (mm)	*P. bicolor*	39	95.64 - 153.67	125.71 (± 14.90)^{b}	4.937	0.10^{ns}
	P. deltodes	19	58.22 - 161.12	120.39 (± 30.39)^{a,b}		
	P. atropurpurea	21	71.83 - 138.38	108.28 (± 18.57)^{a}		
DNL (mm)	*P. bicolor*	39	103.37 - 159.39	133.27 (± 13.79)^{b}	3.471	0.30^{ns}
	P. deltodes	19	61.09 - 169.03	129.13 (± 29.14)^{a,b}		
	P. atropurpurea	21	67.39 - 144.21	118.88 (± 20.69)^{a}		
PAMPDNL (mm)	*P. bicolor*	39	3.47 - 10.80	6.78 (± 2.34)^{b}	19.984	0.00*
	P. deltodes	19	0.07 - 8.07	3.09 (± 2.23)^{a}		
	P. atropurpurea	21	1.63 - 10.47	7.34 (± 2.49)^{b}		
WS (mm)	*P. bicolor*	39	0.72 - 3.64	1.59 (± 0.60)^{a}	65.381	0.00*
	P. deltodes	19	2.16 - 6.07	4.60 (± 1.34)^{b}		
	P. atropurpurea	21	1.15 - 3.14	2.19 (± 1.05)^{a}		

* highly significant at (P<0.05); ns=not significant at (P>0.05)
Mean±SD subjected to Tukey HSD Post-hoc test
Mean±SD in column with dissimilar superscript letter is significantly different (P≤0.05)

Abbreviations : TL=Total Length; WL=Width Length; DML=Dorsal Margin Length; DPML=Dorsal Posterior Margin Length; PAMPSM=Posterior Adductor Muscle to Posterior Shell Margin; APAML=Anterior to Posterior Adductor Muscle Length; NL=Dorsal Nacreous Length; PAMPDNL=Posterior Adductor Muscle to Posterior Dorsal Nacreous Layer; WS=Width of Sulcus

in position of the posterior adductor muscle scar in relation to the dorsal nacreous lobe (*Figs. 2C, 3C* and *4C*).

Analysis of variance (One-way ANOVA) showed that out of nine morphometric data, four characteristics (WL=Width Length, DPML=Dorsal Posterior Margin Length, PAMPDNL=Posterior Adductor Muscle to Posterior Dorsal Nacreous Layer and WS=Width of Sulcus) were highly significant (P<0.05) between *P. bicolor, P. deltodes* and *P. atropurpurea*. The graph of the means boxplots from nine morphometric characteristic also show four (B, D, H and I) morphometric characteristics that were significantly different for mean values of the morphometric characters between *P. bicolor, P. deltodes* and *P. atropurpurea* (*Fig. 5*).

The value of the first three principle components performed on the nine raw morphometric data are presented in Table 2 and *Fig. 6*. The positive and negative values indicate shape variation. The negative value was not considered as a good discriminant as shown by four characters (DPML= -1.02, PAMPSM= -0.35, PAMPDNL= -2.98 and WSL= -2.28) in the first component. The component loadings were also very high for most of the variables accounted for by the first principle component, which described 99.57% cumulative variance within the samples.

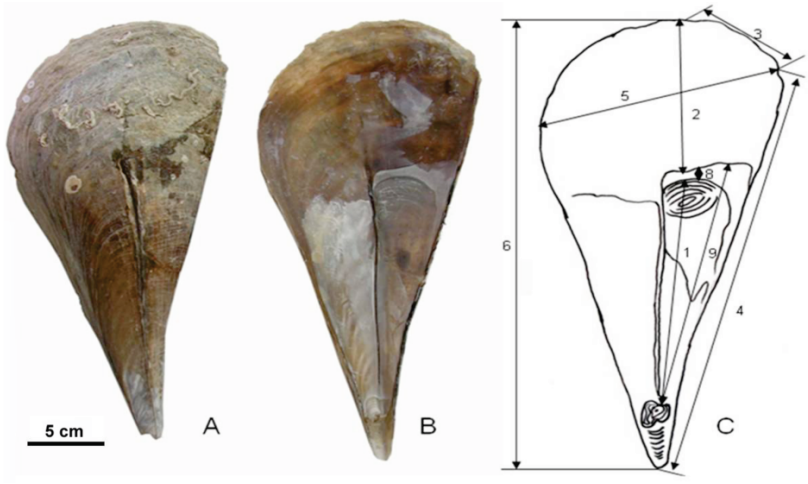

Fig. 2: Exterior of right valve of Pinna bicolor *showing (A) outer and inner surface, (B) internal view of left valve and(C) diagrammatic sketch of internal part of* P. bicolor *and the their characteristics view*

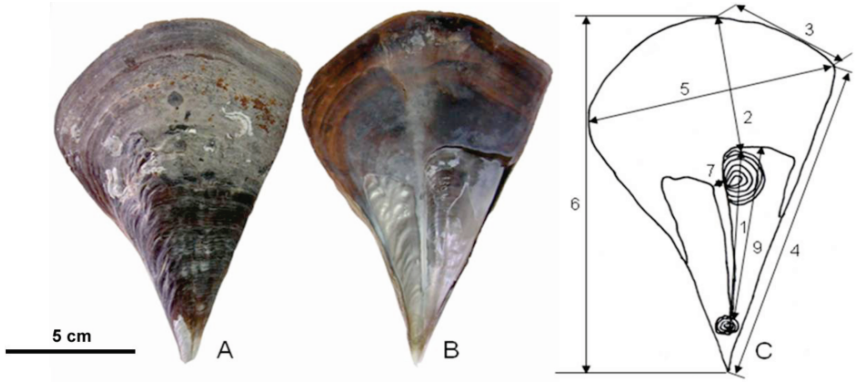

Fig. 3: Exterior of right valve of Pinna deltodes *showing (A) outer and inner surface, (B) internal view of left valve showing the posterior adductor muscle protruded on posterior margin of dorsal lobe and the width of sulcus between dorsal and ventral lobe of nacreous layer and (C) diagrammatic sketch of internal part of* P. deltodes *and the their characteristics view.*

Morphometric Ratio

An analysis of shell measurement and their ratio also showed significant differences ($P<0.05$) between *P. bicolor, P. deltodes* and *P. atropurpurea* (Table 3). The ratios on width length and total length, width of sulcus, posterior adductor muscle to dorsal posterior dorsal nacreous layer and anterior to posterior adductor muscle and posterior adductor muscle to posterior shell margin showed significant differences for the mean values.

Shell width length for *P. deltodes* and *P. atropurpurea* were three quarters of shell length and flared more posteriorly when compared to *P. bicolor*. The mean value of *P. deltodes* and *P. atropurpurea* were higher than *P. bicolor* with 116.68 ± 22.16 mm (n=19), 103.56 ± 19.19 mm (n=21) and 93.64 ± 18.23 mm (n=39) (*Fig. 7*, no. 5). *P. deltodes* also showed a higher value for dorsal posterior margin length with 71.86 ± 16.69 mm when compared to *P. bicolor* with 47.43 ±12.56 mm and *P. atropurpurea* with 52.54 ± 9.82 mm (*Fig. 7*, no. 3).

In all *P. deltodes* examined, the posterior adductor muscle scar was found to touch or near to the posterior edge of the dorsal nacreous lobe (*Fig. 8*, no. 8) whereas in *P. bicolor* and *P. atropurpurea* shells, the posterior adductor muscle

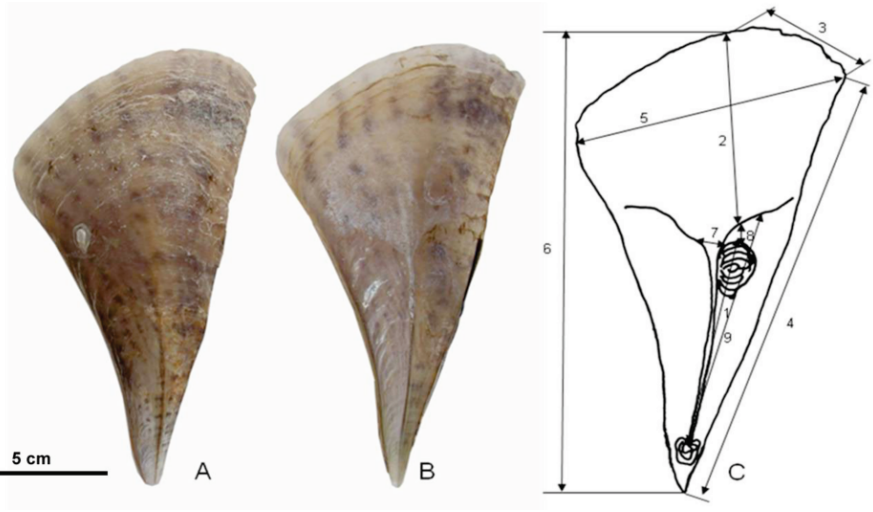

Fig. 4: Exterior of right valve of Pinna atropurpurea *showing (A) outer and inner surface, (B) internal view of left valve, showing dorsal and ventral lobes of nacreous layer forms posteriorly oblique truncated and sloping from sulcus and the nacreous lobes forming deep 'V' shape and (C) diagrammatic sketch of internal part of* P. atropurpurea *and the their characteristics view*

was always found to lie within the dorsal nacreous lobe (*Figs. 2C, 3C, 4C, 8D - F*, no. *8*). The mean values of posterior adductor muscle to posterior dorsal nacreous layer for *P. deltodes* was lower with a value of 3.09 ± 2.23 mm when compared to *P. bicolor* and *P. atropurpurea* with values of 6.78 ± 2.34mm and 7.34 ± 2.49mm respectively (Table 1).

The shape of the dorsal and ventral nacreous lobes was very obvious for the three specimens (*Figs. 2, 3, 4, 8 A-B* and *9*). Nacreous area iridescent, roughly occupying the anterior half of the shell and is divided along most of its length by a longitudinal sulcus. Dorsal and ventral lobes of nacreous area are moderately well separated. The dorsal lobe of the nacreous area for *P. bicolor* (*Figs. 2B, C, 8A*), usually extends farther posteriorly than the ventral lobe near to the sulcus. Its posterior margin is truncated to slightly oblique. Ventral lobe may extend obliquely farther posteriorly near the ventral margin or it is sometimes unevenly truncated and shorter than the dorsal lobe. The width of sulcus for *P. bicolor* was in the range of 0.72 mm - 3.64 mm with a mean value of 1.59 ± 0.60 mm (*Figs. 2C, 8A*, no.7).

In addition, *Pinna deltodes* (*Figs. 3B, C, 8B*) dorsal lobe occupies two-third of the shell. The dorsal nacreous area extends farther posteriorly than the ventral lobe and posterior margin was truncate. The ventral lobe extend obliquely farther posterior but quite far from the ventral margin or sometimes rounded and shorter than the dorsal lobe. The width of sulcus for *P. deltodes* ranged from 2.16 mm - 6.07 mm with a mean value of 4.60 ± 1.34 mm (*Figs. 3C, 8B,* no.7). The dorsal and ventral lobes of nacreous layer for *P. atropurpurea* showed similar high lobes and forming deep 'V' shape (*Figs. 4B, C, 8C*). The dorsal and ventral lobes of nacreous layer forms posteriorly, oblique truncated and sloping from sulcus. The width of sulcus ranged from 1.15 mm - 3.14 mm with a mean value of 2.19 ± 1.05 mm (*Fig. 4C,* no. 7).

DISCUSSION

Some morphometric characters of pen shell have been recorded by Scheltema (1983) based on the specimens of *P. deltode*s and *P. bicolor* from Australia. Only seven characteristics were measured during that study. In this study, two additional characteristics were included totaling nine characters for a more comprehensive conventional morphometric data analyses to determine the most appropriate for pen shell identification.

Physically, colour, size and shape cannot be used as indicators to differentiate these three *Pinna* species. Previously, Rosewater (1961) reported that *P. deltodes* and *P. atropurpurea* were

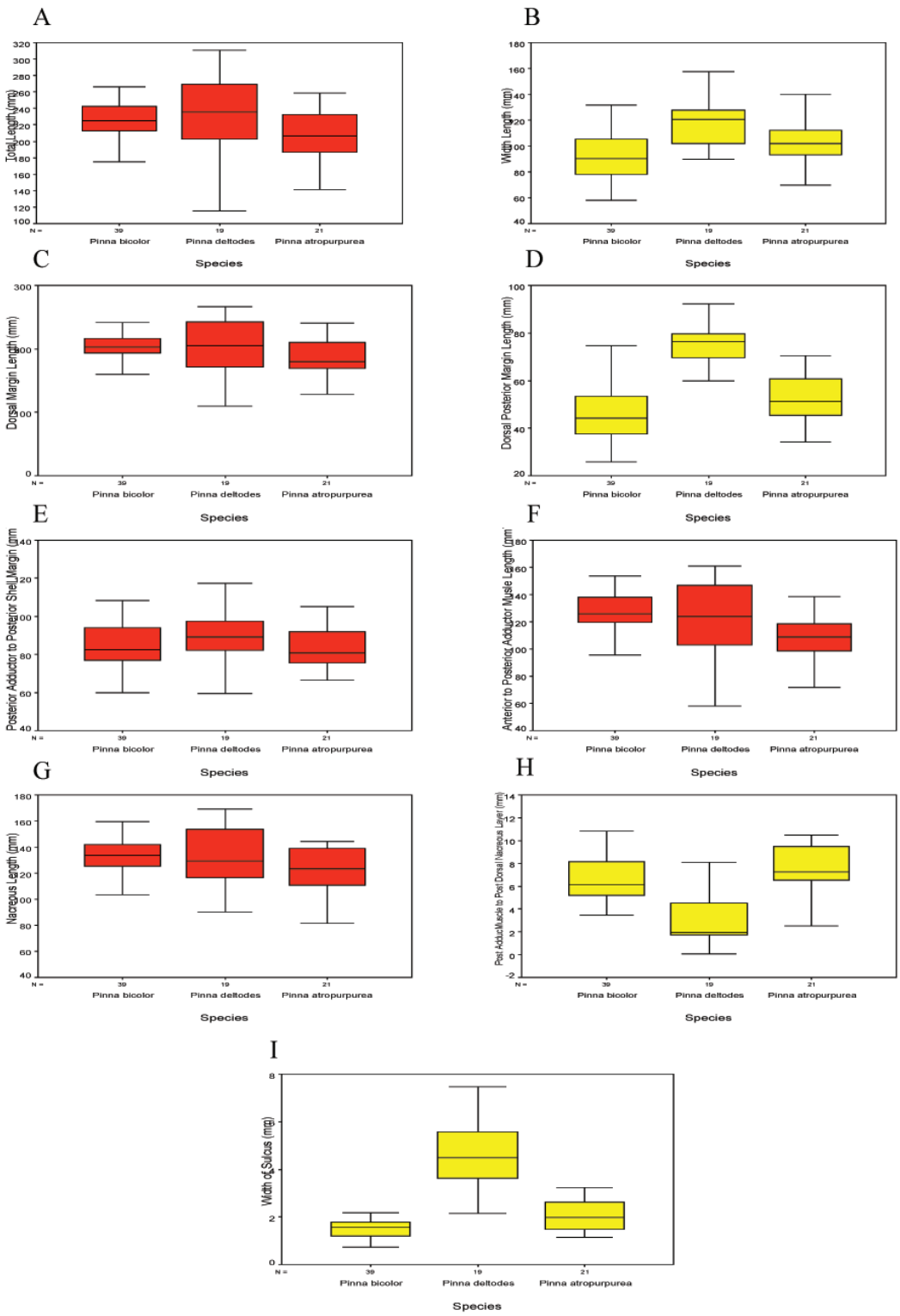

Fig. 5: Mean of boxplots from nine morphometric characteristic difference between P. bicolor, P. deltodes *and* P. atropurpurea

TABLE 2
Value of the first three components obtained through a PCA performed on raw morphometric data of three different species of pen shell

Morphometric Character (mm)	Component		
	1	2	3
TL	2.720	-0.010	-0.004
WL	0.075	-0.185	0.045
DML	2.152	0.049	-0.001
DPML	-1.028	-0.163	-0.064
PAMPSM	-0.358	-0.004	0.053
APAL	0.369	0.095	-0.037
DNL	0.577	0.078	-0.001
PAMPDNL	-2.219	0.096	0.029
WS	-2.289	0.043	-0.019
Eigen value	2.983	0.011	0.001
Cumulative Variance (%)	99.575	0.375	0.050

Abbreviations: TL=Total Length; WL=Width Length; DML=Dorsal Margin Length; DPML=Dorsal Posterior Margin Length; PAMPSM=Posterior Adductor Muscle to Posterior Shell Margin; APAML=Anterior to Posterior Adductor Muscle Length; DNL=Dorsal Nacreous Length; PAMPDNL=Posterior Adductor Muscle to Posterior Dorsal Nacreous Layer; WS=Width of Sulcus

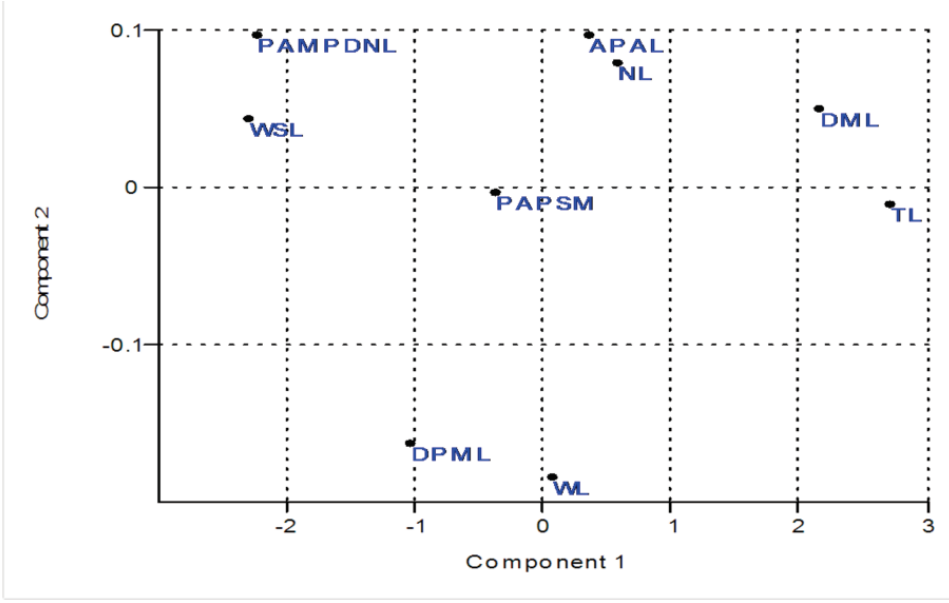

Fig. 6: Plots of coordinates of three different species of Pen Shells according to the first two discriminant functions, obtained from morphometric data

similar to *P. bicolor* based on external and internal characteristics. Some external morphological characters may be absent because of the broken shell especially at the posterior portion of the shell. For this reason we cannot get clear information to identify the specimens.

The results of ANOVA showed that of nine characteristics, four characters were highly significant ($P<0.05$) (width length-WL, dorsal posterior margin length-DPML, posterior adductor muscle to posterior dorsal nacreous layer-PAMPDNL and width of sulcus-WS) between

TABLE 3
Means and range of shell measurement and their ratio of *Pinna bicolor, Pinna deltodes* and *Pinna atropurpurea*

Measurement or Ratio*		*P. bicolor*	*P. deltodes*	*P. atropurpurea*
DPML (3) : TL (6)	Means	0.21 ± 0.45a	0.32 ± 0.50c	0.26 ± 0.39b
	Range	0.14 - 0.31	0.22 - 0.43	0.21 - 0.36
WL (5) : TL (6)	Means	0.42 ± 0.65a	0.52 ± 0.63b	0.50 ± 0.65b
	Range	0.30 - 0.56	0.40 - 0.60	0.39 - 0.65
DML (4) : TL (6)	Means	0.90 ± 0.40a	0.88 ± 0.53a	0.88 ± 0.12a
	Range	0.76 - 1.02	0.75 - 0.95	0.41 - 0.97
APAML (1) : PAMPSM (2)	Means	1.51 ± 0.23b	1.34 ± 0.19a	1.27 ± 0.13a
	Range	1.17 - 2.06	0.92 - 1.74	0.95 - 1.50
WS (7)	Means	1.47 ± 0.37a	4.60 ± 1.35c	2.20 ± 1.04b
	Range	0.94 - 2.17	2.16 - 7.46	1.15 - 3.23
DNL (9) : TL (6)	Means	0.59 ± 0.37b	0.57 ± 0.38a	0.57 ± 0.35a,b
	Range	0.53 - 0.69	0.52 - 0.64	0.48 - 0.64
PAMPDNL (8)	Means	6.79 ± 2.34b	2.68 ± 1.58a	7.35 ± 2.49b
	Range	3.47 - 12.87	0.07 - 5.67	1.68 - 10.47

Mean±SD subjected to Tukey HSD Post-hoc test
Mean±SD in column with dissimilar superscript letter is significantly different (P≤0.05)

Abbreviations: TL=Total Length; WL=Width Length; DML=Dorsal Margin Length; DPML=Dorsal Posterior Margin Length; PAMPSM=Posterior Adductor Muscle to Posterior Shell Margin; APAML=Anterior to Posterior Adductor Muscle Length; DNL=Dorsal Nacreous Length; PAMPDNL=Posterior Adductor Muscle to Posterior Dorsal Nacreous Layer; WS=Width of Sulcus

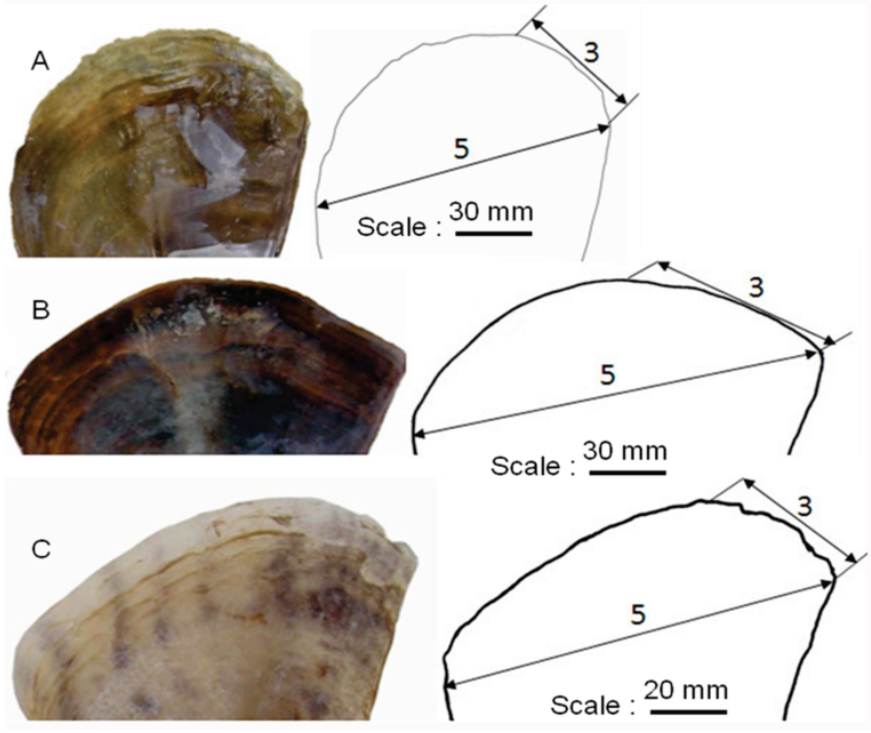

Fig. 7: Photo on posterior margin of the shell (A) P. bicolor; *(B)* P. deltodes; *(C)* P. atropurpurea *and their morphometric characters (no.3) DPML = Dorsal Posterior Margin Length; (no.5) WL = Width Length*

the three species of *Pinna*. The results showed that the same four morphometric characteristics resulted in highly significant (P<0.05) differences between the characters of the three *Pinna* species. From the morphometric analysis, the four characters showed clear characteristics for differentiating the three species (*P. bicolor, P. deltodes* and *P. atropurpurea*) from Malaysian waters.

Although shell shape is markedly variable in the three species, each has a common and distinct shape with *P. deltodes* and *P. atropurpurea* usually nearly symmetrical and *P. bicolor* typically asymmetrical (*Figs. 2, 3* and *4*). The difference in shape can be expressed by the ratio between the length of the dorsal posterior margin and longest shell length (Scheltema, 1983). From the examination, dorsal posterior margin were more than double in *P. deltodes* and *P. atropurpurea* compared to *P. bicolor*. The width of shell is about one-half of the shell length in *P. bicolor* and nearly three-quarter the shell length in *P. deltodes* and *P. atropurpurea* (Table 3).

The pattern of the nacreous layer and the position of the posterior adductor muscle scar determine generic and species membership in the Pinnidae (Turner and Rosewater, 1958; Rosewater, 1961). Unique to the genus *Pinna* is the sulcus which divides the nacreous area into dorsal and ventral lobes (*Figs. 2C, 3C* and *4C*, no. 7). An examination of all *P. bicolor* and *P. deltodes* shells by Scheltema (1983) in several museums showed no overlap of these two character states in specimens from Australia.

The position of the posterior adductor muscle scar of *P. deltodes* lies anterior to the mid-point of the shell in more than one-half of the specimens examined. Usually the posterior adductor muscle scar of *P. deltodes* protruded to the posterior dorsal nacreous layer but not in *P. bicolor*. The greater shell width of *P. deltodes* is reflected internally in the greater width of the sulcus between the nacreous lobes, which is on average twice as wide as in *P. atropurpurea* and triple in *P. bicolor*. The other five morphometric characteristics in these three species were found to be similar to each other.

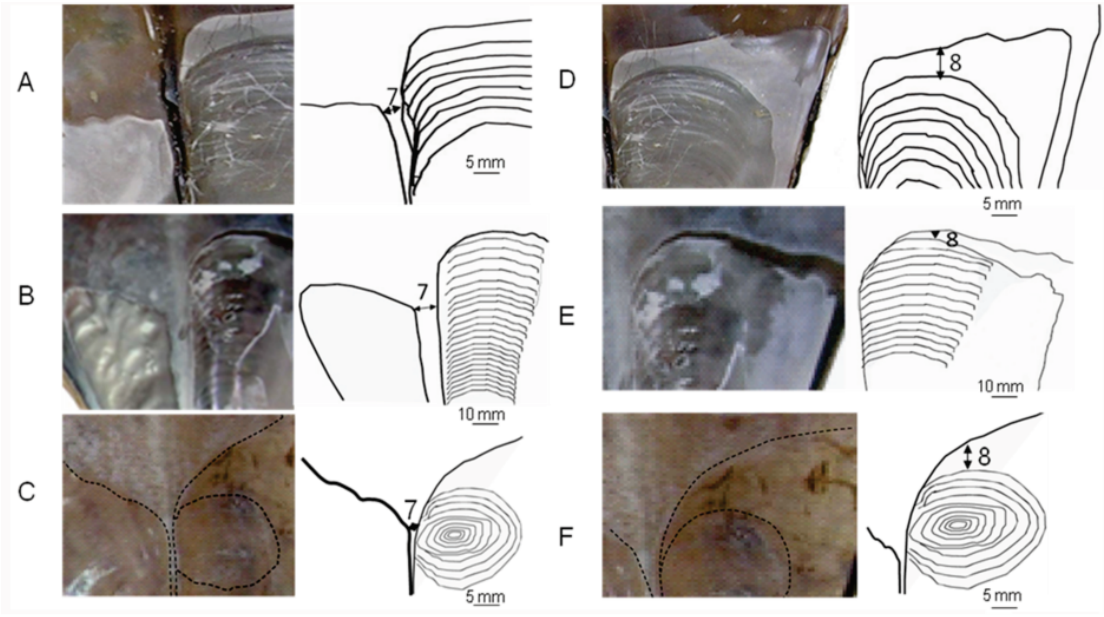

Fig. 8: Photo and diagrammatic sketch of different characters for width of sulcus and posterior adductor muscle scar located. A - C = Width of sulcus (no. 7) (A - P. bicolor, *B* - P. deltodes, *C* - P. atropurpurea*) and D - E = Posterior adductor muscle scar located (no. 8) (D* - P. bicolor, *E* - P. deltodes, *F* - P. atropurpurea*)*

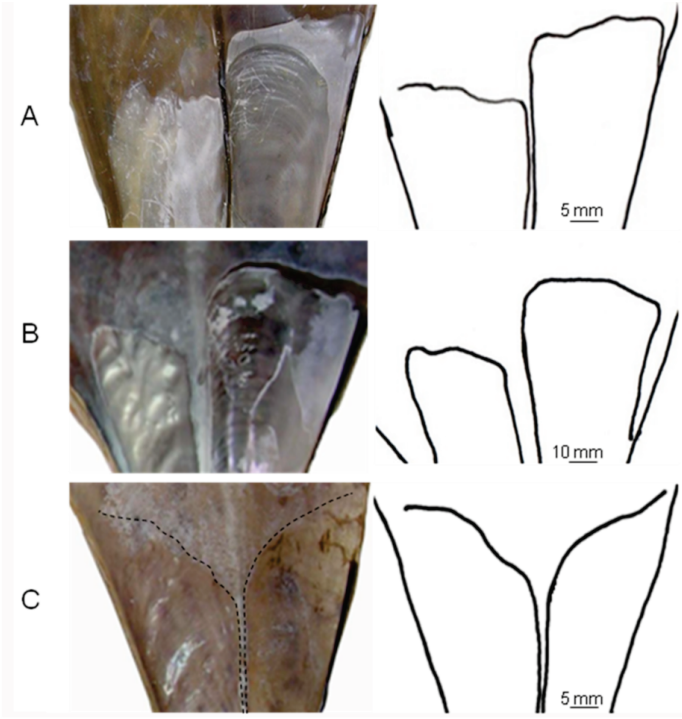

Fig. 9: Different shapes of nacreous lobe of three Malaysian Pen Shells. A - P. bicolor; *B* - P. deltodes *and C* - P. atropurpurea

CONCLUSIONS

From this study it could be concluded that Merambong shoal, Tanjung Adang shoal and Merambong island seagrass beds provided a habitat for *Pinna bicolor*, *Pinna deltodes* and *Pinna atropurpurea*. Nine internal and external morphological characteristics have been established for taxonomic identification. Differences in the four morphometric (width length-WL, dorsal posterior margin length-DPML, posterior adductor muscle to posterior dorsal nacreous layer-PAMPDNL and width of sulcus-WS) characteristics distinguish *Pinna bicolor*, *Pinna deltodes* and *Pinna atropurpurea* found in Malaysian waters.

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Pertanika accepts submission of mainly four types of manuscripts. Each manuscript is classified as **regular** or **original** articles, **short communications**, **reviews**, and proposals for **special issues**. Articles must be in **English** and they must be competently written and argued in clear and concise grammatical English. Acceptable English usage and syntax are expected. Do not use slang, jargon, or obscure abbreviations or phrasing. Metric measurement is preferred; equivalent English measurement may be included in parentheses. Always provide the complete form of an acronym/abbreviation the first time it is presented in the text. Contributors are strongly recommended to have the manuscript checked by a colleague with ample experience in writing English manuscripts or an English language editor. Lingually hopeless manuscripts will be rejected straightaway (e.g., when the language is so poor that one cannot be sure of what the authors really mean). This process, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

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1. Regular article

Definition: Full-length original empirical investigations, consisting of introduction, materials and methods, results and discussion, conclusions. Original work must provide references and an explanation on research findings that contain new and significant findings.

Size: Should not exceed 5000 words or 8-10 printed pages (excluding the abstract, references, tables and/or figures). One printed page is roughly equivalent to 3 type-written pages.

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Size: Should not exceed 2000 words or 4 printed pages, is intended for rapid publication. They are not intended for publishing preliminary results or to be a reduced version of Regular Papers or Rapid Papers.

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Definition: Critical evaluation of materials about current research that had already been published by organizing, integrating, and evaluating previously published materials. Re-analyses as meta-analysis and systemic reviews are encouraged. Review articles should aim to provide systemic overviews, evaluations and interpretations of research in a given field.

Size: Should not exceed 4000 words or 7-8 printed pages.

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Definition: Usually papers from research presented at a conference, seminar, congress or a symposium.

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5. Others
Definition: Brief reports, case studies, comments, Letters to the Editor, and replies on previously published articles may be considered.

Size: Should not exceed 2000 words or up to 4 printed pages.

With few exceptions, original manuscripts should not exceed the recommended length of 6 printed pages (about 18 typed pages, double-spaced and in 12-point font, tables and figures included). Printing is expensive, and, for the Journal, postage doubles when an issue exceeds 80 pages. You can understand then that there is little room for flexibility.

Long articles reduce the Journal's possibility to accept other high-quality contributions because of its 80-page restriction. We would like to publish as many good studies as possible, not only a few lengthy ones. (And, who reads overly long articles anyway?) Therefore, in our competition, short and concise manuscripts have a definite advantage.

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The paper should be formatted in one column format with the figures at the end. A maximum of eight keywords should be indicated below the abstract to describe the contents of the manuscript. Leave a blank line between each paragraph and between each entry in the list of bibliographic references. Tables should preferably be placed in the same electronic file as the text. Authors should consult a recent issue of the Journal for table layout. There is no need to spend time formatting your article so that the printout is visually attractive (e.g. by making headings bold or creating a page layout with figures), as most formatting instructions will be removed upon processing.

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We recommend that authors prepare the text as a Microsoft Word file.

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   - *Authors of Short Communications should state the total number of words (including the Abstract)
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     Footnotes. Current addresses of authors if different from heading.
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   - References should be listed in alphabetical order, by the authors' last names. For the same author, or for the same set of authors, references should be arranged chronologically. If there is more than one publication in the same year for the same author(s), the letters 'a', 'b', etc., should be added to the year.
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     - Jalaludin, S. (1997b). The use of different vegetable oil in chicken ration. *Mal. Agriculturist*, 11, 29-31.
     - Tan, S.G., Omar, M.Y., Mahani, K.W., Rahani, M., Selvaraj, O.S. (1994). Biochemical genetic studies on wild populations of three species of green leafhoppers *Nephotettix* from Peninsular Malaysia. *Biochemical Genetics*, 32, 415 - 422.
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   Chapter in Book: Kanwal, N.D.S. (1992). Role of plantation crops in Papua New Guinea economy. In Angela R. McLean (Eds.), *Introduction of livestock in the Enga province PNG* (p. 221-250). United Kingdom: Oxford Press.

   Proceedings: Kanwal, N.D.S. (2001). Assessing the visual impact of degraded land management with landscape design software. In N.D.S. Kanwal and P. Lecoustre (Eds.), *International forum for Urban Landscape Technologies* (p. 117-127). Lullier, Geneva, Switzerland: CIRAD Press.

9. **Short Communications** should include **Introduction, Materials and Methods, Results and Discussion, Conclusions** in this order. Headings should only be inserted for Materials and Methods. The abstract should be up to 100 words, as stated above. Short Communications must be 5 printed pages or less, including all references, figures and tables. References should be less than 30. A 5 page paper is usually approximately 3000 words plus four figures or tables (if each figure or table is less than 1/4 page).

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4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors submit a revised version of the paper to the executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor.

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

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JTAS is devoted to the publication of original papers that serves as a forum for practical approaches to improving quality in issues pertaining to tropical agricultural research or related fields of study. It is published twice a year in **February** and **August**.

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Papers should be double-spaced 12 point type (Times New Roman fonts preferred). The first page should include the title of the article but no author information. Page 2 should repeat the title of the article together with the names and contact information of the corresponding author as well as all the other authors. Page 3 should contain the abstract only. Page 4 and subsequent pages to have the text - Acknowledgments - References - Tables - Legends to figures – Figures, etc.

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