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Pertanika Editorial Office

Research Management Centre (RMC)
1st Floor, IDEA Tower II, UPM-MTDC Technology Centre
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
Tel: +603 8947 1622, 8947 1620
E-mail: ndeeps@admin.upm.edu.my

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The UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia
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Short Communications

Willingness to Pay a Price Premium for Certified Wood Products among Consumers in Malaysia

Shukri Mohamed* **and Awang Noor Abd Ghani**
Department of Forest Management, Faculty of Forestry,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
**E-mail: shukri@putra.upm.edu.my*

ABSTRACT

Consumers are assumed to be willing to pay price premiums for certified wood products. In this study, Malaysian consumers' willingness to pay a price premium for certified wood products and factors influencing their willingness to pay were investigated. The study made use of the data obtained from 994 systematically selected mall-intercepted respondents. A binomial logit analysis was applied to determine the factors influencing the respondents' willingness to pay. About 74% of the respondents indicated that they would choose wood products made from certified timbers. However, only 57% stated that they were willing to pay a price premium for the products. The respondents' willingness to pay was found to be influenced by their knowledge and perceived importance of forest certification, as well as the inclination to choose wood products made from certified timbers. There is also a positive correlation between the willingness to pay and the respondents' education, income, and current ownership of the wooden furniture items. The opportunity for further research includes determining the amount of premium the consumers are willing to pay and identifying consumer segments where certified wood products can be successfully marketed.

Keywords: Willingness to pay, price premium, certified wood products, consumers

INTRODUCTION

Environmental certification of wood products, as a consequence of environmental certification of forest management practices, has its origin from the global concern for the widespread deforestation in the tropics during the eighties. The recognition that forests must be managed in a sustainable manner was formalised in the deliberations of the United Nations Conference on Environment and Development held in Rio de Janeiro in 1992 (Vertinsky and Zhou, 2000). Wood products entering the international trade are expected to be manufactured from timbers originating from areas certified to be managed on sustainable or environment-friendly practice.

Sustainable forest management, however, are expected to cost more than the present forest management practices (Leslie, 2006). Thus, forest owners and producers of wood products must capture a price premium which allows them to recover the costs of practicing certified sustainable forest management and maintaining certification (Upton and Bass, 1995). It is believed that consumers are willing to pay a premium for products originating from well-managed forests (Carter and Merry, 1998), and this ranges from 5 to 10 percent (Forsyth, 1998, cited in Vertinsky and Zhou, 2000). A number of studies to date have shown that consumers in the more affluent and developed countries are

*Corresponding Author

willing to buy and pay a premium for certified wood products (CWP) (e.g. Ozanne and Vlosky, 1997; Gronroos and Bowyer, 1999; Ozanne *et al*., 1999; Kozak *et al*., 2004; Veisten, 2002).

Malaysia has responded positively towards the call for sustainable forest management and certification of its forest management practice. The country is now operating a voluntary national forest management and chain-of-custody certification under the Malaysian Timber Certification Scheme (MTCS) which was begun in 2001. Currently, an area of about 3.85 million ha of permanent forest reserves, in Peninsular Malaysia and Sarawak, has been certified under the scheme (MTCC, 2007). In addition, the Forest Stewardship Council has awarded six combined forest management and chain-of-custody certificates for various forestry and wood product manufacturing firms in Malaysia (FSC, 2008). Since 2002, Malaysia has exported more than 267,000 m^3 of MTCS-certified wood products, especially to Europe (MTCC, 2008).

Despite being a producer and exporter of CWP, there seems to be no effort to market such wood products domestically. There is, therefore, a need to understand the factors that could accelerate exploitation of the full potential of CWP in Malaysia. In specific, this study addressed two questions; (i) whether the consumers in Malaysia are willing to pay a price premium for CWP, and (ii) what are those factors that influence their willingness to pay?

METHOD

A self-administered questionnaire was used to obtain the data for this study. The questionnaire was distributed to the adults who were systematically selected at four shopping malls in Kuala Lumpur, Malaysia. These adults were selected based on a previously determined criterion that every tenth adult who walked past the enumerators was to be interviewed. However, only those who indicated their willingness to participate were given the questionnaire.

The survey solicited information in relation to the respondents' perception on the importance of forest certification, knowledge of forest certification, and their environmental consciousness. The respondents were also asked to choose, in a hypothetical purchase situation, between a wooden dining furniture set made from certified timbers and an identical one made from the non-certified timbers with response options 'choose set made from certified timbers', 'choose set made from non-certified timbers', 'would choose either set' and 'don't know'. It is important to note that the respondents were informed that the only difference between the two furniture sets was the type of timber used to manufacture the items while price, design, quality, and other attributes were similar. In order to ensure that the respondents fully understood the meaning of forest certification and certified timbers, each questionnaire included the following definition: "Forest certification is a system of forest inspection plus a means of tracking timber through a 'chain of custody' – following the raw material through to the finished product". The goal of forest certification is to ensure that the products have come from forests which are well-managed, i.e. its management takes into account the environmental, social and economic benefits of the forests. "Timbers which come from the certified forests are thus certified timbers". This definition was repeated three times in the questionnaire.

If the respondents indicated a preference for the dining set made from certified timbers, an indifference (a 'would choose either set' response) or uncertainty (a "don't know" response), they were asked if they would be willing to pay a price premium for the CWP with a 'yes' or 'no' response option. A binomial logit analysis for the binary choice responses was then applied to determine the factors influencing the respondents' willingness to pay a price premium for CWP. The respondents' socio-economic information was also collected during the survey as they are reported, although with mixed results (e.g. Schlegelmich *et al*., 1996; Maineiri *et al*., 1997; Laroche *et al*., 2001; Forsyth *et al*., 1999; Laureiro *et al*., 2002) to exert an influence

on the consumers' environmental purchase behaviour and willingness to pay a premium for environment-friendly products. Table 1 presents the variables used in the model and their definitions.

RESULTS AND DISCUSSION

After eliminating incomplete and erroneous surveys, a total of 994 usable questionnaires were used in the analysis. Overall, the respondents were mostly Malays (74%) and there were slightly more women (52%) than men in the sample. The respondents' average age was 32 years and they had spent an average of 15 years in education. Meanwhile, their average monthly income was RM2,371 and almost all these respondents are not members of any environmental organisation or association. More than three-quarters of the respondents currently own wooden dining furniture sets. A large majority of the respondents (74%) showed a preference for CWP. However, only 57% were willing to pay a price premium for such products. Table 2 summarises the respondents' demographic information and responses to selected questions in the survey.

The results of the logit analysis on the willingness to pay a price premium for CWP are shown in Table 3. The positive and significant coefficient on *IMPORTANCE* implies that it is highly probable that a consumer would be willing to pay a premium for the CWP if he/she believes that forest certification is important.

TABLE 1
Definition and descriptive statistics of the variables used in the model

Variable	Definition
IMPORTANCE	There is a need for forest certification in Malaysia yes = 1, otherwise = 0
KNOWLEDGE	I understand the concept of forest certification yes = 1, otherwise = 0
CONSCIOUS	Whenever possible, I buy products that is produced with no adverse impact on the environment yes = 1, otherwise = 0
CHOICE	If you were to buy a wooden dining furniture set, would you choose to buy a set made from certified timber or a set made from non-certified timber (assuming price, design, quality and other attributes are similar)? set made from certified timber = 1, set made from non-certified= 2, would choose either set = 3, don't know = 4
OWNERSHIP	Do you currently own wooden dining furniture set yes = 1, otherwise = 0
GENDER	Male = 0, Female = 1
AGE	Age of the respondent (years)
MARITAL	Bachelor = 1, Married = 2, Others = 3
ETHNIC	Ethnicity of the respondent 1 = Malay, 2 = Chinese, 3 = Indian and others
INCOME	Gross monthly income of the respondent (RM)
EDUCATION	Educational level of the respondent (years)
MEMBER	Membership in an environmental association yes = 1, otherwise = 0

TABLE 2
Respondents' demographic characteristics and responses to selected questions in the survey

Characteristics	Percentage
Gender	
Male	47.79
Female	52.21
Age	
30 years and below	53.82
31 – 40 years	27.16
41 – 50 years	13.78
51 – 60 years	4.73
61 years and above	0.51
Ethnic	
Malay	74.44
Chinese	14.89
Indian and others	10.67
Education	
At least 6 years (primary)	3.82
At least 13 years (secondary)	30.08
At least 15 years (certificate)	13.88
At least 16 years (diploma)	23.64
At least 17 years (university degree)	28.57
Monthly gross income	
RM2000 and below	58.15
RM2001 – 4000	30.88
RM4001 – 6000	5.53
RM6001 and above	3.32
(Missing cases: 51)	
Membership in an environmental organisation	
Yes	1.81
No	98.19
Own wooden dining furniture set	
Yes	76.66
No	23.34
Choice of wooden dining furniture set chosen in a hypothetical purchase decision	
Set made from certified timber (1)	74.04
Set made from non-certified timber (2)	3.32
Would choose either set (3)	15.09
Don't know (4)	7.55
Willingness to pay price premium for certified wood products	
Yes	58.48
No	41.52
(961 cases for response option 1, 3 and 4 above; a 'yes' response for 994 respondents is 57%)	

TABLE 3
Results from the logit model on willingness to pay a premium for CWP

Variable	Coefficient	*z*-values	Marginal probability
CONSTANT	-2.982**	-4.41	
IMPORTANCE	0.541**	2.83	0.1332
KNOWLEDGE	0.583**	3.96	0.1411
CONSCIOUS	-0.855	-0.59	- 0.0206
CHOICE	0.419*	2.49	0.1027
OWNERSHIP	0.502**	3.00	0.1232
GENDER	0.062	0.43	0.0152
AGE	0.001	0.18	0.0004
MARITAL	0.328	1.72	0.0775
MALAY	- 0.206	-0.95	-0.0498
CHINESE	-0.068	-0.26	-0.0166
INCOME	0.000*	2.46	0.0000
EDUCATION	0.107**	3.09	0.0260
MEMBER	-0.379	- 0.72	- 0.0937
Log-likelihood	- 569.36		
Pseudo-R^2	0.078		
X^2 (df=11)	96.61		
Significance level	0.000		

* Significant at 0.05 level
**Significant at 0.01 level

Consumers who showed a strong opinion on the importance of forest certification had been identified to be the most likely group to buy CWP at a premium (Vlosky *et al*., 1999; Ozanne and Vlosky, 2003; Anderson and Hansen, 2004).

A positive and highly significant coefficient on *EDUCATION* implies that an increase in the education level would increase the probability of a consumer's willingness to pay. An intuitive explanation for this could be that consumers with more formal education probably have better understanding of the benefits associated with forest certification and are therefore willing to pay a higher price for CWP. Education, although with mixed results, was found to have an influence on the consumers' willingness to pay price premiums for CWP (Ozanne and Vlosky, 1997; Ozanne and Smith, 1998). The respondent's knowledge of forest certification (*KNOWLEDGE*) also has a similar influence on the willingness to pay. Individuals who are knowledgeable about environmental issues were found to be more willing to pay a price premium for environmental friendly products (Amyx *et al*., 1994, cited in Laroche *et al*., 2001).

The results also show that owning wooden furniture items (*OWNERSHIP*) and having a preference for wood products made from certified timbers (*CHOICE*) have a positive influence on the consumers' willingness to pay a premium for CWP. Meanwhile, no plausible explanation can be offered for the former, as studies have shown that consumers stating a preference for the CWP have also considered paying a premium for these wood products (Ozanne and Vlosky, 1997; Aguilar and Vlosky, 2007). Another variable that has a significant influence on the willingness to pay is the

respondent's income (*INCOME*). Income has always been found to be positively correlated to the willingness to pay a price premium, not only for CWP (Aguilar and Vlosky, 2007) but also for other environmental friendly products (Saphores *et al*., 2007). Meanwhile, individuals with higher incomes are believed to be able to bear a marginal increase in the costs associated with supporting environmental causes and favouring environment-friendly products (Straughan and Roberts, 1999).

CONCLUSIONS

In this study, the willingness of consumers in Malaysia to pay a price premium for certified wood products and the factors influencing their willingness to pay were investigated. The results showed that there were consumers in Malaysia who would choose wood products made from certified timbers. However, not all of them were willing to pay a price premium for such wood products. Equally important is the finding that their willingness to pay is influenced by their knowledge of and the perceived importance of forest certification, and the inclination to choose certified wood products. There is also a positive correlation between the willingness to pay and the respondents' education, income, and current ownership of wooden furniture items.

Considerations of how representative the samples are must be kept in mind when evaluating the broader implications of the findings in 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 might not be representative of the Malaysian population. Even so, this study should still be useful for the wood product industry to evaluate the market potential of certified wood products in Malaysia and develop effective marketing strategies. There are also opportunities for further research, particularly to determine the amount of premium the consumers are willing to pay and identify consumer segments where certified wood products can be successfully marketed.

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Short Communications

Depuration of Gut Contents in the Intertidal Snail *Nerita lineata* is Not Necessary for the Study of Heavy Metal Contamination and Bioavailability: A Laboratory Study

Yap, C.K.* **and Cheng, W.H.**
Department of Biology, Faculty of Science,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
**E-mail: yapckong1973@yahoo.com*

ABSTRACT

Some of the scientific papers in the literature regarding heavy metal concentrations in the soft tissues of molluscs are always rejected because there is no depuration of metals before the molluscs samples are analyzed for heavy metal accumulation, although the acceptance of a paper in a journal is assessed based on many other factors. The depuration of gut contents of molluscs has been the initial step before the metal analysis on the soft tissues of molluscs by many researchers. The depuration process in some molluscs involves holding the animals in clean water or clean sediment for a suitable period (8–24 hrs) to purge their guts after exposing them to contaminated conditions, and before they are analyzed for whole-body contaminant burden (Neumann *et al*., 1999; Gillis *et al*., 2004). The depuration ensures that metal-contaminated particles in the animal's gut do not lead to overestimation of metal bioavailability. Undoubtedly, clearing the gut contents is theoretically a laboratory technique in order to get an accurate estimate of heavy metal concentrations accumulated in the soft tissues of molluscs. In addition, the suggestion on the use of molluscs as biomonitors of metal bioavailability becomes invalid because their soft tissues were not depurated. Consequently, validity on the data of metal concentrations could not be achieved. Therefore, in order to determine if a particular species could be used as biomonitor for metals, depuration of soft tissues of the molluscs is imperative to effectively determine the availability of metal (Riba *et al*., 2005; Wang *et al*., 2005; Szefer *et al*., 1999; Cravo *et al*., 2004; Baldwin and Maher, 1997; Nicholson and Szefer, 2003). In standard protocols (ASTM, 2003), although it is not a standard practice to clear the gut of organisms before analyzing their tissues for whole-body metal accumulation, some investigators do transfer animals to clean water conditions in order to purge their guts after they have been collected from the field. In this study, the snail known as *Nerita lineata* of the *Neritidae* family, were collected to study if there was any significant difference in the concentrations of Cu and Zn in the soft tissues, before and after four weeks of depuration.

Keywords : Depuration, gut contents, *Nerita lineata*, mollluscs

MATERIALS AND METHODS

The snails, *N. lineata* in their adult stage, were collected from Sg. Janggut, in Selangor (N 04º 8'10''; E 101º 22'31'') where water irrigation activities were observed. Aquaria were cleaned and acid-washed before the experiment was carried out. For this purpose, thirty snails of almost similar size and shell length (14.00-32.22 cm) were selected from the collected samples. Meanwhile, fifteen snails were placed in one laboratory aquarium without feeding for 4 weeks, and fifteen other snails were analysed

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*Corresponding Author

for the initial concentrations of Cu and Zn (Yap *et al*., 2003: 2006). After 4 weeks, the depurated snails were analyzed for Cu and Zn concentrations using the established methods (Yap *et al*., 2003: 2006). They were dried in n oven for 72 hours at 105°C to constant dry weights. The dried tissues of the snails were digested in concentrated nitric acid (AnalaR grade, BDH 69%) by placing them in a hot-block digester; first at low temperature for one hour and they were then fully digested at high temperature (140°C) for at least three hours. After that, the digested samples were diluted to 40 mL with double distilled water. After filtration, the samples were determined for their Cu and Zn concentrations 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 metal levels found in the soft tissues of *N. lineata*, before and after 4 weeks of depuration, were statistically analyzed to check for any significant difference using t-test in the SPSS software version 15.0 for Windows.

There was no significant ($P>0.05$) difference in the concentrations of Cu and Zn, before and after 4 weeks of depuration in the intertidal snails *N. lineata* (Table 1). This indicated that the depuration of metals in *N. lineata* can be neglected. Although Cu and Zn are essential metals for the molluscs, since they are incorporated in the enzymes of the organisms, there is regulative mechanism for the essential metals in gastropods (Phillips and Rainbow, 1993). Therefore, these two metals are preferentially found in the soft tissues of the snails (Cravo *et al*., 2004). Moreover, Cu and Zn are likely to be detoxified and this involves metallothioneins and depurated if present in excess (Ng *et al*., 2007). Therefore, the choice for the two metals in this study is reasonable.

There are four important points which can be highlighted to make reasonable arguments to reject the necessity of depuration of gut contents in *N. lineata* besides the significant finding from this laboratory study. First, the depuration process may easily cause metal contamination (Rainbow *et al*., 2004). For this pragmatic reason, the snails should not be allowed to depurate their gut contents (Rainbow *et al*., 1989). This contamination will certainly increase the actual metal concentrations and metal bioavailability. Many studies (e.g. Gunther *et al*., 1999; Rainbow and Blackmore, 2001; Rainbow *et al*., 2004; Sidoumou *et al*., 2006; Espana *et al*., 2007) on molluscs did not carry out depuration prior to the analysis of heavy metals in soft tissues. Nonetheless, these papers are still accepted for publication in international journals. This could be due to the fact the depuration process would reduce the validity of data collected because of errors and

TABLE 1

A comparison of the concentrations (μg/g dry weight) of Cu and Zn in the soft tissues of *Nerita lineata* collected from Sg. Janggut snails between, before, and after weeks of depuration. N= 3 based on 15 individuals of snails

	Before			After 4 weeks			Significance level
Cu shell	2.23	±	0.29	3.54	±	0.01	p>0.05
Cu operculum	5.62	±	0.71	6.04	±	0.22	p>0.05
Cu soft tissues	14.31	±	2.13	11.89	±	0.10	p>0.05
Zn shell	8.07	±	0.64	7.29	±	0.16	p>0.05
Zn operculum	27.85	±	5.54	33.56	±	0.59	p>0.05
Zn soft tissues	91.69	±	1.50	96.12	±	2.88	p>0.05

external contamination. Second, *Nerita* snails are generally strong accumulators of heavy metals. Therefore, snails could potentially contain gut content, but these are considered to represent only a small proportion of the total body metal content, given the characteristic of molluscs (*Nerita*) which are particularly strong heavy metal accumulators (Rainbow, 1987: 1998).

Third, any variability introduced into the results is minimized and accounted for by the pooling technique used during the metal analysis in the laboratory and/or the statistical treatment of data (Rainbow and Blackmore, 2001). Since *Nerita* snails are small in size, the soft tissues must be pooled in order to get enough tissues for the metal analysis, and these pooled tissues would certainly reduce the variability due to gut contents.

Fourth, according to Rainbow and Phillips (1993), deposit-feeding species living in soft muds may exhibit different total body concentrations of metals, before and after gut depuration. Therefore, the depuration of the gut contents of deposit feeders is needed. In contrast, the need for depuration of the gut content is not necessary for all the molluscs, especially for the small intertidal snails and non-deposit filter feeders such as *N. lineata*. The problem of bioavailability overestimation of heavy metals in *N. lineata* without depuration is almost non-existing.

In conclusion, the above four points, plus the results of the present laboratory study, suggest that the determination of the bioavailability and accumulation of metals using the total soft tissues of *N. lineata* without depuration, can still be interpreted with high validity. Therefore, the time consuming depuration of gut contents in the intertidal snail *N. lineata* is not necessary for the study of heavy metal contamination and bioavailability.

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Effects of Defoliation from an Outbreak of the Teak Skeletoniser, *Paliga damastesalis* Walker (Lepidoptera: Crambidae), on the Growth of Teak, *Tectona grandis* L.

Lim Wui Oi[1], Yusof Ibrahim[2*], Laurence G. Kirton[1] and Faizah Abood Harris[3]
[1]Forest Research Institute Malaysia (FRIM), Kepong, 52109 Selangor, Malaysia
[2]Department of Technical and Vocational Education,
Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia
[3]Faculty of Forestry, Universiti Putra Malaysia, 43400 UPM,
Serdang, Selangor, Malaysia
[*]E-mail: yusofib@agri.upm.edu.my

ABSTRACT

The effects of defoliation by the teak skeletoniser *Paliga damastesalis* Walker (Lepidoptera: Crambidae) on a six-month old stand of teak *Tectona grandis* L. was evaluated on the subsequent year-long growth in the field. Defoliation did not exceed 10%. It had a transient negative effect on post-defoliation height increment and new leaf production of up to two and three months of growth. However, defoliation severity significantly affected collar diameter increment up to the seventh month. A peak in the mean monthly growth increments for new leaf production occurred in April, followed by collar diameter increment in June and height in July. In addition, a peak in one of these three growth parameters also corresponded with a trough in the other or both of the other parameters; as such, the height increment peaked with a concomitant trough in collar diameter increment a month later. As for the remaining period of the dry season after July, new leaf production remained at a constant low level, while increments for both the height and collar diameter were found to decrease. Tree recovery appeared rapid with regard to these growth parameters, thus the impact of defoliation was generally and relatively negligible on the growth parameters measured.

Keywords: Collar diameter, defoliation, height increment, *Paliga damastesalis, Tectona grandis*

INTRODUCTION

Teak is an important exotic tree species in the Malaysian forest plantations (Ahmad Zuhaidi *et al*., 2002). However, it is often attacked by the teak skeletoniser, *Paliga damastesalis* Walker (Lepidoptera: Crambidae) (Intachat, 1997). This pest can cause severe defoliation on teak (Tho, 1981; Chey, 1999), with a complete denudation (Intachat, 1999). The effects of natural and simulated defoliation on various aspects of teak growth have been evaluated in several countries, such as India and Bangladesh, but the teak skeletoniser species *Paliga machoeralis* Walker is similar to *P. damastesalis* (Intachat, 1998). For instance, defoliation experienced during three consecutive outbreaks of *P. machoeralis*, within one growing season in India, was found to have caused losses in volume increment ranging from 8.3 – 65% (Tewari, 1992). Likewise, four consecutive annual artificial defoliations of 50% resulted in a 62% volume increment loss (Baksha and Crawley, 1998), while three consecutive artificial defoliations of 25% in one growing season reduced radial growth by 48% (Kirtibutr, 1983).

Information regarding defoliation effects on teak growth in Malaysia is limited to

[*]Corresponding Author

that reported by Hashim (2003), who found significant growth reduction in potted one year-old teak, especially when these seedlings were completely defoliated. The paucity of data on how defoliation affects teak growth precludes developing tools, such as economic threshold that aids in decision-making for control of this pest. The current arbitrary recommendations differ as to the defoliation levels that warrant the application of control measures, e.g. 30 to 50% (Tho, 1981), and exceeding 50% (Intachat, 1997). The baseline information regarding the impact of *P. damastesalis* defoliation on the growth performance of teak throughout this crop's rotation cycle is therefore needed to formulate economic injury levels for a range of tree ages. The present study evaluates the effects of natural defoliation by *P. damastesalis* on the growth of young teak trees in the field for a year.

MATERIALS AND METHODS

Experimental Site

The present study was conducted to determine the effects of defoliation by *P. damastesalis* on a six-month, even-aged stand of 629 teak trees with a mean height of 134.2 cm (range: 30-305 cm) planted on a level area of 0.52 ha of alluvial soil (Penambang series). This stand was maintained by the Mata Ayer Forest Research Station (FRIM), Perlis. The plot was surrounded by mature teaks. The outbreak occurred during the months of January and February 2000 at the onset of the dry season; with the mean annual precipitation ranging from 1800 to 1850 mm (Amir Husni *et al*., 1996). The rainfall data for the station for the year 2000 were also obtained from the Hydrology Unit (FRIM).

Assessment of defoliation

Defoliation assessment was initiated at the end of February 2000, i.e. approximately three weeks after the peak of the outbreak. A visual assessment of defoliation of every tree was also made, whereby every leaf was examined and the defoliation severity was estimated for each of the leaves. Meanwhile, the level of defoliation was categorised into four groups so as to estimate the overall loss in the leaf area; low (<25%), moderate (26-50%), high (51-75%), and very high (>76%).

The effects of varying degrees of defoliation on the subsequent growth of the trees were evaluated on a monthly basis to determine the growth parameters such as height, collar diameter increment, and the production of new leaves. Tree height was measured from the base of the tree to the tip of the apical shoot using a graduated bamboo measuring pole, while a stainless steel vernier caliper was used to measure collar diameter. The stem diameter at the base of the tree was considered as the collar diameter, instead of the diameter at breast height (dbh) as most of the trees were not tall enough for the dbh measurements. The production of new leaves was recorded by counting the number of leaves produced above the previous month's height measurement for the trees concerned. The study was carried out in a period of 10 months.

Statistical Analysis

The data analysis was carried out using the statistical software Minitab 14® (MINITAB, 2007) which included a Chi-square test and a correlation analysis (Neave and Worthington, 1992) with a reduced dataset of 436 trees instead of 629 trees. However, the final assessment excluded 21 dead and 170 damaged (bent and/or snapped trunk) trees mainly because the relationship between the initial defoliation severity and the death or damage of those trees which was recorded at the end of the study was ascertained as not significant ($\chi 2 = 7.145$, $df = 3$, $p < 0.05$).

The final dataset was examined for the auto-correlations between the height, damage, and subsequent growth of trees since taller trees have been reported to experience more insect attacks that need to be controlled when analysing the effect of defoliation on height increment (Cunningham and Floyd, 2006). These were resolved using partial correlation to partition

out the effects of interactions (Sokal and Rohlf, 1981). The partial correlation between defoliation level and monthly growth increment parameters, with initial tree size held constant, was calculated based on the procedure for the partial correlation given in Minitab14®. However, kurtosis in the first two monthly growth increment data could not be corrected using square-root-, log10- or inverse-transformations. Meanwhile, significant correlations among all the defoliation levels in the monthly growth increment parameters were measured over time ($r > 0.95$, $p < 0.001$ for all correlations). Therefore, the results were averaged across the defoliation levels to obtain the mean monthly growth increment for each of the parameters (*Fig. 1*). The residuals, obtained from the different regressing defoliation levels and the monthly growth increment on the initial tree size were correlated to give the corrected result.

The dataset was also examined for the trend of growth increment throughout the study period as averaged across defoliation levels. The defoliation levels were correlated to monthly growth increment for tree height, collar diameter, and new leaf production, while post-defoliation growth patterns were explored in relation to the monthly rainfall.

RESULTS

Insect defoliation never exceeded 10% on any tree at any time after the outbreak. Defoliation had a transient negative effect on height increment and new leaf production of up to 2-3 months; however, negative post-defoliation effects on collar diameter increment were observed up to the seventh month (Table 1). Over the entire study period, there was no significant association between height increment and defoliation severity ($F = 0.62$, d.f. = 1, 437, $p > 0.05$). The collar diameter increment, however, was significantly and negatively related to defoliation severity ($F = 4.99$, d.f. = 1, 437, $p < 0.05$) during that period.

The peak rainfall in April was matched by the peak in new leaf production, and this was followed by a peak in the increment of collar diameter in June. Meanwhile, the increment in height was found to reach its peak in July. A peak in one of these three growth parameters usually corresponded with a trough in the other or both of the other parameters. In March, however, when the new leaf production was at its highest, the increments in the height and collar diameter were found to be low. In April, the production of new leaves was minimal while the increment of collar diameter was at its peak. The height increment peaked with a concomitant trough in collar diameter increment a month later. As for the remaining period of the dry season after July, new leaf production remained low with decreases indicated for both height and collar diameter increments. However, the wet month of November saw a rise in height increment that was not shown in collar diameter increment.

The correlation analysis indicated that both defoliation severity and subsequent tree growth were potentially dependent on the size of trees. The defoliation level was negatively associated with the following month's growth increment, i.e. height increment ($r = -0.17$ and $p < 0.0010$), collar diameter increment ($r = -0.09$ and $p > 0.05$), and new leaf production ($r = -0.07$ and $p > 0.05$). The initial height of trees was positively associated with the height increment and new leaf production in the subsequent month, i.e. $r = 0.323$ and $p < 0.001$; $r = 0.29$ and $p < 0.001$, respectively, but it was found to be negatively associated with collar diameter increment, i.e. $r = -0.10$ and $p < 0.05$.

After the outbreak, other defoliators, such as *Hyblaea puera* Cramer and *Hypomeces squamosus* (Fabricius) (Coleoptera: Curculionidae), were occasionally detected during the monthly assessments. However, *P. damastesalis* was not found during the later part of the study period.

DISCUSSION

Since the ensuing insect defoliation never exceeded 10% on any tree at any time during the study, this incidence was considered as a one-time defoliation caused by *P. damastesalis*. Meanwhile, subsequent post-outbreak defoliation

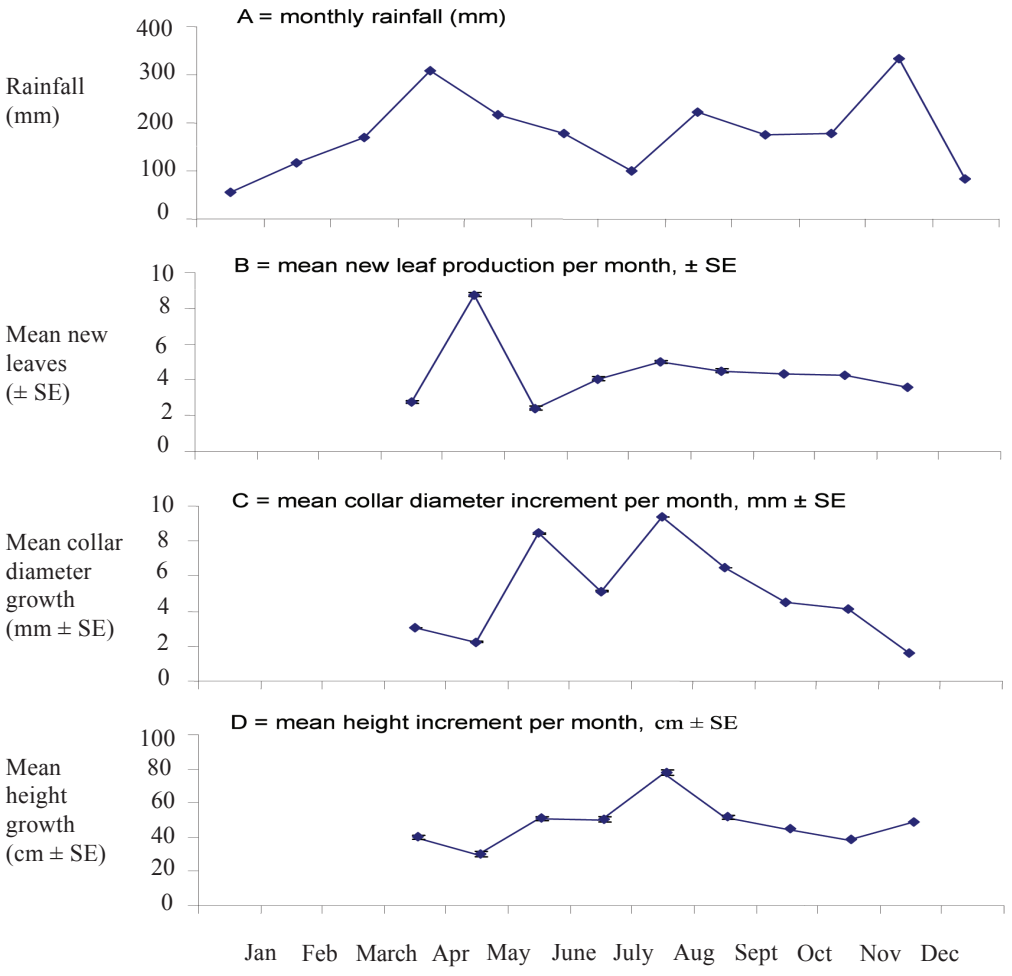

Fig. 1: Growth characteristics of six month-old teak (n = 436) in relation to rainfall after defoliation by P. damastesalis *at Mata Ayer, Perlis*

was shown to have a negligible effect on the measured growth parameters, i.e. height, collar diameter, and new leaf production. Therefore, the level of defoliation during the outbreak was the main factor influencing subsequent growth performance of the trees. No further loss was noted in the leaf area from the leaf fall due to defoliation which particularly affected the heavily skeletonised leaves (76-100% defoliation). Coley (1983) reported that leaves shed after defoliation increased the leaf area lost to insect herbivory in the tropical forests; thus conservative defoliation levels were recorded in the present study.

The occurrence of the outbreak during the dry month of January concurs with that noted by Intachat *et al.* (2000), whereby more severe attacks were reported during the dry spells in Sabah. However, Tho (1981) observed that defoliation was heaviest at the FRIM Mata Ayer field station from April to July, and attributed this to the leaf flush that followed the onset of the southwest monsoon in March. Chey (1999) suggested that pest was more abundant during wetter periods in Sabah, but found no significant correlation between the adult *P. damastesalis* population and rainfall in a year-long study. Although the general consensus

TABLE 1
Effect of *P. damastesalis* defoliation on monthly collar diameter and height increment, and new leaf production of six month-old teak

Month	Partial correlation between*			
	Df and Cd (Initial Cd held constant)	Df and Ht (Initial Ht held constant)	Df and New leaf production	
			Initial Ht held constant	Initial Cd held constant
1	-0.07 0.158	-0.29 0.00	-0.18 0.00	-0.16 0.001
2	-0.15 0.002	-0.19 0.00	-0.13 0.009	-0.11 0.026
3	0.004 0.933	0.02 0.741	-0.11 0.018	-0.12 0.014
4	0.02 0.027	0.07 0.161	0.06 0.233	0.05 0.337
5	-0.11 0.027	0.05 0.240	-0.04 0.455	-0.01 0.812
6	-0.01 0.867	0.04 0.363	-0.09 0.065	-0.07 0.130
7	-0.10 0.029	0.05 0.276	0.03 0.589	0.03 0.533
8	0.05 0.279	0.02 0.705	0.06 0.206	0.07 0.127
9	0.04 0.416	-0.02 0.714	0.02 0.661	0.04 0.468

Cells contain r (Pearson's) and p-value (italics). Significant correlation is given in bold-face.
* Df, defoliation increment; Cd=collar diameter increment; Ht=height increment. Partial correlation controlled for the initial height effect on both defoliation severity and subsequent growth (n = 436)

is that outbreaks of this pest occur seasonally, there remain conflicting observations as to the season in which outbreaks occur. Meanwhile, food availability and weather conditions, brought about by changes in the seasons, are probably two important factors contributing to the *P. damastesalis* outbreaks. Cold, dryness and/or rainfall are also thought to affect the *P. machoeralis* populations in India (Tewari, 1992). Furthermore, seasonal changes affecting weather could have indirectly affected insect populations, while the drought in the present study could have reduced nectar production from flowering weeds and depressed parasitoid populations.

A greater defoliation intensity observed with taller trees could have been due to being the first encountered by wind-borne female moths and/or perhaps as a result of preferential oviposition by the moths on taller trees. Gravid females may exhibit oviposition preferences for suitable host plants based on chemical cues (Hinton, 1981), while *P. damastesalis* females might have selectively oviposited on the healthier and taller trees. *Paliga damastesalis* is not a strong flier (Intachat, 1999), but if present in the plot prior to the outbreak, they could have conceivably reached the taller trees from their daytime ground vegetation refuges. Moths lay several

eggs at a time on different leaves and the even defoliation pattern throughout the affected tree suggests that the moths are capable of flying to a height of at least three metres (Lim, personal communication).

The negative association between defoliation level and post-defoliation height increment and new leaf production for the initial two and three months, respectively, indicate short-term impacts of defoliation on these growth parameters. Tree recovery was fast with regard to these growth parameters. However, the variably depressed collar diameter increment, up to the seventh month of post-outbreak, suggests that girth expansion is affected more severely than height increment or new leaf production. Young trees such as those assessed in the present study could be badly affected by defoliation and/or other stress factors because they have less reserves upon which to draw from compared with older trees (Reekie, 1997). Food reserves stored in plant roots and stems that possibly support cambial growth could have been diverted for new leaf production after denudation. A severely defoliated plant would therefore need to restore such reserves before cambial growth could occur.

According to Hopkins (1999), factors influencing plant growth could be intrinsically mediated through genetic and hormonal reactions, as well as extrinsically through environmental factors such as light and moisture. This may be true in the case of the consecutive peaks in new leaf production, collar diameter and height increment. In addition, the plant is also capable of maximising growth, under variable environmental conditions over the study period, by adjusting within-plant allocation patterns (Bazzaz, 1997). In the present study, it is apparent that rainfall was the environmental factor that influenced tree growth across all the defoliation classes; however, within-plant shifts in allocation patterns could have probably resulted in the interactions among the growth parameters. During the transition period, i.e. from dry to wet season, a light flush of new leaves were produced to replace those shed due to defoliation and/or drought. The subsequent channelling of photoassimilate from maturing leaves to the vascular cambium probably explains the surge in collar diameter increment. Meanwhile, the girth expansion resulting from addition of structural tissue would then provide the mechanical support needed for further height increment or canopy expansion (Grace, 1997). The growth peak in June and the corresponding trough in height and collar diameter increments may reflect an alternating period of activity (photoassimilate uptake) in the apical meristem and vascular cambium. The findings gathered in the present study suggest that rainfall strongly influences the overall growth patterns of teak, the results which contradict with the ones reported by Chowdhury (1940). Fertilizing and irrigating trees following defoliation may boost their growth and aid in the recovery process. A complex interaction between the parameters measured seems to appear and this could be attributed to the inherent shifts in photoassimilate allocation.

CONCLUSIONS

Defoliation was found to have depressed the relative growth rates in relation to the level of defoliation experienced for the first two to three months after the outbreak. The reduction in growth was manifested by a reduced collar diameter increment, height increment, and new leaf production. An increase in one of these growth parameters corresponded with a decrease in either or both of the other parameters. Finally, the trees were observed to recover by the fourth month.

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Morphological and Chemical Characteristics of Black Gram (*Vigna mungo* L.) Sprouts Produced in a Modified Atmosphere Chamber at Four Seeding Densities

S.Y. Choon[1], S.H. Ahmad[1*], P. Ding[1], U.R. Sinniah[1] and A.A. Hamid[2]
[1]Department of Crop Science, Faculty of Agriculture,
[2]Department of Food Science, Faculty of Food Science and Technology,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: hajar@agri.upm.edu.my

ABSTRACT

A modified atmosphere with low oxygen and high carbon dioxide sprouting environments can be used to regulate sprout growth. By monitoring seeding density used for sprouting, stress ethylene is produced due to the compactness within sprouting pot. A study on the morphological and chemical characteristics and the total phenolic compounds of black gram sprouts (*Vigna mungo*), grown in a hermetically sealed chamber, was conducted using four seeding densities (50, 75, 100, and 125 g seeds L^{-1}). For this purpose, the selected pre-soaked seeds were put into a pot and placed in the chamber. The seeds were allowed to sprout for four days and watering was done every three hours for 20 minutes. Sprouts produced with lower seeding density (50 and 75 g seeds L^{-1}) were long and etiolated with long roots and higher sprout weight. In higher seeding density (100 and 125 g seeds L^{-1}), on the contrary, sprout length was shorter with short roots and lower sprout weight, and the ratio between hypocotyl and root length was 1:1. Meanwhile, there were no significant differences in hypocotyl diameter of sprouts produced. Sprouts produced at a lower seeding density had 2.7% lower soluble solids concentration, but they had 18% higher contents of ascorbic acid as compared to the ones produced in higher seeding density. Similarly, there were significant differences in the total phenolic compounds of sprouts and the contents decreased by 43% as the seeding densities increased. Over-crowding and lack of watering produced poor quality sprouts. Thus, seeding density and watering duration during sprouting in a hermetically sealed chamber need to be determined for sustainable sprout production, as well as to produce safe sprouts as demanded by consumers.

Keywords: Ethylene, phenolic compounds, soluble solids concentration, ascorbic acid, cotyledon, sprout length

INTRODUCTION

Bean sprouts or *tauge* are three- to five-day old germinated seedlings of green gram (*Vigna radiata*) or black gram (*Vigna mungo*) which are grown as soilless culture in darkness. The total amount of bean sprouts produced in Malaysia is estimated to be about 12 million metric tons a year, with an annual value of RM144 million. Bean sprouts can be cooked in many ways or eaten raw. They are rich in vitamins, minerals, and have been reported to contain important phytochemicals for disease prevention and health promoting benefits (Fernandez-Orozco *et al*., 2008). Bean sprouts are particularly popular in Asian cuisine and the consumption is increasing in Western countries.

Sprouting activates enzyme processes to sustain the later growth stages (Fernandez-Orozco *et al*., 2006). These chemical changes

[*]Corresponding Author

increase nutritive values and health qualities of sprouting seeds (Plaza *et al*., 2003). Storage nutrients such as concentrated starch and protein are mobilized into simpler carbohydrates and free amino acids (Ghazali and Cheng, 1991), which can be readily used by the human body (Sattar *et al*., 1988). Several studies have reported higher levels of amino acids, digestive protein, and available carbohydrates, as well as lower levels of non-nutritive factors such as trypsin inhibitors, phytic acid, and α-galactosides in legume sprouts as compared to their non-germinated seeds (Fernandez-Orozco *et al*., 2008; Vidal-Velverde *et al*., 2002).

Bean sprouts are conventionally grown in drums (blue-plastic or stainless steel), tanks (fibre-glass) or bins (garbage) and watered manually using rubber hoses or buckets. The sprouts produced are normally of poor quality with long (10-12 cm) and thin (1-1.5 mm) hypocotyls and long (7-8 cm) roots. A non-registered chemical known as 'Santoso' is widely used by local growers to produce bean sprouts with short (5-6 cm), thick (2-3 mm) and crispy hypocotyls, and short (1-2 cm) roots. Such short-rooted sprouts are highly demanded by Malaysians since they can just be cooked directly without having to remove their undesirable looking roots.

A preliminary work to study the use of 2,4-Dichlorophenoxyacetic acid (2,4-D) and Benzylaminopurine (BAP) as soaking solution on the growth characteristics of sprouts resulted in rootless sprouts with abnormal radial expansion at the hypocotyl which was similar to commercial sprouts. Bean sprouts that are too stunted and with over swollen hypocotyls are usually found among a batch of sprouts sold in the market, indicating indiscriminate and excessive use of this particular non-registered chemical. As indicated earlier, the sprouting process takes only three to five days. Thus, sprouts produced contain chemical residues that could be hazardous to human health in the long term.

Seeding density, which is the amount of seeds per litre of container, is important in sprout production as it determines sprout quality (Chang and Yeh, 1984). Low seeding density causes poor growth and produces thin and long sprouts. Meanwhile, high density causes overcrowding which results in short, curved sprouts and non-germinated seeds (Lee *et al*., 2007). Studies showed that ethylene is produced during seed germination and it acts as a growth inhibitor with the triple-response effects of dark-grown seedlings of *Pisum sativum* and *Arabidopsis* (Guo and Ecker, 2004). These responses consist of thickened hypocotyl, inhibition of both hypocotyl and root elongation, as well as exaggerated apical hook formation. The concentration of endogenous ethylene produced affects the quality of sprouts.

A hermetically sealed chamber was designed and constructed (Ahmad and Mohamed, 1988), and it could produce a modified environment for sprout growth. However, the chamber has not been fully tested and utilized commercially. Sprouting in the modified chamber environment has also been shown to produce high phenolic compounds as reported for tomatoes (Kubota *et al*., 2006). In plants, phenolic compounds are secondary metabolites which are synthesized through the glycolytic pathway, pentose phosphate pathway, shikimate and phenylpropanoid pathways (Randhir *et al*., 2004). They function as antimicrobial, antioxidant or chemical toxins in plant and repel would-be predators (Mccue and Shetty, 2001). Phenolics have nutraceutical importance to human who consumed plants containing them (Parr and Bolwell, 2000; Hasler, 1998). They are able to scavenge free radicals through electron-donating properties, generating a relatively stable phenoxyl radical or non-radical species (Lu and Foo, 2001).

The objective of this study was to determine the effects of seeding density, during sprouting in a chamber, on the morphological and chemical characteristics and the total phenolic compounds of sprouts. This is because the seeding density that produces sprouts with the optimum

morphological and chemical characteristics and total phenolic compounds could be used for commercial sprout production, and more importantly, without the use of any unregistered chemical to produce safe and desirable bean sprouts for consumption in Malaysia.

MATERIALS AND METHODS

Seeds and Sprouting Process

The black gram seeds used in the present study were those imported from Myanmar and purchased from a seed supplier in Malacca. These seeds were selected to remove imperfect seeds (e.g. broken or empty seeds) and inert matters (small stones, soil particles, and other debris), washed and sterilized in 10% sodium hypochlorite solution (NaCIO) (Clorox®) for 10 min. Then, the selected seeds were soaked in 150 mgL^{-1} calcium ($CaNO_3$) solution at room temperature for 12 h. Wholesome and fully imbibed seeds were put into a plastic pot (19.5 cm height x 16.5 cm diameter) which was perforated with holes (2 cm distance between the holes of 0.5 cm in diameter) at the bottom for drainage purposes.

Four seeding densities (50, 75, 100, and 125 g seeds L^{-1}) of soaked bean seeds, were put into each pot. The pots were arranged randomly in a stainless steel chamber equipped with an automatic watering system (Ahmad and Mohamed, 1988). Then, the chamber was hermetically sealed. The seeds were let to sprout in the chamber for four days, at 22-25°C and with 95-99% relative humidity. The sprouting seeds were watered for 20 minutes with a total volume of 9.6 L water at every three-hour interval. The sprouts were harvested after four days of sprouting for both the morphological and chemical characteristics measurement. Prior to sampling, the sprouts were separated into three equal layers, namely, top, middle, and bottom parts of each pot. Representative samples for the physical and chemical analysis were taken from the middle and bottom layers only, while the sprouts from the top layer were discarded because they were thin, long, and yellow.

MORPHOLOGICAL CHARACTERISTICS MEASUREMENT

Fifty sprouts were randomly selected in this study. The length of these sprouts was measured from the root to the base of the cotyledon, while the hypocotyl length was measured from just above the root to the base of the cotyledon. The length of root was measured from the end of the hypocotyl to the end of the root, while the hypocotyl diameter (thickness of sprout hypocotyls) was measured at the centre of each hypocotyl using an electronic calliper. The sprout fresh weight (i.e. the average fresh weight of 50 sprouts) and the cotyledon fresh weight (i.e. the average fresh weight of 50 cotyledons) were also determined.

CHEMICAL CHARACTERISTICS MEASUREMENT

Soluble Solids Concentration (SSC)

The soluble solids concentration (SSC) was determined according to Dadzie and Orchard (1997). Twenty gram of samples and 80 mL distilled water were homogenized with a blender (MX-798S, National, Malaysia) and filtered. Soluble solid concentration (SSC) was measured by dropping 1-2 drops of filtrate on the glass prism of a hand refractometer (Model N-1E, Atago, Japan). The reading was recorded and % SSC was calculated using the following formula:

[(refrectometer reading x dilution factor) + 0.28]

Meanwhile, the dilution factor was used as the sample was diluted in distilled water and the value was calculated using the following formula:

$$\frac{1 + \text{water volume (ml)}}{\text{Sample weight (g)}}$$

Titratable Acidity (TA)

For the titratable acidity measurement (Ranganna, 1977), 2 drops of 1% phenolphthalein was added into 5 mL of filtrate remaining from the SSC determination. The filtrate was titrated with

0.1 M NaOH until the colour turned pink. The results were converted to % citric acid using the following formula:

$$\frac{\text{(ml NaOH x 0.1 M NaOH x titrate vol (100 mL) x 64 g (equivalent weight of citric acid) x 100)}}{\text{sample weight (20 g) x sample vol for titration (5 mL) x 1000}}$$

pH

The balance of the filtrate from the determination of titratable acid was also used for the pH measurement using a pH meter (GLP 21, Crison, Barcelona). The glass electrode of pH meter was calibrated with buffer at pH 4.0, and this was followed by pH 7.0 before it was used. The glass electrode was washed with distilled water after calibration and then wiped with a soft tissue paper. It was then placed in the filtrate and a stabilized pH reading was recorded.

Ascorbic Acid (AA)

For the ascorbic acid determination (Ranganna, 1977), 20 g of sprout sample were homogenized with 80 mL of 3% cool metaphosphoric acid in a blender (MX-798S, National, Malaysia). Similarly, five ml from the filtrate was titrated with 2,6-diclorophenol-indophenol dye solution until the colour turned pink. The concentration of ascorbic acid was recorded as ascorbic acid (mg/100 g) using the following formula:

$$\frac{\text{ml-dye used x dye factor x titrate vol (100 mL) x 100}}{\text{sprout weight (20 g) x sample vol for titration (5 mL)}}$$

TOTAL PHENOLIC COMPOUNDS (TPC)

The total phenolic compounds were determined using Folin-Ciocalteau reagent according to the procedure proposed by Lin and Lai (2006). The harvested sprouts were freeze-dried, blended with a blender (MX-798S, National, Malaysia) and the fine powder obtained was stored in darkness in a freezer at -10°C until further use. The sprout powder (5 g) was suspended in 80% methanol solution (100 mL) and extracted at 60°C in a water bath with continuous shaking for 2 hours. After that, the extracted solvent was filtered through a filter paper. The filtrate (100 µL) was diluted with 100 µL of MeOH/0.3% HCl (6:4, v/v), mixed with 2% Na_2CO_3 solution (2 mL) and kept for 2 min. After the addition of 100 µL of 50% Folin-Ciocalteu's reagent to the mixture, the filtrate was incubated for 30 min at room temperature in the dark. The filtrate was vortexed and the absorbance at 750 nm was measured using a spectrophotometer (S1200 spectrawave, Cambridge, England). The determination of the TPC amount was repeated in triplicates and calculated from the standard curve of gallic acid (1-20 mgL^{-1}) (R^2=0.98) to give an absorbance range of 0.326-1.055. The results were expressed as gallic acid equivalents (mg gallic acid per g of dry meal).

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

The experiment was laid out in a completely randomized block design in order to determine the effects of seeding density on the morphological and chemical characteristics, as well as the total phenolic compounds of sprouts produced. Three out of the ten pots produced in the chamber of each seeding density were used for the analysis. The data were analyzed using the analysis of variance and the means were separated using the Duncan's multiple range tests at $P \leq 0.05$. Meanwhile, the regression analysis was carried out to describe the relationship between the morphological and chemical characteristics at different seeding densities. The correlation analysis was performed to indicate the strength of relationship within the morphological and chemical characteristics.

RESULTS AND DISCUSSION

Based on the data gathered in the experiment, there was a significant ($P \leq 0.01$) quadratic decrease in the length of sprouts as seeding density was increased (*Fig. 1A*). Initially, the sprout length showed a linear decrease when

50 to 75 g seeds L^{-1} were used for sprouting. However, as the seeding densities increased, the sprout length gradually decreased. There were also significant ($P \leq 0.01$) quadratic decreases of hypocotyl and root length (*Figs. 1B* and *1C*). Similarly, the lengths of hypocotyl and root showed linear decreases as seeding density was increased from 50 to 75 g seeds L^{-1} seeds and the length gradually grew shorter as the seeding densities increased.

The significant positive correlation (r^2=0.98) between the length of hypocotyls and root length indicated that a decrease in the hypocotyl length was followed by a decrease in the root length (Table 1). From the data obtained, the ratio between hypocotyl and root length was 1:1. These findings showed that during sprouting, reserves from cotyledon were used for both the growth of hypocotyl and root. In the correlation analysis, the length of sprouts had significant and positive correlations with the length of root

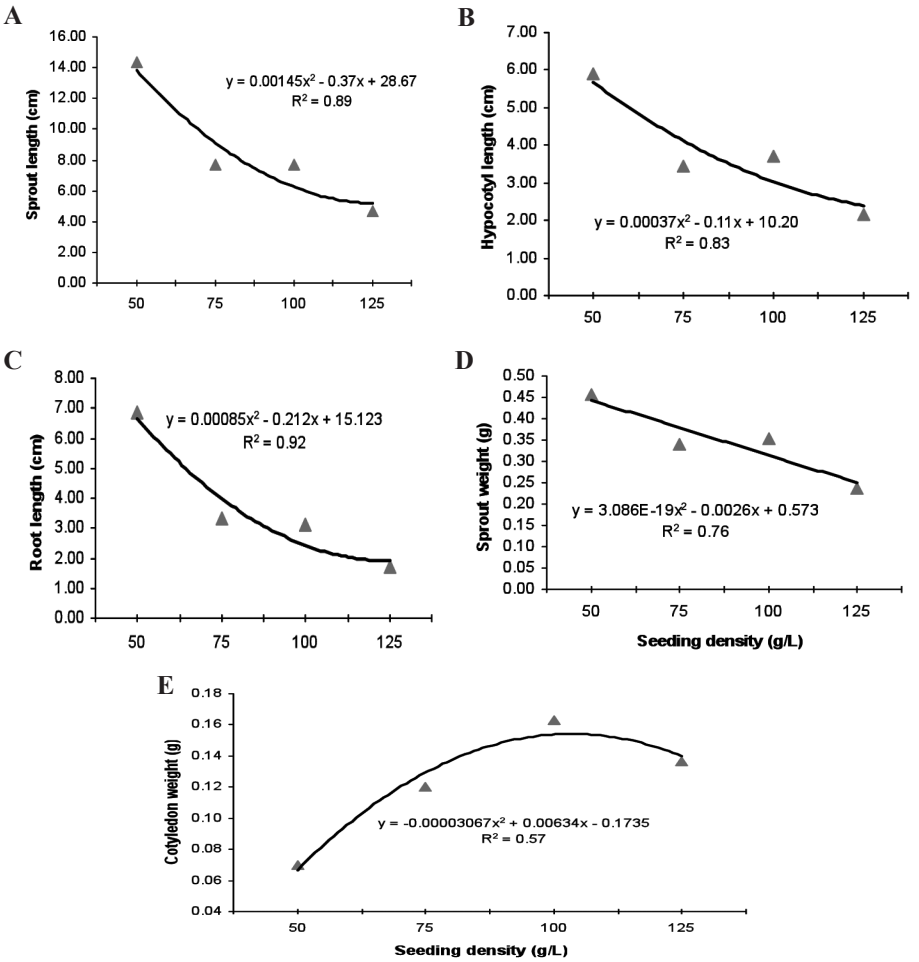

Fig. 1: The physical characteristics of sprouts produced; (A) sprout length, (B) hypocotyl length, (C) root length, (D) sprout weight, and (E) cotyledon weight. Data shown are the mean of 3 replicates of 100 sprouts (per replicate) collected from the two layers of sprouts in the sprouting pot

(r^2=0.99) and hypocotyl (r^2=0.98). Therefore, in this study, the increase in seeding densities resulted in shorter hypocotyl and root lengths of sprouts which affect the length of sprouts produced.

The decrease in the length of sprouts could be related to the ethylene effect during sprouting. When seeding density was increased, the sprouts grew closer to one another, and this led to compaction within the sprouting pot, and induced production of stress ethylene (Ahmad, 1985). The higher the seeding density used, the higher the production of stress ethylene would be. Several studies showed that restricting the elongation of soybean seedlings with a strain gauge resulted in a 3-7 fold increase in the ethylene production rate from hypocotyl which retarded elongation and increased hypocotyl expansion (Abeles *et al*., 1992). Meanwhile, pea epicotyls that were enclosed in the chambers, where the elongation was restricted by glass beads, were found to increase ethylene evolution that in turn induced radial expansion and decreased internode length (Abeles *et al*., 1992).

THE EFFECT OF SEEDING DENSITY AND WATERING DURATION ON HYPOCOTYL DIAMETER

Nonetheless, hypocotyl diameter was not affected by seeding densities as there was no significant linear or quadratic relationship found between hypocotyl diameter of sprout and seeding density used. The correlation analysis revealed that the relationships between the hypocotyl diameter with sprout length (r^2=0.42), hypocotyl length (r^2=0.44), root length (r^2=0.43), sprout weight (r^2=0.56), and cotyledon weight (r^2=0.15) were low (Table 1).

THE EFFECT OF SEEDING DENSITY AND WATERING DURATION ON SPROUT AND COTYLEDON FRESH WEIGHT

The results obtained indicated that there was a significant ($P \leq 0.01$) quadratic decrease of sprout weight as seeding density increased (*Fig. 1D*). When the seeding densities increased, the sprout weight reduced. The decrease of sprout weight was found to be related to the lengths of

TABLE 1
Correlation coefficients between sprout length (SL), hypocotyl length (HL), root length (RL), hypocotyl diameter (HD), sprout weight (SW) and cotyledon weight (CW) of the sprouts produced in four seeding densities

	SL	HL	RL	HD	SW	CW
SL	-					
HL	0.99*	-				
RL	0.99*	0.98*	-			
HD	0.42^{ns}	0.44^{ns}	0.43^{ns}	-		
SW	0.92*	0.95*	0.91*	0.56^{ns}	-	
CW	-0.60*	-0.55^{ns}	-0.61*	0.15^{ns}	-0.36^{ns}	-

For correlation coefficient, n=12.

^{ns} and * non-significant, significant at $P \leq 0.05$, respectively

sprouts, hypocotyl, and root. The correlation analysis also demonstrated that there were significant positive correlations between the weight of sprouts and sprout length (r^2=0.92), hypocotyl length (r^2=0.95), and root length (r^2=0.91). However, no significant correlation (r^2=0.56) was shown between sprout weight and hypocotyl diameter. This finding indicated that sprout weight was not affected by the diameter of hypocotyl.

Meanwhile, the decrease in sprout weight as a result of the increase in seeding densities indicated that when more seeds were used in the study, shorter sprouts were produced. Shorter sprout length in higher seeding densities occurred when there were more seeds in the containers during sprouting and led to over-crowding. In more specific, seeds from the bottom layers were unable to get sufficient water for growth and cooling purposes. In addition, the metabolic heat created during sprouting and the endogenous ethylene produced could also be the reasons that inhibited the sprout growth.

Apparently, there were significant negative correlations between the cotyledon weight with sprout length (r^2=-0.60) and root length (r^2=-0.61) (Table 1). On the other hand, the weight of cotyledon had no significant relationships with the weight of sprouts (r^2=0.36), hypocotyl length (r^2=0.55), and hypocotyl diameter (r^2=0.15). It is important to note that cotyledons are the food storage tissues of the embryo in dicots. The function of cotyledon is to supply the growing seedlings with nutrients until they are capable of producing their own food. In most species, the storage tissues shrivel and drop off as their reserve nutrients deplete (Elias, 2006).

The results from the study showed that the weight of cotyledon was heavier in sprouts from higher seeding densities (*Fig. 1E*). Meanwhile, the correlation analysis revealed that there were significant negative correlations (r^2=-0.60) between the weight of cotyledon and sprout length. In addition, there was also a significant negative correlation (r^2=-0.61) between the weight of cotyledon and length of roots (Table 1).

The decrease in the weight of cotyledon indicated that there was a translocation of soluble products to the axis for respiration (Karunagaran and Ramakrishna Rao, 1991). In the present study, however, no decrease was observed in the weight of cotyledon in higher seeding densities. These could have resulted from reduced mobilization of reserves from cotyledon or from their reduced utilization by the embryo axis (Promila and Kumar, 2000).

Sprouts which are produced under modified environment have shorter and thicker hypocotyls and shorter roots as compared to conventionally grown sprouts (Ahmad, 1985). It was reported that sprouting in an enclosed system restricted oxygen supply by the surrounding structures causing anaerobic respiration in sprouts, and resulting in reduced respiration rate. Peppelenbos and van't Leven (1996) found that bean sprouts displayed a high respiration rate during sprouting. However, high carbon dioxide concentrations, at ambient oxygen concentrations, were found to decrease respiration rates in mungbean sprouts. This was because the internal oxygen was consumed by the respiration of both cells and tissues with carbon dioxide production in the chamber (Varoquaux *et al*., 1996). When the respiration rate of sprouts slowed down, the reserves in the cotyledon were not fully utilized, and thus inhibited sprout growth (Bewley and Black, 1978).

THE EFFECT OF SEEDING DENSITY AND WATERING DURATION ON SOLUBLE SOLID CONCENTRATION, TITRATABLE ACID AND PH

There were significant ($P \leq 0.05$) quadratic increases of soluble solid concentration and pH in the sprouts produced (*Figs. 2A* and *2C*), while titratable acidity showed a significant ($P \leq 0.05$) quadratic decrease with increasing seeding density (*Fig. 2B*). The higher percentage of soluble solids concentration of sprouts, due to the increased seeding densities, also indicated an increased sweetness in sprouts. However, as the seeding densities increased, sprouts

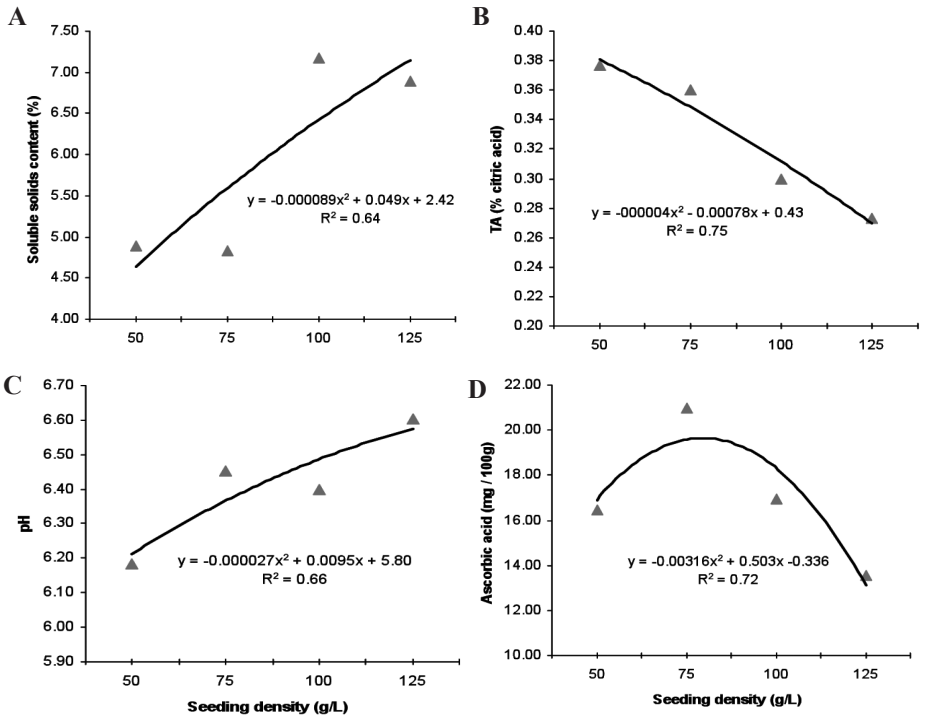

Fig. 2: The chemical characteristics of sprouts; (A) soluble solids concentration, (B) titratable acid, (C) pH, and (D) ascorbic acid. Data shown are the mean of 3 replicates of each treatment

produced showed a decrease of titratable acidity and an increase of the pH value. There was a significant negative correlation (r^2=-0.72) between the soluble solids concentration and titratable acidity (Table 2). The percentage of titratable acidity usually decreases as soluble solid concentration increases because they are respired and converted to starch and sugars (Knee and Smith, 1989).

During germination, the principal reserve materials (starch, proteins, and lipids) are degraded in the cotyledon and the products released are translocated to the developing axis (Karunagaran and Ramakrishna Rao, 1991). From the results, the sweetness of the sprouts was contributed by the contents of soluble sugar in the cotyledon. In more specific, higher cotyledon weight shows that more reserves are contained in it since cotyledon is the part where all the reserves are stored. Meanwhile, sprouting preceded by soaking the seeds in water activates the hydrolytic enzymes (Elias, 2006) and causes the reserve materials of the seeds to be degraded.

α-amylase is the major enzyme involved in the initial degradation of starch into the more soluble forms, while ß-amylase and phosphorylase assist in converting into free sugars in the cotyledon of germinated black gram seeds (Koshiba and Minamikawa, 1981) and mungbean seeds (Toyooka *et al*., 2001). During germination, amylase activity contributes significantly to soluble sugar of the cotyledon.

In horse gram, sugar presents in the cotyledon during the early period of growth is primarily responsible for growth of the embryo axis (Promila and Kumar, 2000) that controls the reserve mobilization in dicotyledonous seeds by affecting α-amylase activities of the cotyledon (De Klark, 1986). A similar situation was also found in mungbean sprouts (Morohashi,

TABLE 2
Correlation coefficients between soluble solids concentration (SSC), titratable acidity (TA), ascorbic acid content (AA) and pH of sprouts produced in four seeding densities

	SSC	TA	AA	pH
SSC	-			
TA	-0.72*	-		
AA	-0.49^{ns}	0.57^{ns}	-	
pH	0.55^{ns}	-0.64*	-0.23^{ns}	-

For correlation coefficient, n=12.
^{ns} * non-significant and significant at P≤0.05, respectively

1982). The degraded reserves are partly used for the respiration and synthesis of the new cell constituents of the developing embryonic axis during sprouting (Vidal-Valverde *et al*., 2002). It is important to note that this process causes important changes in biochemical, nutritional, and sensory characteristics in the sprouts.

In black gram seeds, starch constitutes 47.9% of the seed composition (Srinivasa, 1976). Ghazali and Cheng (1991) reported that the germination of black gram seeds led to a progressive increase in the total sugar content (glucose, sucrose, and fructose) in sprouts. The increase in the total sugar contents has been suggested to be the result of mobilization of oligosaccharides and starch into simple sugar by enzymes (Jaya and Venkataraman, 1980).

In buckwheat sprouts, Kim *et al*. (2004) reported that as seeding days progress, the contents of monosaccharides (fructose and glucose) increased while disaccharides (sucrose and maltose), trisaccharides and tetrasaccharide were decreased. These results illustrated that the di-, tri-, and tetrasaccharide were degraded to monosaccharides during sprouting in the cotyledon to provide the energy for seedling development. Based on the results gathered in the present study, higher cotyledon weight indicated that the soluble sugar in the cotyledon had not been fully used for sprout growth.

THE EFFECT OF SEEDING DENSITY AND WATERING DURATION ON ASCORBIC ACID

There was a significant (P≤0.05) quadratic decrease in the content of ascorbic acid as seeding density increased (*Fig. 2D*). Ascorbic acid is a water-soluble vitamin that acts as an antioxidant protecting against oxidative damage to DNA, membrane lipids, and proteins (Dekkers *et al*., 1996) by reducing small molecule antioxidants such as glutathione, tocopherols, and carotenes (Fernandez-Orozco *et al*., 2006). Germination has been found to increase ascorbic acid level (Kim *et al*., 2004). The increase in ascorbic acid level is due to the consequence of the reactivation of ascorbic acid biosynthesis in the seeds during germination (Mao *et al*., 2005; Xu *et al*., 2005).

In this study, the percentage of ascorbic acid showed the highest level at 75g seeds L^{-1}, while the sprouts from the other three seeding densities yielded lower levels. This might be due to the metabolic heat produced by the sprouting seeds that affected the ascorbic acid content of the sprouts. As the seeding density increased, less water could reach the bottom part of the sprout pot. At the same time, the metabolic heat produced inhibited the biosynthesis of the ascorbic acid, causing low ascorbic acid content of the sprouts produced in higher

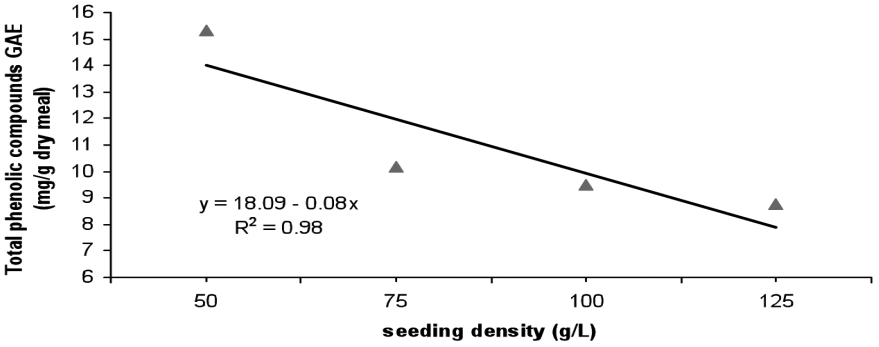

Fig. 3: The total phenolic compounds as GAE (mg/g dry meal) of sprouts. The sprout samples of each treatment from 3 replicates were analyzed in triplicates

seeding densities. Ghazali and Cheng (1991) reported that dry black gram seeds contained only trace amounts of ascorbic acid. However, the content of ascorbic acid increased as the germination proceeded. In lupin sprouts, Frias *et al*. (2005) found that the content of ascorbic acid was increased by 322% after 2 days and this continued to gradually rise up to 9 days where an increment of 866% was observed. In other legumes, such as soybean, Xu *et al*. (2005) found that the germination of soybean seeds for 4 days increased the content of ascorbic acid, but a subsequent germination up to 9 days caused it to decrease. Sattar *et al*. (1988) observed that sprouting temperature affected nutrients (protein, ascorbic acid, riboflavin, and thiamine) and anti-nutrients (phytate and trypsin inhibitor) of mungbean sprouts. Ascorbic acid of mungbean sprouts reached the maximum value after 48 hours of germination, i.e. at 20-35°C.

THE EFFECT OF SEEDING DENSITY AND WATERING DURATION ON THE TOTAL PHENOLIC COMPOUNDS

The TPC estimation is important to determine the antioxidant activity of the sprouts produced. TPC of sprouts produced at 50, 75, 100, and 125 g seeds L^{-1} were 15.3, 10.2, 9.5, and 8.7 of GAE (mg/g dry meal), respectively (*Fig. 3*). There were significant differences ($P \leq 0.05$) in terms of TPC in the sprouts produced using the different seeding densities. The variations of TPC in sprouts were apparently affected by cultivars, test method and environment stresses, such as sprouting temperature and drought (Kim *et al*., 2006). Fernandez-Orozco *et al*. (2008) found that mungbean seeds germinated for 7 days had increased the content of TPC in sprouts.

Randhir *et al*. (2004) stated that germination caused a decrease in TPC of mungbean seeds after 1 day of germination. The higher phenolics produced on Day 1 was utilized for guaiacol peroxidase-mediated polymerization to form polymeric phenolics and lignin during germination. The reduced TPC in the mungbean sprouts in the later stage of germination was due to the partitioning of lignifications and other developmental needs. In this study, sprouts in the higher densities produced lower TPC as compared to sprouts in the lower seeding densities, although they were subjected to a greater stress due to overcrowding. The reduced TPC could be attributed to the high demand for oxygen and phenolic compounds used to protect the cells from oxidation-induced deterioration (Randhir *et al*., 2004).

Xu and Chang (2008) reported that in common beans, the loss of TPC increased with the increasing hydration rate. Meanwhile, longer soaking time caused some polyphenols in the seed coat to be hydrolyzed and diffused into

the water. Thus, further studies on the effect of soaking duration on the sprouts of black bean should be carried out in order to maintain TPC content in sprouts produced.

CONCLUSIONS

In this study, the seeding densities used did not produce the desired sprouts having the characteristics of fat and thick hypocotyl diameter. At the same time, lower seeding densities (50 and 75 g seeds L^{-1}) used were not able to create the compactness in the pot, and thus less ethylene stress was produced. Instead, thin and etiolated sprouts were produced. At high seeding density (i.e. 125 g seeds L^{-1}) the sprouts growth were inhibited. The poor growth and quality of sprouts were due to the over-crowding of the sprouts in the pot, in addition to the condition whereby irrigation water could possibly not reach the bottom part of the sprouting pot and led to partially germinated or non-germinated seeds. Therefore, more studies need to be carried out on seeding density and watering duration during sprouting in a hermetically sealed chamber for sustainable sprout production, as well as produce safe sprouts for consumers.

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Management of Mixed Weeds in Young Oil-palm Plantation with Selected Broad-Spectrum Herbicides

Rosli B. Mohamad[1*], Wahyu Wibawa[1], Mohd Ghazali Mohayidin[2], Adam B. Puteh[1], Abdul Shukor Juraimi[1], Yahya Awang[1] and Mohammad B. Mohd Lassim[1]

[1]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

[2]Faculty of Business and Management, Open University Malaysia, 50480 Kuala Lumpur, Malaysia

[*]E-mail: rosli@agri.upm.edu.my

ABSTRACT

A field study to evaluate the efficacy of three broad spectrum herbicides on mixed weed in a young (2 year-old) oil palm plantation was conducted. Experimental plots, with the size of 4.8 x 20.5 m^2 each, were laid in a randomized complete block design with 4 replications. Weed vegetation analysis was conducted before herbicide treatments were applied. Herbicide treatments used were paraquat and glufosinate-ammonium (200, 400, 600, 800 g a.i. ha^{-1}), and glyphosate (400, 800, 1200, 1600 g a.i. ha^{-1}), with untreated control. The experimental locality indicated a composite of mixed weeds of broadleaf and grasses. The growth of broadleaf was more dominant with 25 species (relative dominance of 82.1%) than the grasses with only 7 species (relative dominance of 17%). The three most dominant species were the broadleaves of *Croton hirtus* and *Asystasia gigantica*, and a grass, *Paspalum commersonii*. The percentage of the mixed weed composite killed was found to be significantly affected by the treatments of paraquat, glufosinate–ammonium and glyphosate, relative to the untreated control, with more than 50 percent weed killed taken at 2 and 4 WAT. Meanwhile, glyphosate and glufosinate-ammonium produced greater efficacy (more than 90 percent killed) as compared to paraquat which produced lower total weed killed (50 to 83%). There were positive correlations between the percentages of weed killed and weed growth reduction. Increased percentage of weed killed was followed by the increase in the percentage of weed growth reduction, with the indication that weeds were recovering and began to produce new shoots at 16 WAT. Treatments producing fewer efficacies caused weeds to regrow and recover faster or in a shorter time. Increased rates of paraquat treatments, i.e. from 200 to 600 and 800 g a.i. ha^{-1}, were found to increase the duration of effective weed control. The duration of effective weed control produced by glufosinate-ammonium at 200 to 800 g a.i. ha^{-1} and glyphosate at 400 to 1600 g a.i. ha^{-1} ranged from 14.5 to 15 weeks, which were significantly longer than the paraquat treatments. The increased rates of glufosinate-ammonium and glyphosate did not necessarily increase the duration of effective control.

Keywords: Mixed weeds, young oil-palm plantation, broad spectrum herbicides

INTRODUCTION

Weed is a major component in oil palm production system. The composition of weeds is a mixture of grasses, sedges, and broadleaves which often changes according to the crop growth stages which provide specific climatic and environmental conditions suitable for specific weed growth. The shade provided by the palm canopy influences the nature of weed composition, and grass species tend to dominate as the oil palms get bigger (Wan Mohamed *et al*., 1987). The effect of weeds on oil palm

[*]Corresponding Author

is difficult to quantify because of their long economic life (i.e. 20-30 years) but they can affect the growth of crops or cause yield losses (Kuan *et al*., 1991).

Weeds in plantation are managed using several methods such as cultural, mechanical, integrated production system of using livestock to control the weeds, or chemical (herbicides). Weed management with the use of chemical herbicides is the most common practice in oil palm plantations at some stages of crop development. Broad spectrum herbicide, paraquat, was used for more than 40 years, and was the only mostly used herbicide in Malaysian plantations. The use of this herbicide, however, has been halted since 2002 by the Government of Malaysia, for reasons of toxicity and hazards to humans, but was lifted in 2006 to allow for more comprehensive study. The prohibition of paraquat use left an open option to users for replacement. Several other common broad-spectrum herbicides are available in the Malaysian market. Among these herbicides are glufosinate-ammonium and glyphosate. All these herbicides are foliar applied, with paraquat activity being through contact (Turner and Gilbanks, 2003), glufosinate-ammonium being partially systemic (Collins, 1991), and glyphosate being systemic (Chang and Liao, 2002).

Most herbicide efficacy studies in oil palm plantation have reported on certain specific noxious weeds (Chung and Balasubramanian, 1991; Pin and Lang, 1991; Ikuenobe and Ayeni, 1998; Utulu, 1998). Meanwhile, a study evaluating some new herbicides for general weed control in young oil palm was reported by Khairudin and Teoh (1990). However, a single predominant weed is rarely found under field condition. Instead, predominant weeds comprised of a few weed species (or rather it is a mixed weed situation) (Aldrich, 1984), and weed population, particularly in crop areas, are never constant, but are in dynamic state of flux due to changes in climatic and environmental conditions, husbandry methods, and the use of herbicides (Cobb, 1992). This study was undertaken to gather more current information on the control of natural mixed weeds in immature oil palm, whereby the use of broad spectrum herbicides is the most appropriate. It evaluates the efficacy and duration of effectiveness in using the broad spectrum herbicides of paraquat, glufosinate-ammonium and glyphosate on the total mixed weed population found in a young (2 year-old) oil palm plantation.

MATERIALS AND METHODS

Field Experimental Set-up and Initial Vegetation Analysis

The experiment was conducted in a two-year old oil-palm plantation at MAB Agriculture-Horticulture Sdn. Bhd. in Sepang, Selangor, Malaysia. The plots in the size of 4.8 x 20.5 m^2 each were set up in a randomized complete block experimental design with four replications.

An initial weed vegetation analysis was conducted in the experimental plots prior to the application of the herbicide treatments. It was conducted to determine the composition of weeds based on their species, density of growth, and species dominance in the experimental locality. The identification of the weed species was done according to Bernes and Lus (1990) and Sahid and Chan (2000). Meanwhile, the square method (0.5 x 0.5 m2) was used to sample and classify the weed species. Weeds were counted in 10 quadrates to determine their species, as well as to obtain the density and dominance of each species expressed in relative terms, using the following formulae (Derksen et al., 1993):

$$\text{Relative (X) of a species} = \frac{\text{Absolute (X) of the species}}{\text{Total absolute (X) of all species}} \times 100\%$$

where, X = density or dominance

Above ground weed vegetation was harvested and separated by species (for identification), sun-dried for 4 days and then oven-dried at 75^{0}C for 48 h (for dominance evaluation) (Felix and Owen, 1999).

Herbicide Treatments

The plots were sprayed in the manner of controlling weeds (at 200 L ha^{-1}), with four rates of each: paraquat (200, 400, 600, and 800 g a.i. ha^{-1}), glufosinate-ammonium (200, 400, 600, and 800 g a.i. ha^{-1}), and glyphosate (400, 800, 1200, and 1600 g a.i. ha^{-1}), and untreated control plots. The rates cover the range of the recommended rates for the field applications, namely paraquat (400-600g a.i. ha^{-1}), glufosinate-ammonium (500g a.i. ha^{-1}), and glyphosate (1000g a.i. ha^{-1}). The herbicide formulations used were Gramoxone^{R} (200g paraquat $liter^{-1}$, Syngenta Crop Protection), Basta 15^{R} (150g glufosinate-ammonium $liter^{-1}$, Bayer Cropscience), and Roundup^{R} (360g glyphosate $liter^{-1}$, Monsanto). The spraying was done manually using a knapsack sprayer fitted with AN 2.5 deflector nozzle.

Evaluation of Treatment Efficacy

The square method was also used to evaluate the degree of weeds controlled (killed, growth reduction, and duration) by the herbicide treatments. The samples were randomly taken at three locations in each experimental unit. Destructive and non-destructive samplings were used for this purpose. The percentages of the total weed killed and the total weed growth reduction relative to the untreated control plot, and the duration of effective control were evaluated. The percentage of weeds killed was taken at 2 and 4 weeks after the treatment (WAT) by counting the plants with all the tissues from the growing point to the soil surface being completely dead. The percentage of weed growth reduction was calculated from the dry weight of the weeds sampled at 8, 12, and 16 weeks after the treatments using the following formula (Pritchard, 2002; Chuah *et al*., 2004):

$$\% \text{ growth reduction} = 100 - \frac{\text{Dry weight of samples from treated plot}}{\text{Dry weight of samples from untreated plots}} \times 100$$

Duration of effective weed control (i.e. a period when a treatment is able to suppress weed growth >50% relative to untreated plot) was deduced from the percentage of weed growth reduction. The data were obtained for 8, 12, and 16 WAT. The data between the narrowest observations, i.e. from 8 to 12 and 12 to 16 weeks after the treatments, were predicted using the regression analysis to estimate the correlations between the percentage weed killed to weed growth reduction, and the duration of effective weed control.

RESULTS AND DISCUSSION

Initial Weed Vegetation Analysis

Initial weed vegetation analysis is needed to determine the weed species present, and their density and dominance of growth at the locality of the experiment. Krueger *et al*. (2000) noticed that one of the keys for a successful post-emergent weed management strategy is the knowledge of weeds present in the field, and the density of each species. In fact, a single predominant weed is rarely found under field condition, and yet predominant weed is composed of few weed species (Aldrich, 1984). Weed populations, especially in crop areas, are never constant, but they are in dynamic state of flux due to the changes in climatic and environmental conditions and husbandry methods (Cobb, 1992). In the present study, the weed vegetation analysis recorded 32 weed species, indicating a composition of mixed weed species in the experimental area (Table 1). The composition is dominated by broadleaf species with 25 species (including remnants of cover crop species such as *Calopogonium* sp. and *Centrosema* sp.), and only 7 grass species.

The dominance of broadleaf species covering the area is obvious with their total relative density of 82.9% and relative dominance of 82.4% (Table 1). About 80% of the composition is represented by 10 species in term of their relative density and dominance. Among these, eight species were represented by the broadleaf, with only 2 species of grasses. Meanwhile, three

TABLE 1
Weed species composition of the experimental locality

Weed species	Type*	Relative density (%)	Relative dominance (%)
Ageratum conyzoides	B	6.6	3.4
Asystasia gangetica	B	11.4	12.9
Hedyotis verticillata	B	5.0	5.2
Borreria latifolia	B	3.8	7.5
Borreris repens	B	2.4	1.0
Cardiospermum halicacabum	B	0.39	0.1
Calopogoniun mucunoides	B	1.2	3.6
Centrosema pubescen	B	7.8	9.3
Cleome rutidosperma	B	2.5	3.1
Clidemia hirta	B	0.2	0.7
Croton hirtus	B	33.6	23.6
Emilia sonchifolia	B	1.6	0.8
Melastoma malabathricum	B	0.1	0.4
Cassia tora	B	0.2	0.3
Melochia corchorifolia	B	0.2	0.9
Mimosa invisa	B	0.2	0.2
Mimosa pigra	B	0.1	0.1
Mimosa pudica	B	1.0	1.9
Mikania micrantha	B	1.3	2.1
Oxalis barrelieri	B	1.4	1.1
Passiflora foetida	B	0.2	0.2
Phyllanthus amurus	B	1.2	2.4
Porophyllum ruderale	B	0.3	0.1
Rubus moluccanus	B	0.1	0.6
Synedrela nodiflora	B	0.1	0.9
Axonopus compressus	G	0.2	0.1
Paspalum commersonii	G	12.4	9.7
Brachiaria miliformis	G	0.2	0.1
Digitaria ciliaris	G	1.5	0.7
Eleusine indica	G	0.7	1.2
Ischaemum timorense	G	0.3	0.2
Pennisetum polystachyon	G	2.6	5.9

*B: broadleaf; G: grass

species were found to dominate the locality based on their relative density and dominance. These are two broadleaves of *Croton hirtus* and *Asystasia gigentica*, and one species of grass, *Paspalum commersonii*. The dominance of *Croton hirtus* was obvious with the relative density (33.6%) and dominance (23.6%) being much higher than the other species present, followed by the values for *Asystasia gigentica* (11.4% and 12.9%), and *Paspalum commersonii* (12.3% and 9.7%). Two species could be rated as being the least dominant. These were *Mimosa pigra* (broadleaf) with 0.1% and 0.1%, and *Bracharia miliformis* (grass) with 0.2% and 0.1%, respectively. Therefore, the experimental locality indicated a composite of mixed weeds of broadleaves and grasses, with the broadleaves being more dominant over the grasses. In this study, these are the weed species which were found to be growing with this state of relative density and dominance at this stage of oil palm growth and under the environmental condition in the plantation. The situation, therefore, necessitates the use of general post-emergent herbicides for chemical weed management in the locality of this experimentation.

Herbicide Efficacy

The efficacy of the treatments was evaluated as the effects on the total weed population (mixed weed situation of broadleaf and grasses). The percentage of the weeds killed was significantly affected by the treatments of paraquat (200 to 800g a.i. ha^{-1}), glufosinate –ammonium (200 to 800g a.i. ha^{-1}), and glyphosate (400 to 1600g a.i. ha^{-1}) relative to the untreated control, with at least more than 50% weed killed observed at 2 and 4 WAT. However, when the efficacy of these three herbicides was compared, paraquat was found to produce a lower weed killed (50 to 83%) in total than those by glufosinate-ammonium (between 91 to 98%), and glyphosate (between 95 to 100%) (*see* Table 2). Using the weed control rating proposed by Burill *et al*. (1976), i.e. where 70% killed was considered as the minimum acceptable level of control and more than 90% killed as an excellent level of control, paraquat at lower rates of 200 and 400 g a.i. ha^{-1} was considered to be less effective in killing the weeds in this mixed weed condition. At higher rates of 600 and 800 g a.i. ha^{-1}, paraquat was found to produce considerably good kill of the weeds (74 to 83%) over the 4 WAT period. These results, however, were lower than those produced by glufosinate-ammonium and glyphosate at all levels of treatments, which gave the kill of between 91 to 100% at the 4 WAT. Meanwhile, treatments at lower rates of each herbicide produced lower kill than those of the higher doses of the same chemical, except for glyphosate where only the lowest rate was significantly lower than the higher rates. Sampling times (i.e. at 2 and 4 WAT) did not indicate any differences in the percentage of weed killed for the herbicides at all the treatment rates. A significant difference in the weed killed was observed between the herbicides, with glyphosate giving the highest kill, and paraquat the lowest kill. Meanwhile, glyphosate and glufosinate-ammonium showed greater efficacy on this mixed weed population than paraquat. As mentioned earlier, the weed composition of the area suggested certain influence on the outcome of the treatments. The efficacy of these herbicides was affected by the nature of the weed composition of the area and the surrounding environment (especially light penetration) of the locality. The effect of paraquat is reduced in high light intensity because the leaves are damaged, particularly at the site of application (Ipor and Price, 1991). Herbicide treatments, in general, are affected by dominance of weed species, crop cultivated and environment, whereby they can be effectively controlled for several months (Hoerlein, 1994). Paraquat is a non-selective (broad-spectrum) and contact herbicide which has a tendency to injure broadleaf plants somewhat more than grasses at a given rate (Ashton and Crafts, 1981; Calderbank and Slade, 1975). Its greatest efficacy is on weed species with restricted root system or which are still small (Turner and Gillbanks, 2003), and inversely proportionate to moisture where its effect increases under moisture stress (Turner and Gillbanks, 2003). Glufosinate-ammonium

TABLE 2
The percentage of weed killed after the treatments with paraquat, glufosinate-ammonium and glyphosate to mixed weed composition

Treatment (g a.i. ha^{-1})	Mean percent weed killed*	
	2 weeks after treatment	4 weeks after treatment
Untreated	0 f	0 g
Paraquat 200	50.7 e	50.9 f
Paraquat 400	65.8 d	66.4 e
Paraquat 600	74.1 cd	74.1 e
Paraquat 800	81.6 c	82.6 d
Glufosinate amm. 200	82.0 c	91.6 c
Glufosinate amm. 400	83.6 c	95.1 bc
Glufosinate amm. 600	95.0 b	97.9 b
Glufosinate amm. 800	91.2 b	98.0 b
Glyphosate 400	83.1 c	95.8 b
Glyphosate 800	98.5 a	100 a
Glyphosate 1200	98.8 a	99.7 a
Glyphosate 1600	100 a	100 a
Standard error	0.65	0.60

*Means within the same column, followed by the same letter, are not significantly different at P<0.05 by DMRT

is a broad-spectrum (Maschoff *et al*., 2000) and partially systemic that tends to give more persistent control of grasses than paraquat (Collins, 1991), apart from the effect on the broadleaf weeds. Glyphosate, however, is a non-selective systemic herbicide. When applied to the foliage, it is absorbed by the leaves and readily translocated to other parts of the plant, making it very effective to even the perennial weeds (Collins, 1991). A complete translocation of this herbicide confers remarkably with the efficacy on most weeds, broadleaves, grasses, or sedges (Kataoka *et al*., 1996).

The weed dry weight influenced the reduction of weed growth, which reflected the relative capability of a treatment to suppress weed growth relative to the untreated control. The dry weight of weeds measured the productivity of the weed community. The dry weight of weeds recorded for all the treatments with paraquat, glufosinate-ammonium, and glyphosate was significantly ($p<0.05$) lower relative to the untreated check (Table 2). However, this degree of significance was observed until 12 WAT for the paraquat treatments, after which there was a sign of weed growth recovery. The recovery for paraquat treatments is faster because the herbicide fails to make contact with some parts of the plant, especially the enclosed growing point or shoot, making immediate regrowth of the weeds possible. Paraquat, which is widely used for broadleaf control, is a quick acting, non-selective compound that destroys green plant tissue; nonetheless, it has been reported

to possibly temporarily suppress some annual grasses only because the low and enclosed growing points are not contacted by the spray (Collins, 1991). Ipor and Price (1991) reported that the effect of paraquat is reduced in high light intensity because the leaves are damaged, particularly at the site of application. Light intensity is high in the inter-rows of young oil palm plantation. Glufosinate-ammonium and glyphosate treatments, however, are more lasting with low weed dry weight recorded until 16 WAT (45.3 and 45.9 g, respectively) as compared to the untreated check at 76.2g. The glufosinate-ammonium and glyphosate treatments, therefore, have higher ability to reduce the weed population dry weight than paraquat. In particular, glufosinate-ammonium is more persistent in controlling grasses than paraquat (Collins, 1991), and the treatment rates are affected by the dominance of the weed species, crop cultivated and environment, whereby they can be effectively controlled for several months (Hoerlein, 1994). As for glyphosate, the treated plants were found to die in 1-3 weeks (Chang and Liao, 2002), while the development of symptom was also shown to be slow but irreversible (Collins, 1991). Shift in weed composition is a consequence of differential effectiveness of herbicides (Wrucke and Arnold, 1985; Swanton *et al*., 1993).

Based on the dry weight, weed growth reduction was observed to be significantly affected ($p<0.05$) by paraquat, glufosinate-ammonium and glyphosate treatments at 8, 12, and 16 WAT relative to the untreated control. The glufosinate-ammonium and glyphosate treatments recorded similar results within each sampling time, which ranged between 73 to 82% and 69 to 71%, respectively, for the 8 and 12 WAT (*see* Table 3). However, the paraquat treatment recorded lower growth reductions of 27 to 65% and 38 to 55%, respectively. The results also showed the tendency of weed growth recovery for all the treatments from the 8 to 12 to 16 WAT. The growth of weeds was increased by 25 to 40% over these sampling periods for all the treatments. There were positive correlations between the percentages of weed killed and reduction of weed growth. The increase in the percentage of weed killed was followed by the increase in the percentage of weed growth reduction, as indicated by the regressions $y = 0.0022x^2 + 0.4752x + 1.7461$, $y = 0.0014x^2 + 0.6446X + 1.5315$, $y = 0.0027x^2 + 0.1464x + 0.7233$, and R^2 values of 0.81, 0.87 and 0.66 at 8, 12 and 16 WAT, respectively (see *Fig. 1*). The degree of correlation was stronger for the 8 and 12 WAT, showing that the weeds were still dying until 12 WAT. Meanwhile, the correlation at 16 WAT was lower, and this indicated that the weeds were recovering and began to produce shoots even though the initial weed killed was 70%. These findings prove that the treatments of less efficacy could cause weed to grow and

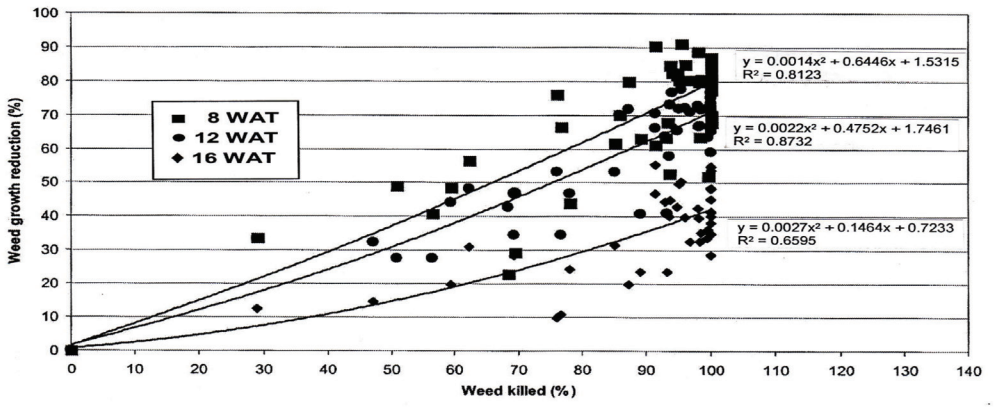

Fig. 1:Relationship between weed growth reduction and weed killed

TABLE 3
Weed dry weight and growth reduction after treatments with paraquat, glufosinate-ammonium and glyphosate

Treatment (g a.i. ha^{-1})	Dry weight (g/0.25m^2)*			Growth reduction (%)*		
	8WAT	12WAT	16WAT	8WAT	12WAT	16WAT
Untreated	60.9 a	73.2 a	76.2 a	0e	0d	0c
Paraquat 200	42.6 ab	44.7 b	66.5 a	26.9 d	38.2 c	12.4 b
Paraquat 400	33.7 bc	44.4 b	67.9 a	39.7 cd	38.8 c	11.5 b
Paraquat 600	28.0 bc	39.9 bc	62.7 a	53.3 bc	44.5	17.5 b
Paraquat 800	21.4 cd	32.4 c	60.9 a	64.5 ab	54.6 b	20.2 b
Glufosinate-amm. 200	12.9 e	22.1 d	42.0 b	76.5 a	69.3 a	45.6 a
Glufosinate-amm. 400	11.9 e	20.7 d	44.5 b	80.0 a	71.2 a	41.8 a
Glufosinate-amm. 600	12.5 e	20.5 d	44.5 b	78.1 a	71.3 a	41.5 a
Glufosinate-amm. 800	14.8 de	21.6 d	45.3 b	73.2 a	70.2 a	40.5 a
Glyphosate 400	13.5 e	22.3 d	45.5 b	76.9 a	69.6 a	40.5 a
Glyphosate 800	11.0 e	21.0 d	45.9 b	81.5 a	72.8 a	39.6a
Glyphosate 1200	11.7 e	21.7 d	42.0 b	79.7 a	69.8 a	44.8 a
Glyphosate 1600	12.4 e	21.2 d	44.7 b	78.1 a	71.3 a	41.3 a
Standard error	0.02	0.00	0.01	1.02	0.51	1.02

*Means within the same column followed by the same letter are not significantly different at P<0.05 by DMRT

recover faster or in shorter times. Chung and Balasubramaniam (1991) reported that the high biomass recorded after the treatment with certain herbicides was caused by a rapid regeneration of the weeds.

The percentage weed killed, weed dry weight, and percentage of weed growth, as components of efficacy evaluation, also reflects the duration of effective weed control. The higher percentage of weed killed lead to longer duration of effective control as indicated by the value of regression $y = 0.0013x^2 + 0.0215x + 0.219$, and R^2 of 0.84 (see *Fig. 2*). When weed killed was at 70% (i.e. the minimum acceptable control according to Burill *et al*., 1976), the control only lasted for 8 weeks, as compared to more than 12 weeks when it was at 90% weed killed. The paraquat treatments had a duration of effective weed control that ranged from 4 to 11.75 weeks. The increased rates of paraquat treatment, i.e. from 200 to 600 and 800g a.i. ha^{-1} were found to increase the duration of effective weed control (*see* Table 4). The effective weed control produced by glufosinate-ammonium (at 200 to 800g a.i. ha^{-1}) and glyphosate (at 400 to 1600g a.i. ha^{-1}) ranged from 14.5 to 15 weeks, and these were significantly longer than the paraquat treatments. The increased rates of

TABLE 4
Duration of effective control after treatment with paraquat, glufosinate-ammonium and glyphosate on mixed weed in young oil palm plantation

Treatment (g a.i. ha^{-1})	Duration of weed control (week)*
Untreated	0 e
Paraquat 200	4.00 d
Paraquat 400	6.00 d
Paraquat 600	8.75 c
Paraquat 800	11.75 b
Glufosinate-ammonium 200	15.00 a
Glufosinate-ammonium 400	14.75 a
Glufosinate-ammonium 600	14.75 a
Glufosinate-ammonium 800	14.75 a
Glyphosate 400	14.50 a
Glyphosate 800	14.75 a
Glyphosate 1200	14.75 a
Glyphosate 1600	14.75 a
Standard error	0.24

*Means within the same column followed by the same letter are not significantly different at P<0.05 by DMRT

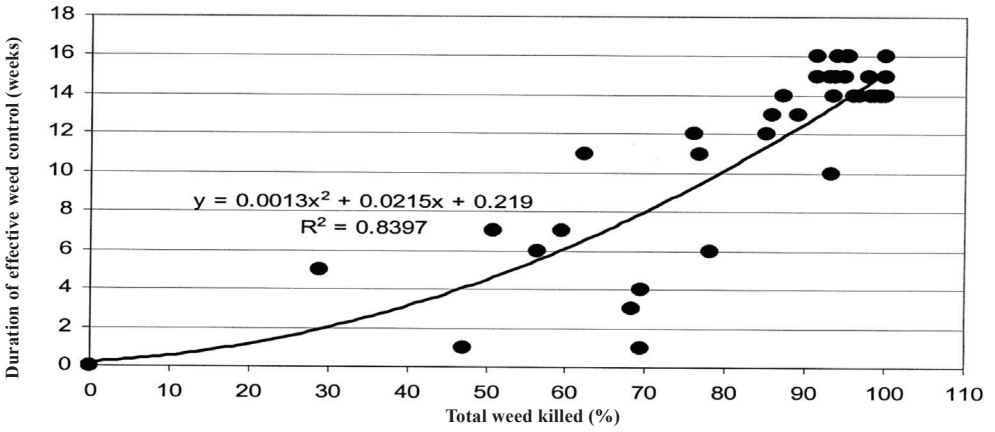

Fig. 2: Relationship between duration of effective weed control and weed killed

glufosinate-ammonium and glyphosate were not necessarily followed by their ability to increase the duration of effective weed control. This indicated that the percentage of weed killed plays an important role in evaluating the efficacy of herbicides applied because it affects the percentage of weed growth reduction and duration of effective weed control.

CONCLUSIONS

Weeds present in young oil palm plantation are of the mixed species of broadleawves and grasses. Broadleaves grow with greater density and dominance than grasses. In this study, treatments of the weeds using broad-spectrum, contact herbicides of paraquat, or partially systemic glufosinate-ammonium, or systemic glyphosate at around their recommended field application rates were found to provide effective kill, growth reduction, and duration of control in the mixed weed population. However, glufosinate-ammonium and glyphosate were found to be more effective than paraquat. Similarly, increasing the rates (200 to 800 g a.i. ha^{-1}) increased the efficacy for paraquat, but increasing the rates for glufosinate-ammonium (200 to 800 g a.i. ha^{-1}) and glyphosate (400 to 1600 g a.i. ha^{-1}) produced the same effect. Herbicide treatments with poor efficacy caused weeds to grow and recover faster or within shorter periods. The higher percentage of weed killed leads to longer duration of effective weed control.

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The Effect of Dexamethasone on Immune Responses of Calves to Intranasal Exposures to Live Attenuated *gdhA* Derivative of *Pasteurella multocida* B:2

Khin, M.N. and Zamri-Saad, M.*
Department of Veterinary Pathology and Microbiology,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
**E-mail: zamri@vet.upm.edu.my*

ABSTRACT

The effect of stress, created using dexamethasone on immune response by calves to intranasal exposures to *gdhA* derivative of *Pasteurella multocida* B:2 was studied. For the purpose of this study, twelve calves of 6 months old were selected and divided into 4 groups. At the start of the experiment, calves of groups 1 and 2 were intramuscularly injected with dexamethasone at the rate of 1 mg/kg body weight for 3 consecutive days. Then, the calves of groups 2 and 3 were exposed intranasal to 5 ml of the inoculums containing 10^6 cfu/ml of the *gdhA* derivative of *P. multocida* B:2. Calves of groups 1 and 4 remained unexposed control. Serum samples were collected prior to the start of the experiment and at weekly interval for a period of 7 weeks. At the end of the 7-week period, all the calves were sacrificed before the lungs were lavaged using 1L of sterile phosphate buffered saline (PBS). The sera and lung lavage fluid were subjected to ELISA to determine the levels of IgG and IgA. Significant increase in both serum and lavage IgG and IgA were observed only in group 3, which were exposed without any dexamethasone treatment. The dexamethasone-treated and exposed group 2 failed to respond to the exposures when the levels remained insignificant to those of the control untreated and unexposed group 4. The calves of group 1, which were treated with dexamethasone but remained unexposed, failed to show any response at all. In conclusion, intranasal exposures to live attenuated *gdhA* derivative of *P. multocida* B:2 must be given only to unstressed calves, since stressful condition has been found to prevent calves from responding significantly to the antigen.

Keywords: Intranasal, *Pasteurella multocida* B:2, dexamethasone, immune response

INTRODUCTION

Haemorrhagic septicaemia (HS) is an infectious disease of cattle and buffalo caused by *Pasteurella multocida* B:2 (Adler *et al.*, 1996). It is commonly fatal and is considered as one of the most economically important livestock diseases in Southeast Asia (Benkirane and De Alwis, 2002).

Subcutaneous injections of alum-precipitated or oil adjuvant vaccines have been used to control the disease. However, the vaccines were able to provide short-term immunity, approximately 4 to 6 months and up to 1 year for alum adjuvant and oil adjuvant vaccines, respectively (Verma and Jaiswal, 1997). Furthermore, the high viscosity of the oil adjuvant vaccines makes them unpopular among field users. Therefore, vaccine development was highlighted as a major area for investigation at the last International Workshop on HS (FAO, 1991). Subsequently, an attenuated *P. multocida* B:2 was successfully created following disruption

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*Corresponding Author

of the *gdhA* gene, a housekeeping gene, of the wild-type *P. multocida* B:2 (Sarah *et al.*, 2006). The attenuated organism was found to be able to stimulate both the mucosal and systemic immunities that protect animals from challenges (Zamri-Saad *et al.*, 2006).

Immunosuppression due to stress can increase the susceptibility to infectious disease (Concordet and Ferry, 1993; Anderson *et al.*, 1999). Similarly, outbreaks of HS have been associated with stressful conditions, particularly changes in weather (Saharee *et al.*, 2005). The administration of dexamethasone, either alone or in combination with stressful conditions, results in a systemic suppression of the immune response (Collins and Suarez-Guemes, 1985).

This study was carried out to determine the suppressive effects of dexamethasone on the systemic and mucosal antibody responses to intranasal exposures of live attenuated *gdhA* derivative of *P. multocida* B:2.

MATERIALS AND METHODS

Animals

Twelve, clinically healthy, calves (approximately 6-month-old) were randomly divided into four groups. Anthelmintic (Ivomec, MSD) was administered at the rate of 0.2 mg/kg body weight upon arrival to control internal parasitism, which might influence the development of immunity responses (Zamri-Saad *et al.*, 1994). Nasal swabs were collected at the same time after the arrival to ensure that the calves were free of *Pasteurella multocida*, prior to the start of the experiment (Zamri-Saad *et al*., 1999). The different groups were kept in separate housing and fed cut grasses and supplement feed at the rate of 1 kg/animal/day, while drinking water was provided *ad-libitum*.

Inoculums

The *gdhA* derivative of *P. multocida* B:2, created earlier by Sarah *et al*. (2006), was used in this study. The inoculums were prepared by resuscitating the stock culture onto blood agar plates containing kanamycin (50 µg/mL) and streptomycin (60 µg/mL), and this was followed by incubation for 46 h at 37°C. At the end of the incubation period, six uniform-sized colonies were selected and inoculated into antibiotic containing 100 ml of brain-heart infusion broth, which was then incubated at 37°C for 18 h before the bacterial concentration was determined using the plate count technique method of Alcamo *et al.* (1997). The concentration was readjusted using phosphate-buffered saline (PBS) to give a final concentration of 10^6 colony-forming unit (cfu)/ml.

Experimental Procedure

At the start of the experiment, all calves of groups 1 and 2 were administered intramuscularly with dexamethasone, at the rate of 1 mg/kg body weight, for 3 consecutive days. On the day after the last dexamethasone injection, the calves of groups 2 and 3 were exposed intranasal to 5 ml of the live *gdhA* derivative of *P. multocida* B:2 inoculums which had been prepared earlier. Two weeks after the initial intranasal exposure, the calves of groups 2 and 3 were once again exposed intranasal to the same inoculums of live attenuated *gdhA* derivative of *P. multocida* B:2. Meanwhile, the calves of groups 1 and 4 remained unexposed.

All the calves were observed for signs of illnesses, while the serum samples were collected from all the calves prior to dexamethasone treatment and on weekly intervals thereafter. On week 7, i.e. after the 1st intranasal inoculation, all the calves were sacrificed and post-mortem examinations were carried out immediately on the respiratory tract. The trachea was exposed and clamped to avoid intratracheal contamination with blood, removed, and the presence, extent, and nature of superficial lesions were determined using a method developed for sheep (Gilmour *et al.*, 1983).

All lungs were lavaged by introducing 1 L cold and sterile phosphate buffered saline (PBS) into the lungs through the trachea, massaged gently before the fluid was re-collected. The

lung lavage fluid was centrifuged at 1000xg for 15 min to remove the debris before the supernatant was collected and kept at -20°C until further use.

Determination of Antibody Levels

The serum and broncho-alveolar lavage samples were used to determine the systemic and mucosal immunoglobulin IgG and IgA levels, respectively, by using the ELISA technique developed by Zamri-Saad *et al.* (2006). In brief, the microtiter plates were coated with 50 µl per well of whole cell inactivated *P. multocida* B:2 suspension prepared earlier at a concentration of 10^9 cfu/ml in carbonate-bicarbonate buffer pH9.6. The plates were dried at 37°C in an incubator for 1 hr. After 2 times washing using a washing buffer, the reactions were blocked with 200 µl PBS/BSA/Tween 20 and incubated for 1hr. Then, 50 µl of either serum or broncho-alveolar lavage samples were introduced and incubated again for 1 hr and washed 3 times. After that, 50 µl of conjugate were put, incubated for 1 hr and washed 3 times. This was followed by the addition of substrate and incubated again. The reaction was stopped by 2 *M* H_2SO_4 and the optical density values were measured at absorbance 450 nm wavelength in an Anthos Zenyth 340 st reader.

Statistical Analysis

All the data were statistically analyzed by using the ANOVA. The probable (p) value of <0.05 was considered as significant.

RESULTS

Serum IgA Levels

Fig. 1 shows the serum IgA response, following intranasal exposure of calves, to *gdhA* derivative of *P. multocida* B:2. Following the dexamethasone treatment, all the groups showed insignificant differences (p>0.05) in the IgA level at week 1. However, after the first

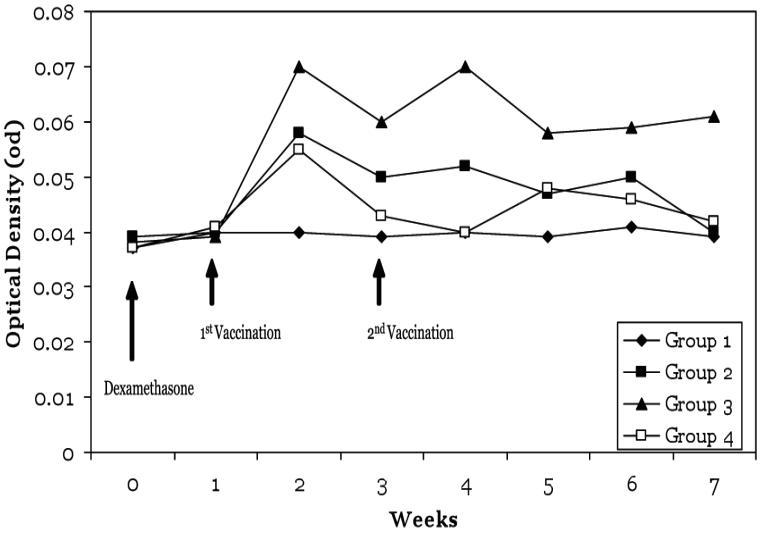

Fig. 1. The serum IgA levels in groups of calves exposed to gdhA *derivative of* P. multocida *B:2 with or without dexamethasone treatment. Group 1 was treated with dexamethasone without exposure to* P. multocida*, group 2 was treated with dexamethasone and exposed to* P. multocida*, group 3 was exposed to* P. multocida *without dexamethasone treatment and group 4 was not treated with dexamethasone and not exposed to* P. multocida

exposure, the calves of groups 2 and 3 showed a significant ($p<0.05$) increase in the IgA level as compared to group 1, while the level showed by group 2 was insignificantly ($p>0.05$) different with those of the unexposed, untreated calves of group 4.

Following the second exposure, the calves of group 3 which were without the dexamethasone treatment showed a significant ($p<0.05$) increase in the IgA level, which remained significantly ($p<0.05$) higher than all other groups (*Fig. 1*). The calves of group 2, which were treated with dexamethasone and exposed to *P. multocida*, failed to record any increment in the level of IgA after the second exposure and remained insignificantly ($p>0.05$) higher than the unexposed, untreated control group 4. Meanwhile, the calves of group 1, which were treated with dexamethasone but unexposed, showed no response throughout the experimental period.

Serum IgG Levels

Fig. 2 shows the serum IgG responses by the three groups. Generally, the IgG patterns were similar to those observed for serum IgA. Following the dexamethasone treatment, the serum IgG levels at the time of the first exposure were insignificant ($p>0.05$) among the three groups. However, after the first exposure, the calves in group 3 showed significantly ($p<0.05$) high IgG level, which remained until the second exposure. The levels in group 2, which was treated with dexamethasone and exposed to *P. multocida* and group 4, but not treated and exposed, remained significantly ($p<0.05$) low even after the second exposure until the end of this 7-week experimental period (*Fig. 2*). Group 1, which was treated with dexamethasone but not exposed to *P. multocida,* did not show any reaction throughout the study period (*Fig. 2*).

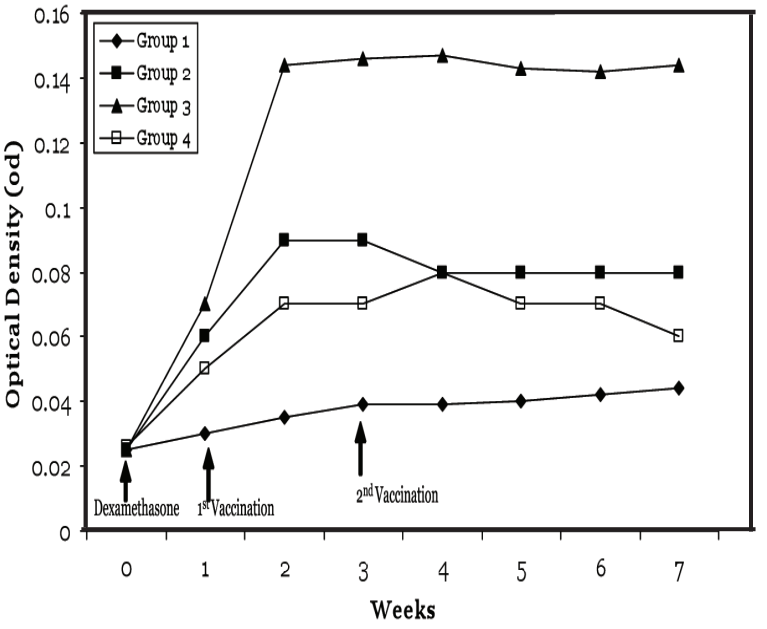

Fig. 2: The serum IgG levels in groups of calves exposed to gdhA *derivative of* P. multocida *B:2 with or without dexamethasone treatment. Group 1 was treated with dexamethasone without exposure to* P. multocida*, group 2 was treated with dexamethasone and exposed to* P. multocida*, group 3 was exposed to* P. multocida *without dexamethasone treatment and group 4 was not treated with dexamethasone and not exposed to* P. multocida

Lung Lavage IgA Levels

Fig. 3 shows the IgA response in the lung lavage fluid of calves at week 4, after the second intranasal exposure to *gdhA* derivative of *P. multocida* B:2. The IgA level in the lung lavage fluid of the calves of group 3 was significantly ($p<0.05$) higher than those of the calves in other groups, which appeared to be insignificantly ($p>0.05$) different.

Lung Lavage IgG Levels

Fig. 4 shows the IgG response in the lung lavage fluid of calves at week 4, after the second intranasal exposure to *gdhA* derivative of *P. multocida* B:2. As observed in the IgA response, the IgG level in group 3 was significantly ($p< 0.001$) higher than the other groups, which were not significantly ($p>0.05$) different.

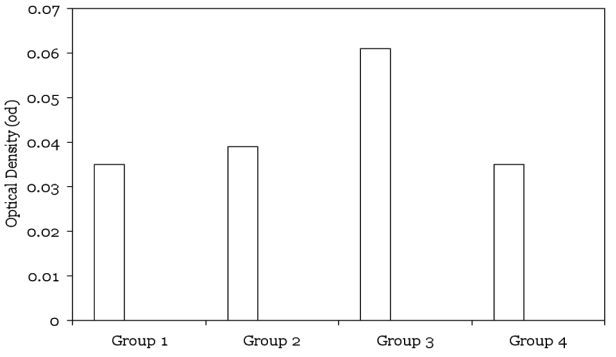

Fig. 3: The IgA level in the lung lavage fluid of calves exposed to gdhA *derivative of* P. multocida *B:2 with or without dexamethasone treatment. Group 1 was treated with dexamethasone without exposure to* P. multocida*, group 2 was treated with dexamethasone and exposed to* P. multocida*, group 3 was exposed to* P. multocida *without dexamethasone treatment and group 4 was not treated with dexamethasone and not exposed to* P. multocida

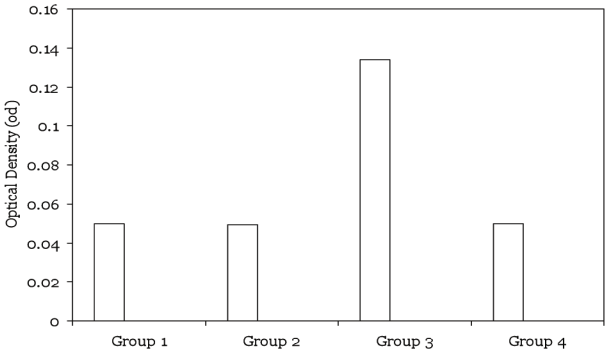

Fig. 4: The IgG level in the lung lavage fluid of calves exposed to gdhA *derivative of* P. multocida *B:2 with or without dexamethasone treatment. Group 1 was treated with dexamethasone without exposure to* P. multocida*, group 2 was treated with dexamethasone and exposed to* P. multocida*, group 3 was exposed to* P. multocida *without dexamethasone treatment and group 4 was not treated with dexamethasone and not exposed to* P. multocida

DISCUSSION

Infection by *P. multocida* B:2 has been associated with stressful situations. During rainy season and at the start of paddy planting season, cattle and buffaloes in many Asian countries are under extreme stressful condition (Interior, 1993). These stresses increase the level of hydrocortisone in the serum which leads to immuno-suppression and subsequent colonisation of bacteria, particularly in the respiratory tract (Zamri-Saad *et al.*, 1991). Dexamethasone injections have been used to replace natural hydrocortisone that mimics stressful condition on animals (Zamri-Saad *et al.*, 1991). The effect of gluco-corticoid administration has resulted in statistically significant reduction in the IgA levels and impaired the mucosal immunity (Alverdy *et al.*, 1997). Other than the local IgA level, serum IgG levels are also affected by dexamethasone administrations which lead to vaccination failure against pneumonic mannheimiosis (Zamri-Saad and Effendy, 1999). This study revealed that intranasal exposures of stressed calves to *gdhA* derivative of *P. multocida* B:2 had failed to stimulate both the mucosal and systemic immunities. This was obvious when the serum IgA and IgG levels of the exposed, stressed calves were similar to that of the unexposed calves.

On the other hand, the unstressed, dexamethasone-free calves showed significantly higher serum IgA and IgG responses following intranasal exposures to the *gdhA* derivative of *P. multocida* B:2. Similarly, the IgA and IgG levels in the lung lavage fluid of calves, which were treated with dexamethaosne, were significantly lower than those without any dexamethasone treatments. Similar poor responses of stressful animals to vaccination, including to intranasal exposures to *Mannheimia* (*Pasteurella*) *haemolytica* in goats (Zamri-Saad and Effendy, 1999) and to *Pasteurella multocida* B:2 in goats (Shafarin *et al.*, 2008), have also been reported. It was concluded that dexamethasone reduced the availability of immune cells, thus reducing immune responses.

In conclusion, exposures of calves during stressful period are of insignificant use since they remain similar as those of the unexposed calves and, therefore, are susceptible to infection. The findings gathered in this study revealed the extreme importance of vaccination against haemorrhagic septicaemia, which had been carried out prior to the stressful period. Many researchers have suggested that vaccination against haemorrhagic septicaemia in the field is done prior to the beginning of a rainy season so that a better and longer antibody response can be achieved to protect the animals during the stressful rainy season (De Alwis, 1993; Jamaludin, 1993; Phuong, 1993).

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Diversity and Distribution of Fish in Irrigation Water Derived from Recycled and Uncontrolled Flow Water Sources in the Muda Ricefields

Shah, A.S.R.M.[1,2], Ismail, B.S.[2*], Mansor, M.[1] and Othman, R.[2]
[1]School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia
[2]School of Environment and Natural Resource Sciences,
Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, 43600 UKM, Bangi, Selangor, Malaysia
[*]E-mail: ismail@pkrisc.cc.ukm.my

ABSTRACT

Study on fish diversity and distribution in rice plots supplied with recycled and uncontrolled flow water was carried out from Season 1/2002 to Season II/2004 (August, 2002 to August, 2004) using a modified electro-shocker. A total of 13 species of fish were identified, with *Cyprinidae* being the dominant family in terms of the number caught (39.5%), followed by *Aplocheilidae* (18.2%), and *Osphronemidae* (12.0%). The number of species identified was lower as compared to those obtained in previous studies at selected irrigation canals in the Muda agro-ecosystem, and this due to different methodology employed. In the rice field plots irrigated by uncontrolled water flow, 13 species were recorded, whereas only 11 species were recorded in the plots irrigated by recycled water. However, based on the Shannon-Weiner Diversity and Evenness Indices, the values for the recycled water plots were higher (0.8764 and 0.8416, respectively) compared to the values obtained for the uncontrolled flow plots (0.8131 and 0.7300, respectively). The clustering analysis showed that the similarity in term of the fish species in both plots is high at 0.8462. In particular, *Esomus metallicus* was the dominant "species catch" (36.4%), followed by *Aplocheilus panchax* (18.2%) and *Anabas testudineus* (10.6%). Most of the species identified were well-adapted to survive under the extreme conditions of the rice fields, such as the lower and higher D.O. and water temperature readings of 0.3-14.8 mg/L and 14.0-41.0ºC, respectively. However, floods that occurred in Season 1/2004 were found to have influenced the fish diversity as some riverine species, such as *Barbodes gonionotus* and *Cyclocheilichthys apogon*, were caught in the studied rice field plots.

Keywords: Fish, distribution, rice fields, flow, uncontrolled flow, MADA

INTRODUCTION

According to Pimental *et al*. (1992), the study of biodiversity associated with agro-ecosystems such as rice fields is of significance for agroecologists and conservation biologists, since maintenance of biological diversity is essential for productive agriculture, and ecologically sustainable agriculture is in turn essential for maintaining biological diversity. In Malaysia, rice crop is cultivated twice a year. The largest rice cultivation area in Malaysia is the Muda rice granary area, which is located in the north east of Peninsular Malaysia (Azmi, 1994). The main season for rice cultivation at the Muda rice agroecosystem is from September to January, whereas the second or "off" season is from March to August (MADA, 2004).

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[*]Corresponding Author

The Muda rice agroecosystem depends on rainfall as the main source of water supply (56%), followed by dam released water (30%), uncontrolled flow (rivers below the dam) (13%) and recycled water (5%) (Lau and Yeow, 1995). The recycling system was initiated in 1982 to overcome the deficiency of water in the Muda irrigation scheme (Lau and Yeow, 1995). However, the introduction of the recycling system was of great concern to the public, particularly with regard to the safety of re-used water and its impacts on agro-biodiversity (Maimon *et al*., 1998; Sani *et al*., 1992; Shah *et al*., 2006).

It is important to note that the source of water supply influences floodwater chemistry and composition of aquatic biota (Bambaradeniya and Amerasinghe, 2003). Sani *et al*. (1992) noted that the *Escherichia coli* count was higher at recycled areas than non-recycled areas. Similarly, Maimon *et al*. (1998) reported that recycled water harboured higher numbers and taxa of insects and arachnids as compared to non-recycled water. On the other hand, a comparative study on the diversity and composition of zooplankton has shown no significant difference ($p>0.05$) between recycled water and uncontrolled flow rice plots (Shah *et al*., 2008).

The status of fish in the rice agro-ecosystem in Malaysia is poorly understood. Several researchers have studied the importance of sump-ponds and their relation to rice-fish productivity (Ali, 1990: 1992; Shah and Ali, 1994; Tan *et al*., 1973). Other studies included fish taxonomy and distribution (Soong, 1948; Shah and Ali, 1998; Shamsudin, 2001), the effects of double cropping and pesticide/herbicide spraying on the rice-fish community (Tan *et al*., 1973), residual pesticides in fish tissues and their association with farmers' health risk (Chen *et al*., 1984) and toxicity studies on rice-fish (Kok, 1972; Shah *et al*., 2007). However, studies on the impact(s) of different water management systems in rice-field plots on fish distribution and compositions have yet to be carried out.

Thus, the objective of this study was to analyze the relationship between the environmental factors and the fish community, and subsequently to compare fish abundance and diversity in rice plots which are irrigated with recycled and uncontrolled flow water.

MATERIALS AND METHODS

Sampling was carried out at Kampung Alur Sekawan, Mukim Tajar CIII, Pendang, in Kedah, Malaysia (see *Fig. 1* for the location). The study area was divided into two treatment plots based on the different irrigation systems present, i.e. recycled and uncontrolled flow (non-recycled) water. Each plot was further divided into four sub-plots (station) to provide a representative sample of the population and the structure of the rice-fish communities (*Fig. 1*). Meanwhile, the sampling was conducted twice a month (i.e. from August 2002 to August 2004) at each station.

The fish samples were obtained using a modified electro-shocker powered by a 12 V dry battery. The operator walked in a transect line (about 100 m long) on the bund adjacent to each treatment plot and electrocuted the fish at the same time. All the specimens were immediately collected by the co-worker using a scoop net.

All the specimens caught were identified using the standard taxonomic keys (Inger and Chin, 1962; Kottelat *et al.*, 1993; Mohsin and Ambak, 1983; Rainboth, 1996), measured to total length (mm) and weighed to the nearest g.

The Jaccards Coefficient of Similarity (JCS), using the Unweighted Paired Group Method (UPGMA), was applied to cluster the fish community groups between the types of water supplied to the plots, using the Multi Variate Statistical Package (MVSP) Version 3.11 (Tongeren, 1987). The Shannon-Wiener Diversity and Evenness Indices were also calculated using the same statistical programme. A rapid appraisal survey, with the plot owner, was carried out to determine the frequency in the use of pesticides.

Prior to fish sample collection, *in-situ* water physico-chemical readings such as dissolved oxygen (D.O. mg/L) and temperature (°C) were measured using the YSI meter (Model 57), whereas the conductivity (µS/cm) and total

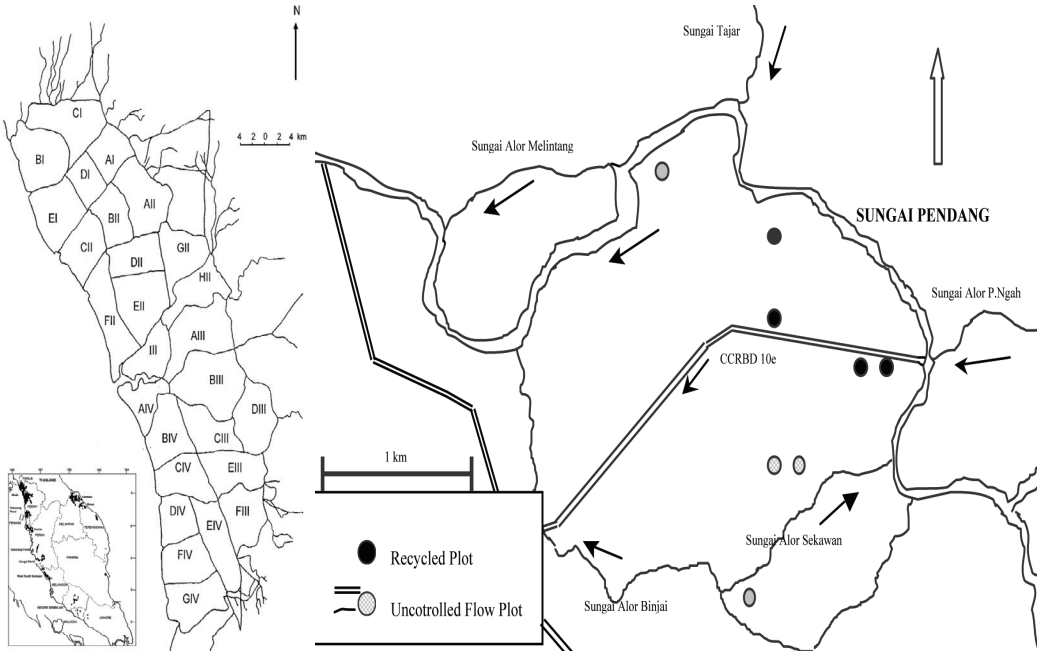

Fig. 1: The location of the main rice granary areas in Peninsular Malaysia, the Muda irrigation scheme and sampling plots at the Kg Alor Sekawan, Mukim Tajar CIII during the study period

dissolved solids (TDS) (mg/L) were recorded with the Hach (Model C0150), and pH with the Orion (Model 230A). A fixed ruler was used to measure the water level at each sampling station.

RESULTS AND DISCUSSION

The mean physico-chemical characteristics of each treatment plot are summarized in Table 1. The recycled plots had higher water levels compared to the uncontrolled flow plots with the means of 8.2 ± 4.5 cm and 7.9 ± 4.6 cm, respectively. The results showed that the dissolved oxygen, conductivity, total dissolved solids, and total suspended solids were relatively higher in the plots with uncontrolled flow than in the recycled plots: 4.8 ± 2.9 mg/L, 66.3 ± 63.1 µS/cm, 33.3 ± 36.3 mg/L, 183.2 ± 443.4 mg/L and 4.4 ± 2.2 mg/L, 62.9 ± 44.3 µS/cm, 29.9 ± 22.0 mg/L, 168.0 ± 270.3 mg/L, respectively (Table 1). Meanwhile, the plots with the uncontrolled flow were slightly more acidic (pH 5.4 ± 0.8) than those with recycled water (5.6 ± 0.7) (Table 1). This might be related to the slightly higher alkalinity readings in the recycled plots (44.0 ± 40.5 mg/L) compared to those in the uncontrolled flow plots (39.8 ± 24.8 mg/L) (Table 1). Generally, the quality of water in the studied plots could be considered as moderate and was able to support a great variety of aquatic organisms such as zooplankton, aquatic insects, and fish (Ali, 1992: 1998; Shamsudin, 2001; Bambaradeniya and Amerasinghe, 2003; Shah *et al*., 2008; Che Salmah and Abu Hassan, 2009). Based on the statistical analysis, there was no significant difference ($p>0.05$) found between the physico-chemical properties of the water for the different irrigation systems (Shah *et al*., 2006). Similar results were also reported by Sani *et al*. (1992).

There are several reports made on the relationship between the environmental factors and fish abundance or fish diversity in rivers (Healy and Lonzarich, 2000; Matthias *et al*., 1996; Stoneman and Jones, 2000). A number of environmental factors, such as current

TABLE 1
Summary of the physico-chemical properties of the water in the recycled and uncontrolled flow rice field plots during the study periods (August 2002 to August 2004)

Plot	Recycle water (n=79)		Range		Uncontrolled flow (n=84)		Range	
	Mean	± s.d.	Min.	Max.	Mean	± s.d.	Min.	Max.
Water level (cm)	8.2	4.5	1	19	7.9	4.6	1	20
Dissolved oxygen (mg/L)	4.4	2.2	0.5	10	4.8	2.9	0.3	14.8
Temperature (°C)	31	4.4	23	41	30.6	4.4	14	39
pH	5.6	0.7	3.6	7.6	5.4	0.8	3.8	8.4
Conductivity (µS/cm)	62.9	44.3	3.4	247	66.3	63.1	13.5	371
Total dissolved suspended (mg/L)	29.9	22	2	115	33.3	36.3	6	221
Total suspended solid (mg/L)	168	270.3	4.8	1548.8	183.2	443.4	2.8	3088.8
Alkalinity (mg/L)	44	40.5	6.9	325.3	39.8	24.8	10	160.1
Orhtophosphate (mg/L)	0.0262	0.0207	0.0001	0.1455	0.0353	0.0456	0.0001	0.2943
Nitrogen-nitrite (mg/L)	0.0003	0.0005	0.0001	0.0037	0.0007	0.0021	0.0001	0.016
Nitrogen-nitrate (mg/L)	0.2379	0.2236	0.0005	0.9644	0.1833	0.1661	0.0002	0.9384

velocity, water depth, degree of plant cover, and water temperature, have been known to affect fish communities. Meanwhile, there is a positive relationship between fish abundance or diversity and the increasing water depth and plant coverage. However, the relationships between fish communities in rice fields are poorly documented.

A total of 13 fish species, belonging to nine families, were recorded in the present study. The number of fish species and their distribution in recycled water and the uncontrolled flow rice plots are shown in Table 2. The list included the "black" fish (e.g. *Channa striata*, *Anabas testudineus* and *Clarias* sp.) and the "white" fish (*Barbodes gonionotus* and *Cyclocheilichthys apogon*) (Welcomme, 1985).

Overall, *Esomus metallicus* was the dominant species (36.4%), followed by *Aplocheilus panchax* (18.2%), and *A. testudineus* (10.6%) (*Fig. 2*). The two former species were found to be dominant in the uncontrolled flow plots, whereas *Anabas testudineus* was dominant in the plots with recycled water (*Fig. 2*). In term of density, *Cyprinidae* was the dominant family (39.5%), followed by *Aplocheilidae* (18.2%), *Osphronemidae* (12.0%), *Anabantidae* (10.6%), *Channidae* (10.4%), *Clariidae* and *Bagridae* (3.4%), *Synbranchidae* (1.4%), and *Notopteridae* (1.1%) (*Fig. 3*). Most of the cyprinids were caught from the uncontrolled flow plots, whereas the other families were more concentrated in the plots with recycled water (*Fig. 3*).

TABLE 2
The fish checklist and its distribution in the recycled irrigation water and uncontrolled flow rice plots

Species	Family	Local name	Recycled	Uncontrolled flow
Anabas testudineus	Anabantidae	Puyu	+	+
Mystus vittatus	Bagridae	Baung	+	+
Betta splendens	Osphronemidae	Karin	+	+
Trichogaster pectoralis	Osphronemidae	Sepat siam	+	+
Trichogaster trichopterus	Osphronemidae	Sepat kedah	+	+
Channa striata	Channidae	Haruan	+	+
Clarias macrocephalus	Clariidae	Keli kayu	+	+
Aplocheilus panchax	Aplocheilidae	Tahi timah	+	+
Esomus metallicus	Cyprinidae	Seluang	+	+
Cyclocheilichthys apogon	Cyprinidae	Temperas	-	+
Barbodes gonionotus	Cyprinidae	Lampam jawa	-	+
Monopterus albus	Synbranchidae	Belut	+	+
Notopterus notopterus	Notopteridae	Selat	+	+

Note: - = absent; + = present

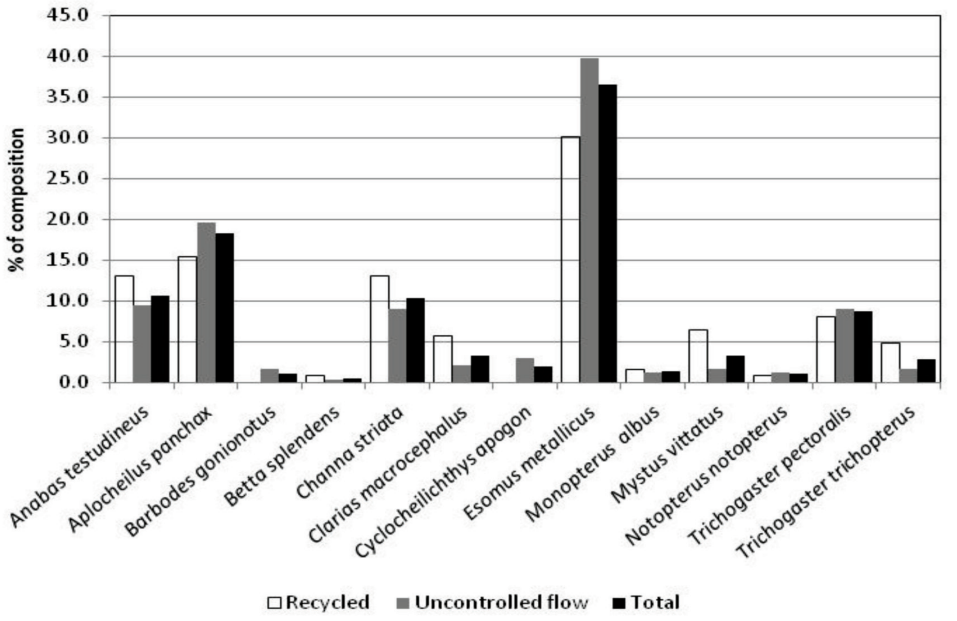

Fig. 2: The percentages in the composition of fish species and the type of water supplied during the study period

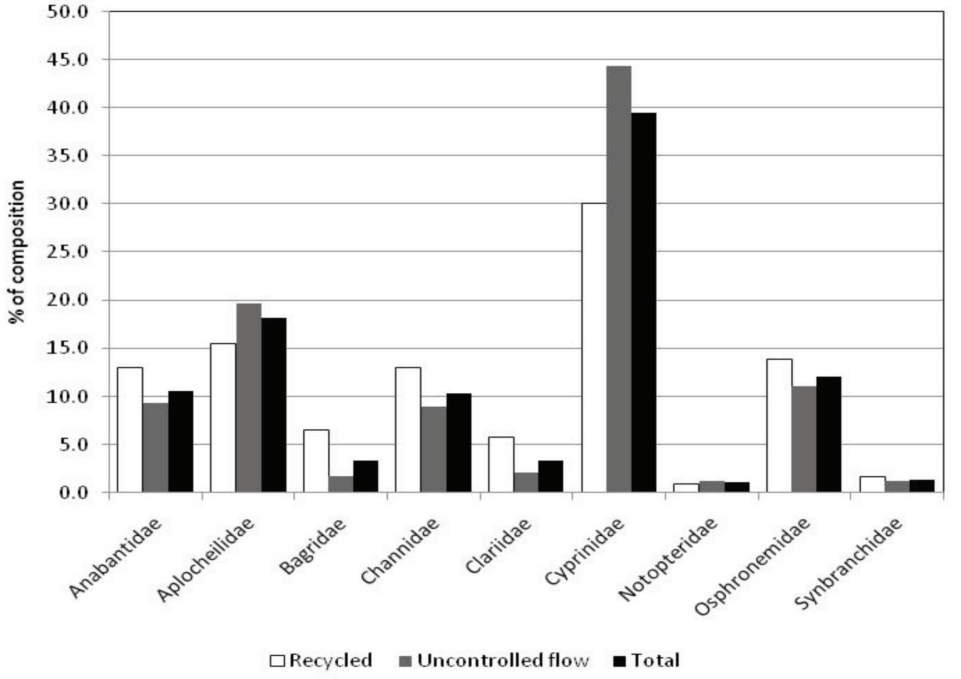

Fig. 3: The percentages in the composition of fish family and the type of water supplied management during the study period

The number of species recorded in this study was slightly higher than those previously reported by Ali (1990) and Shamsudin (2001), whereby only nine and eleven species were recorded in the rice field ecosystem, respectively. Other species such as the walking catfish (*Clarias* sp.), snakehead (*Channa striata*), and climbing perch (*A. testudineus*) are known to have a unique type of respiratory organ that enables them to live under extremely low D.O. levels of 0.3 mg/L and high water temperature conditions of up to 41.0ºC in rice fields (Mohsin and Ambak, 1983; Soong, 1948; Welcomme, 1985).

In the uncontrolled flow plots, thirteen fish species were recorded, whereas only eleven species were recorded in the plots with recycled water. As shown in Table 3, the Shannon-Wiener and Evenness Indices in the recycled water plots were high (0.8764 and 0.8416, respectively) compared to those recorded in the uncontrolled flow plots (0.8131 and 0.7300, respectively) (Table 3). Based on the similarity value, using the JCS analysis, the fish species in both the plots was found to be high at 0.8462.

Based on water quality, the pattern of fish activity in ponds and their adjacent rice fields has not been fully understood. According to Iguchi *et al*. (1999), no component of water quality could fully explain the variation in the fish species diversity in the water systems of the Anji rice field ecosystem. Similar results have been recorded by Matthias *et al*. (1996). Bambaradeniya *et al*. (2004) showed the diversity of biota changes with hydrological regime, where more species recorded during the aquatic phase out of three ecological changes during a single rice cultivation cycle. The distance of the rice plots from the source of water supply, such as irrigation canals or rivers, may also influence the diversity of fish. More species are expected to be found in nearby rivers and canals compared to other sites in the fields. As most of the uncontrolled flow plots are located less than 200 m away from the existing rivers, more fish species were expected to be found there as compared to the number of the fish species in the recycled irrigation canals. Katano *et al*. (2003) reported that the richness and diversity of the fish species in the irrigation ditches with a good connection to rice fields were higher than those in the rice fields. This might be due to the higher water level in the irrigation ditches as compared to that in the rice fields.

Flooding in Season 1/2004 might also have contributed to this factor, as more fish from the Pendang River could freely swim into the nearby rice plots. Two species considered as riverine species or "white" fish, namely *Cyclocheilichthys apogon* (temperas) and *Barbodes gonionotus* (lampam jawa), were caught in the uncontrolled flow plots. However, the total number of both species caught was small (i.e. not more 11 specimens). According to Welcomme (1985), some of the "white" fish species make lateral migration for spawning, especially during the rainy season. This is because the flooded rice fields or other terrestrial habitats which are located near the river provide more food and shelter for the fish for spawning (Fernando, 1993). Katano *et al*. (2003) also recorded that several riverine fish species, such

TABLE 3
The Shannon-Wiener Diversity and Evenness Index of fish in the recycled and uncontrolled flow plots (n = number of species)

Plot	Shannon-Wiener Index	Evenness Index
Recycled (n=11)	0.8764	0.8416
Uncontrolled flow (n=13)	0.8131	0.7300

as *Silurus asotus*, *Cobitis* sp. and *Carassius* spp., are known to spawn in rice fields. Katano *et al*. (2003) reported that the richness and diversity of fish species in ditches were high as fish were able to easily invade the rice fields from the nearby rivers.

The depth of water was also found to influence the diversity of fish species. Among other, Iguchi *et al*. (1999) showed that the species diversity was high in the deeper portions of the rivers and they were gradually reduced in canals, ditches and rice fields, with 25, 12, 8 and 3 species, respectively. Meanwhile, Katano *et al*. (2003) and Shah and Ali (1998) had recorded 19 and 39 species in selected irrigation canals and ditches of Japan and in the Malaysian rice agroecosystem, respectively. On the other hand, Ali (1990) and Shamsudin (2001) recorded only seven and eleven species at the North Krian and Muda rice fields, respectively. The difference in the number of species reported by Ali (1990) and Shamsudin (2001) might be due to the different types of sampling gear used, whereby hand nets were used, along with visual observations, in the first study, as compared to the use of electro-shocker in the second study.

As there were no concrete irrigation canals in the study area, the total number of species in both the recycled irrigation water and uncontrolled flow plots was not very different. Katano *et al*. (2003) showed that the natural stream beds which were not covered by concrete had greater number and biomass of fish than the irrigation ditches made of concrete in the Japanese rice agroecosystem. This is because the natural stream bed provides more niches and food sources for fish as compared to concrete irrigation ditches which have a more homogenous environment.

Nonetheless, the list of species is not complete as some introduced (exotic) species, such as *Oreochromis* sp. and the African catfish (*Clarias gariepinus*), reported to have been released into the rice plots as part of the Integrated Pest Management (IPM) scheme were not caught in the study. According to Matthias *et al*. (1996), *Oreohromis* sp. and *Cyprinus carpio*, which are both exotic species in South East Asia, are popularly cultured in the rice fields to assist in pest management programmes. Therefore, further studies that cover more areas should be carried out in order to obtain more accurate information on the success of these released species in the MADA rice fields.

Based on the species richness and the Shannon-Weiner Diversity Indices, it can be concluded that the water quality of the Muda rice fields is considered moderate with low D.O. (0.3 mg/L) and high TSS (3088.8 mg/L) and it can support several fish species. The uncontrolled flow plots had a slightly higher number of fish species, and only two species are different from the plots with recycled water. This is because most of the uncontrolled flow plots are located near the rivers with larger number of fish species. Therefore, the presence or the absence of such species may closely be related to the changes in water levels and the distance from the sources of water supply. Meanwhile, the flood event in the middle of Season 1/2004 resulted in some riverine species being caught in the uncontrolled flow plots as they got trapped when the water level receded.

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Evaluating the Variability of Gafsa Phosphate Rock Uptake by Oil Palm Genotypes at Nursery Stage

N.P. Tan[1*], A.R. Zaharah[1], A. Siti Nor Akma[2] and N. Jamaluddin[3]
[1]Department of Land Management,
[2]Department of Agriculture Technology, Faculty of Agriculture,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[3]Sime Darby Research Sdn. Bhd., P.O. Box 207, 42700 Banting, Selangor, Malaysia
[*]E-mail: ngaipaingtan@yahoo.com

ABSTRACT

The application of phosphate fertilizer in crops accounts for 90% of the total world's mineral phosphate mined and the reserve of phosphate rock in the world continues to deplete. Thus, it is imperative to understand the potential of different oil palm genotypes in taking up added phosphate fertilizers in order to conserve this particular non-renewable resource. In this study, the P-32 reverse isotope dilution method was used in a greenhouse to assess nine different oil palm genotypes in taking up phosphate fertilizer (Gafsa Phosphate Rock) for a period of 6 months. The measurements of the dry weight and P accumulation in plant were conducted during the course of the study. However, the two measurements did not clearly show the difference in the phosphate uptake after the application of Gafsa Phosphate Rock. In more specific, the accumulation of phosphate in the different genotypes only demonstrated significant difference between the genotypes (25/49 x 2367/17 and 9/103 x 2318/17) in the first three months. However, the P-32 reverse dilution method revealed a significant difference in the phosphate fertilizer uptake for the genotypes at 0-3 and 3-6 months. Over the six months' period, the genotypes were observed to take up around 14 to 46 percent of the phosphate added fertilizer. The potential of the different oil palm genotypes to take up phosphate from fertilizer, according to the P-32 reverse isotope dilution method, could be ranked as 2/35 x 2367/17 ≥ 19/19 x 2367/17 ≥ 2/209 x 2367/17 > 25/49 x 2367/17 ≥ 9/103 x 2318/17 ≥ 33/17 x 2318/17 > 14/34 x 2367/17 ≥ 23/34 x 2367/17 ≥ 1/39 x 2318/17. Therefore, the P-32 reverse isotope dilution method could serve as a useful means of assessing the phosphate uptake potential of oil palm seedlings. This study implies that oil palm seedlings, with better phosphate uptake efficiency from fertilizer, can help to reduce fertilizer wastage and contamination of water source, and obtain a better ability to coup with phosphate deficiency.

Keywords: Oil palm, Gafsa Phosphate Rock, P uptake, P-32 reverse isotope dilution

INTRODUCTION

Phosphorus is one of the most essential elements for growth and functioning of plants. Phosphorus deficiency is considered to be one of the major limitations in the crop production on a global scale, especially in the tropical acidic soil (George *et al.*, 2006; Raghothama and Karthikeyan, 2005). Malaysian soils (namely, Ultisols and Oxisols), like most other tropical soils, are known to be highly weathered, acidic, and inherently low in P and have high P fixing capacities. Ferric oxide and aluminium hydroxide are the primary sorbents for phosphate in soil and these could lead to substantial P-fixation (Zaharah and Sharifuddin, 1979; Goh and Chiew, 1995; Sallade and Sims, 1997; Wilson *et al.*, 2004). Although oil palm requires a smaller amount of P than nitrogen and potassium, this element has been reported

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[*]Corresponding Author

to have a synergistic effect with other nutrients on oil palm yield (Foster *et al.*, 1988; Foster and Prabowo, 1996). High yield and increase in the production of oil palm in Malaysia are largely dependent upon the application of fertilizers because oil palm requires high demand, uptake, and removal of nutrient (Von Uexkull and Fairhurst, 1991; Goh and Härdter, 2003). A direct application of phosphate rock in oil palm plantation has been a standard practice since the 1930s (Zaharah *et al.*, 1997). In particular, phosphate rock is preferred in acidic soil as it is nearly as effective as water-soluble P fertilizer and more cost effective (Chien and Menon, 1995). Runge Metzger (1995) claimed that the consumption of world high-grade phosphate rocks might cause depletion within 60 to 90 years. Therefore, to improve and conserve the phosphate deposit left in this world, it is important to understand the ability of different oil palm genotypes in taking up phosphate in soil. Even at the nursery stage, the requirement for fertilizer by oil palm seedlings with 2 to 5 leaves is around 30 g of NPK12-12-17 fertilizers for every 4 weeks (Gillbanks, 2003). Many studies have reported that the efficiencies of plants to extract and uptake P markedly vary between the cultivars of various crops (Narang *et al.*, 2000; Gahoonia and Nielsen, 1996; Horst *et al.*, 1996; Manske *et al.*, 2000; Osborne and Rengel, 2002). Thus, this experiment was carried out to evaluate the potential of various oil palm genotypes in taking up phosphate fertilizer using the P-32 reverse dilution method.

MATERIALS AND METHODS

The genotypes of oil palm seedlings obtained in this study were the new varieties developed by Sime Darby Research Sendirian Berhad (Table 1). The seedlings used were germinated from seeds and maintained according to generic fertilizer programme for oil palm seedlings (Gillbanks, 2003) before subjected to treatment application. Five-months-old oil palm seedlings from nine genotypes were arranged in Randomized Complete Block Design (RCBD) inside a greenhouse, with and without phosphate fertilizer treatments in four replicates for each genotype. The fertilizer used was Gafsa Phosphate Rock (GPR) (Table 1). A total of 144 bags containing 30 kilograms of soil (Serdang Series) were prepared. The fertilization programme to cover three month's period of the study consists of 4.9 g nitrogen, 5.0 g potassium, and 3.9 g magnesium applied as urea, Muriate of Potash and Kieserite, respectively and micronutrients from AJIB® at 20 g. Only half of 144 bags were applied with 6.7 g phosphorus as Gafsa Phosphate Rock (13.2% P), while the remaining were without any addition of P to be used as control for each oil palm genotypes and to reflect the P derived from the soil. The seedlings were left to grow for a period of three months so as to allow for the uptake of fertilizer. Using the same procedure, another set was prepared with 5-months-old seedlings to be harvested at 6 months after planting. Hence, the duration for the observation of the study was 6 months, comprising two harvests. For the set to be harvested at 3 months, 72 bags of soil were labelled as 100 μCi of P-32 with 2 mg of KH_2PO_4 added as a carrier. Meanwhile, for the set to be harvested at 6 months, 150 μCi of P-32 with 1 mg of KH_2PO_4 as carrier was added to each oil palm seedling for the remaining 72 plants after the first set had been harvested. It is important to note that destructive sampling was conducted at three and six months after planting. Only the shoot of the seedlings were harvested. The samples of the plant tissues were separated into rachis and leaves, prior to drying at 70°C until constant weights were achieved. The dry weight of each sample was recorded using balance. Five grams of finely cut samples from rachis and leaves were ashed at 350°C for two hours and subsequently at 500°C for three hours. The ash was dissolved in 20 ml 2N HCl and filtered (Advantec 5C, Toyo Roshi Kaisha, Ltd., Japan). The P-32 activity was measured using Cerenkov counting with Liquid Scintillization Counter (LSC) (model Tri-Carb 3100TR by Packard-Packard BioScience Co). The inorganic P in the plant tissue was determined using the method proposed by Scheel (1936).

TABLE 1
9 new varieties developed by
Sime Darby Research Sdn. Bhd.

No.	Genotypes
1	14/34 x 2367/17
2	2/35 x 2367/17
3	2/209 x 2367/17
4	19/19 x 2367/17
5	25/49 x 2367/17
6	9/103 x 2318/17
7	23/34 x 2367/17
8	1/39 x 2318/17
9	33/17 x 2318/17

The specific activity was calculated by the disintegrations per minute (DPM) of the sample divided by the total inorganic P in the sample (IAEA, 2001). Phosphorus derived from the fertilizers (PdfF) was calculated using the isotope dilution formula described by Zapata and Axmann (1995), whereby:

PdfF (%) = 100 [1-(specific activity of plants with fertilizers)/specific activity of plants without fertilizer)]

The percentage of phosphate derived from fertilizer (PdfF) was subjected to arc sine transformation. The P accumulation in plant was calculated as the product of rachis P and leaves P concentration times the respective dry biomass of the rachis and leaves. A two-way analysis of variance (ANOVA) was used to analyze the experimental data. For separation of means, TUKEY's HSD (honestly significantly different) test was carried out. All the analysis was done using the SAS package version 9.0 for Windows.

RESULTS AND DISCUSSION

Dry Matter Yield and P Accumulation

The soil has a $pH_{(water)}$ of 4.5, Bray-2 extractable P of 5.4 mg/kg, organic carbon of 0.85%, exchangeable K 0.1 cmol(+)/kg, exchangeable Ca 0.8 cmol(+)/kg, exchangeable Mg 0.2 cmol(+)/kg, and Cation Exchange Capacity of 4.3 cmol(+)/kg. The soil texture analysis showed 38% clay, 10% silt, and 52% which gave a textural class of sandy clay. The application of Gafsa Phosphate Rock to nine genotypes gave a similar weight accumulation over six months (Table 3) which yielded the total weight between 114.1 g to 139.4 g. There was no significant difference (p <0.05) in term of weight at the intervals of 0-3 months and 3-6 months, and this suggested that there was no difference in the response of growth for all the genotypes assessed. However, the growth response of the genotypes at 3 months showed a significant difference (p <0.05) in the accumulation of P (Table 4) between the genotypes (25/49 x 2367/17 and 19/19 x 2367/17) which accumulated 177.2 mg P per plant and 116.2 mg P per plant, respectively. Genotype 19/19 x 2367/17 gave the lowest P accumulation in dry weight. At 6 months (Table 4), the differences in the P accumulation between the genotypes were not statistically detected. At six months, the response of P accumulation in the genotypes was similar to that at 3 months, with 25/49 x 2367/17 (highest mean) being significantly different (p <0.05) and 19/19 x 2367/17 (lowest mean) as the least. According to Fong and Lee (1998), the level of P in plants may not be a good indication of crop performance due to the fact that the P applied will no longer increase the amount of P in plant once the threshold of P level is attained. Overall, there was an increase in the dry weight for all the

TABLE 2
Details of Gafsa Rock Phosphate

P source	Total P (%)	Total Ca (%)	Solubility as percent of rock	
			2% Formic acid	2% Citric acid
Gafsa Phosphate Rock	13.4 ± 0.3	31.8 ± 0.6	20.8 ± 0.2	11.7 ± 0.1

TABLE 3
Dry matter weight of oil palm seedlings accumulated at different times of harvest

Genotype	g per plant					
	0-3 months		3-6 months		Total (1-6 months)	
14/34 x 2367/17	53.53	A	85.83	A	139.36	A
2/35 x 2367/17	52.06	A	70.65	A	114.13	A
2/209 x 2367/17	51.75	A	72.67	A	119.45	A
19/19 x 2367/17	44.34	A	80.94	A	120.99	A
25/49 x 2367/17	56.68	A	73.26	A	129.94	A
9/103 x 2318/17	58.32	A	80.65	A	138.97	A
23/34 x 2367/17	55.68	A	75.05	A	130.73	A
1/39 x 2318/17	47.38	A	71.68	A	115.82	A
33/17 x 2318/17	52.37	A	71.83	A	122.8	A

* Mean with the same letter is not significantly different at 5% level by TUKEY

TABLE 4
Total P accumulated by oil palm seedlings at different times of harvest

Genotype	mgP per plant					
	0-3 months		3-6 months		Total (1-6 months)	
14/34 x 2367/17	162.69	A B	171.19	A	333.89	A
2/35 x 2367/17	140.80	A B	192.94	A	333.74	A
2/209 x 2367/17	148.62	A B	200.40	A	349.03	A
19/19 x 2367/17	116.15	B	166.65	A	282.80	B
25/49 x 2367/17	177.22	A	211.37	A	388.60	A
9/103 x 2318/17	164.30	A B	172.20	A	336.50	A
23/34 x 2367/17	155.57	A B	216.89	A	372.45	A
1/39 x 2318/17	147.40	A B	205.27	A	352.67	A
33/17 x 2318/17	154.20	A B	161.49	A	315.70	A

* Mean with the same letter is not significantly different at 5% level by TUKEY

genotypes, i.e. approximately 20 percent from 3 to 6 months. The P accumulated dry weight also demonstrated an increase of approximately 30 percent for the genotypes 2/35 x 2367/17, 2/209 x 2367/17, 23/34 x 2367/17, 1/39 x 2318/17, and approximately 5 percent increase for the genotype 14/34 x 2367/17, 9/103 x 2318/17, and 33/17 x2318/17.

PHOSPHATE DERIVED FROM FERTILIZER (PDFF)

According to Zapata and Axmann (1995), isotopic technique is an effective method to study crop genotypic differences in phosphate uptake from phosphate efficiency. Isotopic data such as PdfF is a yield-independent parameter that provides an accurate measurement of the available P to a crop from the applied phosphate source (Broeshart, 1974; Kucey and Bole, 1984). Gahoonia and Nielsen (1998) reported that the approach involving the use of the P-32 reverse dilution method provides information on nutrient uptake of plants is evident and reliable. Among other, the study by Zaharah *et al*. (1997) revealed that the P-32 isotope technique provided a good understanding of evaluating the efficacy of various phosphate fertilizer sources for oil palm seedlings. Other similar studies using P-32 were also done to assess phosphorus efficiency in wheat (Hayes *et al*., 2004). There was a significant difference (p <0.05) in the phosphate uptake from fertilizer for the genotypes assessed (Table 5). The accumulations of P in the dry weight of genotypes only demonstrated a significant difference (p <0.05) between the genotypes 25/49 x 2367/17 and 9/103 x 2318/17 in the first three months. The technique of using P-32 reverse isotope dilution method (Zapata and Axmann, 1995) to examine the phosphate derived from fertilizer of various oil palm genotypes showed significant difference (p <0.05) at 3 and 6 months (Table 5). Over the six months, 14% to 46% of the P uptake from fertilizer were observed. The highest phosphate uptake at 3 month was by genotype 2/209 x 2367/17, which was 46.82% or 66.75 mg P per plant. At 6 months, the highest uptake was shown by the genotype 2/35 x 2367/17. Six months of observation revealed that the percentages of the phosphate uptake by the oil palm genotypes could be ranked as in Table 5 (i.e. 2/35 x 2367/17 ≥ 19/19 x 2367/17 ≥ 2/209 x 2367/17 > 25/49 x 2367/17 ≥ 9/103 x 2318/17 ≥ 33/17 x 2318/17 > 14/34 x 2367/17 ≥ 23/34 x 2367/17 ≥ 1/39 x 2318/17), which ranged from 12% to 46%. A relative comparison in the amounts of phosphate derived from fertilizer (mg P per plant) among genotypes over 6 months was ranked similar to the P uptake percentage. 2/35 x 2367/17 recorded 153mg P per plant as the highest P in the plant or 2.3 percent of the P added at the beginning of experiment, while 1/39 x 2318/17 recorded 74.05 mg P per plant or 1.1 percent of the added P.

Implication of the Study

Phosphorus availability is defined as the phosphate in soil solution that is available by desorption and dissolution processes for the uptake by plants in the terrestrial and aquatic ecosystem (Sharpley, 2000). However, the fate of Phosphate in soil is normally precipitated and unavailable for plant use (Marschner, 1995). In acidic condition, aluminium and iron are the most important elements responsible for the immobilization of P. These properties could result in substantial P-fixation with dissolved Fe, Al, and Mn ions to form insoluble hydroxy phosphate precipitates (Fontes *et al*., 1996; Brady *et al.*, 2004). In addressing this problem, the availability of phosphorous can be maintained or built up by adding phosphate fertilizers such as phosphate fertilizer. Interestingly, the application of Gafsa Phosphate Rock is subjected to the same fate of P-fixation in soil, yet this study has shown different genotypes are able to uptake phosphate fertilizer at a variable rate, and 10% to 40% of phosphate from fertilizer was also observed. This finding suggests that fertilizer is more available for uptake in some plants and thus possesses higher phosphate uptake efficiency. Thus, this could be interpreted as part of the measure to reduce fertilizer wastage at the nursery stage.

TABLE 5
P derived from fertilizers (percentage and mg/plant) in oil palm seedlings at different times of harvest

Genotypes	PdfF (%)						PdfF (mg/plant)					
	0-3 months		0-6 months		Average 1-6 months		0-3 months		0-6 months		Total 1-6 months	
14/34 x 2367/17	22.57	B C	8.96	D	15.66	D	36.36	C D E	15.34	D E	52.29	D E
2/35 x 2367/17	44.48	A B	48.92	A	45.96	A	60.53	A B	94.39	A	153.39	A
2/209 x 2367/17	46.82	A	23.59	B C D	34.25	A B	66.75	A	47.27	C D	119.54	B
19/19 x 2367/17	45.4	A B	48.07	A	45.93	A	50.86	A B C	80.09	A B	129.88	A B
25/49 x 2367/17	29.06	A B C	31.66	A B C	30.08	B C	50.51	A B C	66.92	A B C	116.89	B
9/103 x 2318/17	28.42	A B C	31.90	A B C	29.96	B C	46.05	B C D	54.93	B C	100.83	B C
23/34 x 2367/17	20.42	C	7.22	D	13.73	D	31.49	E D F	15.66	D E	51.137	E D
1/39 x 2318/17	14.12	C	10.61	C D	12.32	D	20.68	E F	21.78	D E	43.46	E D
33/17 x 2318/17	8.51	C	38.46	A B	23.46	B C D	13.04	F	62.09	B C	74.05	C D

* Mean with the same letter is not significantly different at 5% level by TUKEY
PdfF: Phosphate derived from fertilizer

Phosphate moves in liquid phase through soil via mass flow and diffusion before it is taken up by roots (Tinker, 1976; Barber, 1995). According to Gillbanks (2003), a normal practice at the nursery stage requires 8 mm to 10 mm of irrigated water to seedlings each day. The movement of water in a seedling polybag often experiences infiltration, percolation, and runoff. Thus, phosphorus fertilizer applied closed to the surface is susceptible to loss by surface runoff (Goh and Hardter, 2003). If a considerable amount of phosphate accumulates in soil solution, there will be eutrophication to other water source (Johnston and Dawson, 2005) and this leads to water problem in/near nursery area. Therefore, seedlings with better phosphate uptake from fertilizer might help to mitigate this problem.

In addition, selecting seedlings with better phosphate uptake may help to rectify phosphate deficiency at a much faster rate to ensure a proper growth of the seedlings. In dealing with soils having low availability of phosphate, the current management practice of oil palm and other crops includes the application of phosphate fertilizer (Zaharah *et al.*, 1997). Phosphate acquisition and translocation greatly influence the quality and yield of crops. Moreover, insufficient P application during the establishment may lead to poor palm development such as stunting (Goh and Hardter, 2003).

The different uptake potentials in phosphate by various genotypes can be explained in several perspectives. In general, it is believed that P-efficient cultivars are able to acquire more phosphate via the induction of phosphate Pi transporters with a higher affinity for phosphate uptake in roots allowing the plant to absorb more P from lower concentrations in the soil solutions (Mimura, 1999; Smith *et al.*, 1997). Moreover, the exudation of compounds into rhizosphere can increase the mobility of P in soil, and this includes the increase of extarcellualar acid phosphatase activity of roots and other organic acid including carboxylates (Bhadraray *et al*., 2002; McLachaln, 1980; Rovira, 1969; Hayes *et al.*, 2004). Meanwhile, the changes in the root morphology and architecture such as the root system enlargement, increased root-length system, and arbuscular mycorrhiza establishment to permit the plant to explore a greater volume of soil can also increase phosphate uptake efficiency (Romer *et al.*, 1988; Williamson *et al.*, 2001; Yan *et al.*, 2001). Therefore, more studies on the physiologies of oil palm should be carried out to understand the reasons for better phosphate uptake in some genotypes, as it has been demonstrated in this study.

CONCLUSIONS

The dry weights and P accumulation of different genotypes did not give a clear estimation on the phosphate uptake by the oil palm seedlings. The dry weight of different genotypes was rather similar while the P accumulation in plant showed only a significant difference between two genotypes over six months (namely, genotype 25/49 x 2367/17 and genotype 19/19 x 2367/17). Apparently, these measurements are not good enough to differentiate the phosphate uptake from fertilizer or soil and ultimately cannot be used to determine the potential of oil palm genotypes to take up phosphate from fertilizer. Meanwhile, the P-32 reverse dilution method provides a way to differentiate the phosphate derived from fertilizer or soil. The potential of different oil palm genotypes to take up phosphate from fertilizer, according to the P-32 reverse isotope dilution method, can be ranked as 2/35 x 2367/17 $\geq$ 19/19 x 2367/17 $\geq$ 2/209 x 2367/17 > 25/49 x 2367/17 $\geq$ 9/103 x 2318/17 $\geq$ 33/17 x 2318/17 > 14/34 x 2367/17 $\geq$ 23/34 x 2367/17 $\geq$ 1/39 x 2318/17, ranging from 46 percent to 12 percent. Such ranking is also similar for the mg of phosphate uptake per plant. Therefore, it is evident that the potential of phosphate uptake by various palm oil genotypes cannot be measured using conventional dry weight and P accumulation in plant, but the radioisotope technique provides a better insight into the potential of various oil palm genotype seedlings to uptake phosphate from fertilizer. This study implies that oil palm seedlings with better phosphate uptake efficiency from fertilizer can help to reduce fertilizer wastage and

contamination of water source, in addition to a better ability to overcome phosphate deficiency. Nonetheless, more studies are required to fully understand the physiologies of oil palm so as to reveal a better phosphate uptake of some genotypes.

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Efficacy and Cost-Effectiveness of Three Broad-Spectrum Herbicides to Control Weeds in Immature Oil Palm Plantation

Wahyu Wibawa[1], Mohd Ghazali Mohayidin[2*], Rosli B. Mohamad[3], Abdul Shukor Juraimi[3] and Dzolkhifli Omar[4]

[1]Balai Pengkajian Teknologi Pertanian Bengkulu, Indonesia
[2]Faculty of Business and Management, Open University Malaysia, 50480 Kuala Lumpur, Malaysia
[3]Department of Crop Science,
[4]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: mohd_ghazali@oum.edu.my

ABSTRACT

Efficacy and cost-effectiveness of three herbicides (paraquat, glufosinate ammonium, and glyphosate) were evaluated at the MAB Agriculture-Horticulture Sdn. Bhd. Plantation, in Sepang, Malaysia from February 2004 to October 2005. The experimental design was RCBD with four replications. Each plot size, with the measurement of 4.8 x 20.5 m, was used for three oil palm plants. There were 13 treatments applied at the respective rates (namely, paraquat at 200, 400, 600, 800 g a.i. ha^{-1}, glufosinate ammonium at 200, 400, 600, 800 g a.i. ha^{-1}, glyphosate at 400, 800, 1200, 1600 g a.i. ha^{-1}) and an untreated check as a control. The rates for the herbicides cover their field recommended rates (paraquat at 400-600 g a.i. ha^{-1}, glufosinate ammonium at 500 g a.i. $ha^{-}1$, and glyphosate at 1000 g a.i. $ha^{-}1$). Results showed that glufosinate ammonium and glyphosate gave better efficacies than paraquat as revealed by the data on the percentage of weeds killed, the percentage of weed growth reduction and the duration of effective weed control. Nonetheless, a similar efficacy did not always produce the same cost-effectiveness. The most cost-effective treatment was glyphosate (at 400 g a.i. ha^{-1}), followed by glyphosate (at 800 g a.i. ha^{-1}) and glufosinate ammonium (200 g a.i. ha^{-1}) with the costs around RM108.95, RM160.70, and RM214.19 $ha^{-1}year^{-1}$, respectively. Meanwhile, glyphosate has the ideal criteria as the most cost-effective herbicide because it is cheap (at the current price of RM13.75 L^{-1}), good efficacy at low dose, produces long duration of effective weed control, and lesser spraying rounds required $year^{-1}$.

Keywords: Efficacy, cost-effectiveness, oil palm, herbicides

INTRODUCTION

Weed control is an important and expensive component of plantation crop management (Khairudin and Teoh, 1990). Azahari *et al.* (2004) stated that the cost incurred to control weed may account for 17 to 27 percent of the total upkeep cost in immature or mature oil palm. Herbicides are not problem-free, but there are many reasons why they are such a popular form of weed control (Esterninos and Moody, 1988). Among other, paraquat, glyphosate, and glufosinate ammonium are the most commonly used herbicides in oil palm plantation (Chung and Sharma, 1999; Madeley, 2003). In Malaysia, the use of herbicides in 2004 contributed to 67.49% of the total pesticides used, and herbicide use was predicted at 15.6 million

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[*]Corresponding Author

litres in oil palm in 2005 (Malaysia Agricultural Directory and Index, 2003/2004).

Efficacy is the ability of pesticides to produce a desired effect on a target organism (Kamrin, 1997). Terms such as "mode of action", "injury", and "weed killed" should be well understood to avert improper perception. Sometimes, growers are confused with the use of the terms "injury" and "weed killed". So far, most applicators and growers have perceived efficacy based on the period required by herbicides to show injury on the controlled weeds. The efficacy alone is not enough to determine suitable herbicides in weed management. Cost-effectiveness of herbicides applied is another factor to be taken into consideration when making any decision. Orme (2001), and Turner and Gillbanks (2003) calculated the weed control cost ha^{-1} $year^{-1}$ by summing 3 or 4 cost components, including herbicide cost, labour cost, number of spray round $year^{-1}$, and water transport. According to Atkin and Leisinger (2000), growers prefer an effective herbicide with acceptable cost.

In order to determine how efficient a treatment is when it is applied, the efficacy and cost-effectiveness of each herbicide should be calculated. It is important to note that the treatments with good efficacy are not always the most cost-effective because efficiency is affected by many factors. The objective of the present study was to compare the efficiency of herbicides applied for general weed control in immature oil palm based on their efficacies and cost-effectiveness.

MATERIALS AND METHODS

Experimental Site and Treatments

In this study, two-year old oil palms were used for the experiment. Dominant weed species found in this area include broad-leaves (*Croton* sp., *Asystasia gangetica, Centrosema pubescen, Borreria latifolia, Hedyotis* verticillata) and narrow-leaves (*Paspalum commersonii. Pennisetum polystachyon, Eleusine indica, Digitaria ciliaris, Ischaemum* timorense) (Wibawa, 2007). The experimental design was RCBD with four replications. There were 13 treatments applied (namely, paraquat at 200, 400, 600, 800 g a.i. ha^{-1}, glufosinate ammonium at 200, 400, 600, 800 g a.i. ha^{-1}, glyphosate at 400, 800, 1200, 1600 g a.i. ha^{-1}, and an untreated check as a control). The rates of the herbicides used were inclusive of their recommended field application rates (i.e. paraquat 400-600 g a.i. ha^{-1}, glufosinate ammonium 500 g a.i. ha^{-1}, and glyphosate 1000 g a.i. ha^{-1}). The herbicide formulations used were Gramoxone® (200 g paraquat L^{-1}), Basta 15® (150 g glufosinate ammonium L^{-1}) and Roundup® (360 g glyphosate L^{-1}). Knapsack sprayer, fitted with AN 2.5 deflector nozzle, was used to deliver 200 L ha^{-1} of herbicide solution. In this study, blanket spray was applied for the experiment.

Efficacy

The square method was used to evaluate weed control as a result of herbicide application. The percentage of weed killed was calculated according to the method described in Alloub *et al*. (2000) and Pritchard (2002). Weeds killed meant that all tissues from the growing points to the soil surface were completely dead. The evaluation of weed dry weight, at 8, 12, and 16 weeks after the treatment (WAT), was done as described in Felix and Owen (1999), while the percentage of weed growth reduction was calculated using the method described in Lanie *et al*. (1993), Lanie *et al*. (1994), Murray *et al*. (1994), Utulu (1998), Pritchard (2002), and Chuah *et al*. (2004). The weed dry weight values, between the narrowest observations from 8 to 12, 12 to 16 WAT, were predicted using the regression. The formula used to calculate the reduction in the percentage of growth is as follows:

$$\% \text{ growth reduction} = 100 - \frac{\text{Dry weight of samples from treated plot}}{\text{Dry weight of samples from untreated plots}} \times 100$$

Whereby,

0 percent of growth reduction = no weed control

1-10 percent of growth reduction = very poor weed control

11-20 percent of growth reduction = poor weed control

21-30 percent of growth reduction = poor to deficient weed control

31-40 percent of growth reduction = deficient weed control

41-50 percent of growth reduction = deficient to moderate weed control

51-60 percent of growth reduction = moderate weed control

61-70 percent of growth reduction = weed control somewhat less than satisfactory

71-80 percent of growth reduction = satisfactory to good weed control

81-90 percent of growth reduction = very good to excellent weed control

91-100 percent of growth reduction = complete weed control

The duration of effective weed control is the period where a treatment was able to suppress weed growth, in term of weed dry weight, i.e. ≥50 percent relative to the untreated check. This characteristic was calculated based on the percentage of weed growth reduction values at 8, 12, and 16 WAT.

Meanwhile, the number of the actual spraying round/year refers to the re-spraying needed to get a satisfactory weed control. Note that the unit used for the duration of effective weed control is week and there are 52 weeks in a year.

$$\text{Spraying round year}^{-1} = \frac{52 \text{ weeks}}{\text{Duration of effective weed control (week)}}$$

Cost Effectiveness

The major operational costs to manage weeds include herbicide cost, labour cost, and the actual number of spray round/year. Thus, the cost for controlling weeds/ha/year can be formulated as follows:

$$\text{Cost ha}^{-1}\text{ year}^{-1} = [(\text{herbicide price L}^{-1} \times \text{herbicide dose L ha}^{-1}) + \text{labour cost} + \text{water cost}] \times \text{number of actual spraying round year}^{-1}$$

Based on the survey data, the labour cost at the MAB plantation was RM15 ha^{-1}, while the capacity of workers ranged from 1 to 3 ha^{-1} man/day. Cost for water transportation was sometimes not computed as cost component because water supply is available in the field like ponds, streams, or drain water. For the current study, the price of herbicides was based on the price recorded in July 2005.

RESULTS AND DISCUSSION

Efficacy

Apparently, the percentages of weed killed, weed growth reduction, and duration of effective weed control were significantly affected by the treatments used (Table 1). The percentage of weed killed is good and important indicator to determine the efficacy of herbicide applied. In particular, the paraquat treatments were found to produce lower amount of weeds killed (50.94 – 82.58 percent), than the glufosinate ammonium and glyphosate treatments which destroyed around 91.55 – 97.97 percent and 95.78 – 100.00 percent of weeds, respectively (Table 1). These findings showed that the glufosinate ammonium and glyphosate herbicides are better or more efficient than paraquat in destroying weeds. The paraquat treatments at 200 and 400g ha^{-1} were not effective in controlling mixed weeds in immature oil palm because the ability to control weeds was below 70 percent. Burrill *et al*. (1976) stated that 70 percent of weed killed is the minimum acceptable level of control, while more than 90 percent weeds killed is an excellent level of control. Ashton and Crafts (1981) stated that paraquat is not considered to be selective herbicide, although broadleaf plants are injured somewhat more than grasses at a given low rate. Collins (1991) reported that paraquat has limited efficacy on perennial weeds, but it is more effective on weeds which

are small and in early establishing or vegetative phase of growth. Some annual grasses may only be temporarily suppressed because the low and enclosed growing points are reached by the spray. Turner and Gillbanks (2003) stated that greatest paraquat efficacy is found where the weed species to be controlled have restricted root system or are still young.

Weed growth reduction reflects the capability percentage of a particular treatment to suppress weed growth relative to the untreated check. A higher percentage of weed growth reduction leads to a higher ability to suppress weed growth. As indicated earlier, paraquat has been found to reduce weed growth less efficiently than glufosinate ammonium and glyphosate, particularly at 8, 12, and 16 WAT (Table 1).

In this study, the treatments using paraquat produced shorter duration of effective weed control (i.e. within 4.00 – 11.75 weeks) than glufosinate ammonium and glyphosate which gave 14.50 – 15.00 weeks. Consequently, shorter duration of weed effective control leads to a more frequent spraying round/year (Table 1). In order to get satisfactory weed control, 4.44 – 13.04 of paraquat treatment spraying rounds year^{-1} are needed, whereas only 3.48 – 3.60 of spraying rounds year^{-1} are required when glufosinate ammonium is used. Kuan *et*

TABLE 1
The effects of paraquat, glufosinate ammonium, and glyphosate on the percentages of weeds killed, weed growth reduction, duration of effective weed control and spraying rounds year^{-1}

Treatment	Total weed killed (%)	Weed growth reduction (%)			Duration of effective weed control (weeks)	Spraying rounds year^{-1}
		8 WAT	12 WAT	16 WAT		
Untreated check	0.0 h	0.0 e	0.0 d	0.0 c	0.0 e	0.0
Paraquat 200 g ha^{-1}	50.9 g	26.9 d	38.2 c	12.4 b	4.0 d	13.0
Paraquat 400 g ha^{-1}	66.4 f	40.0 cd	38.8 c	11.6 b	6.0 d	8.7
Paraquat 600 g ha^{-1}	74.1 f	53.3 bc	40.6 c	17.5 b	8.8 c	6.0
Paraquat 800 g ha^{-1}	82.6 e	64.5 ab	49.8 b	20.2 b	11.8 b	4.4
Gluf. ammonium 200 g ha^{-1}	91.6 d	76.4 a	69.3 a	45.5 a	15.0 a	3.5
Gluf. ammonium 400 g ha^{-1}	95.3 cd	89.0 a	71.2 a	41.8 a	14.8 a	3.5
Gluf. ammonium 600 g ha^{-1}	97.9 bc	78.1 a	71.4 a	41.5 a	14.8 a	3.5
Gluf. ammonium 800 g ha^{-1}	98.0 bc	75.3 a	70.2 a	40.5 a	14.8 a	3.5
Glyphosate 400 g ha^{-1}	95.8 bc	76.9 a	69.6 a	40.0 a	14.5 a	3.6
Glyphosate 800 g ha^{-1}	100.0 a	81.4 a	70.7 a	39.6 a	14.8 a	3.5
Glyphosate 1200 g ha^{-1}	99.7 ba	79.7 a	69.8 a	44.8 a	14.8 a	3.5
Glyphosate 1600 g ha^{-1}	100.0 a	78.1 a	71.3 a	41.3 a	14.8 a	3.5

Means within the columns followed by the same letter are not significantly different at 5 percent by the DMRT

al. (1991) reported that the number of spraying rounds year^{-1} for weed control in immature oil palm (<3 year old) ranged from 5 to 6.67 rounds, while Chung and Sharma (1999) reported that the frequency of weeding ranged from 4 to 6 round year^{-1}.

The spraying round/year has a close relationship with the efficacy of herbicides applied (Table 1). In more specific, the efficacy of glufosinate ammonium and glyphosate treatments was apparently much better than the paraquat treatments; their uses in the treatments increase the duration of effective weed control and reduce the number of spraying rounds year^{-1}.

Meanwhile, there is a positive correlation and regression between the percentages of weeds killed and weeds growth reduction (*Fig. 1*). The increase in the percentage of weeds killed is always followed by the increase in the percentage of weed growth reduction.

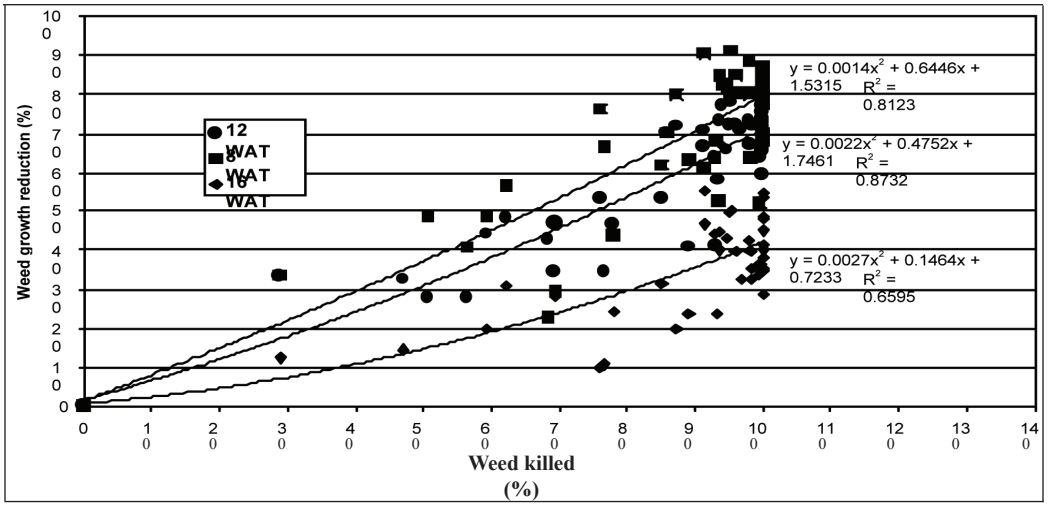

Fig. 1: Regression of the percentage of weed growth reduction on the percentage of weeds killed (same data were used in Rosli Mohamad et al., 2010. Pertanika J. Trop. Agric. Sc. 33(2): 193, same issue)

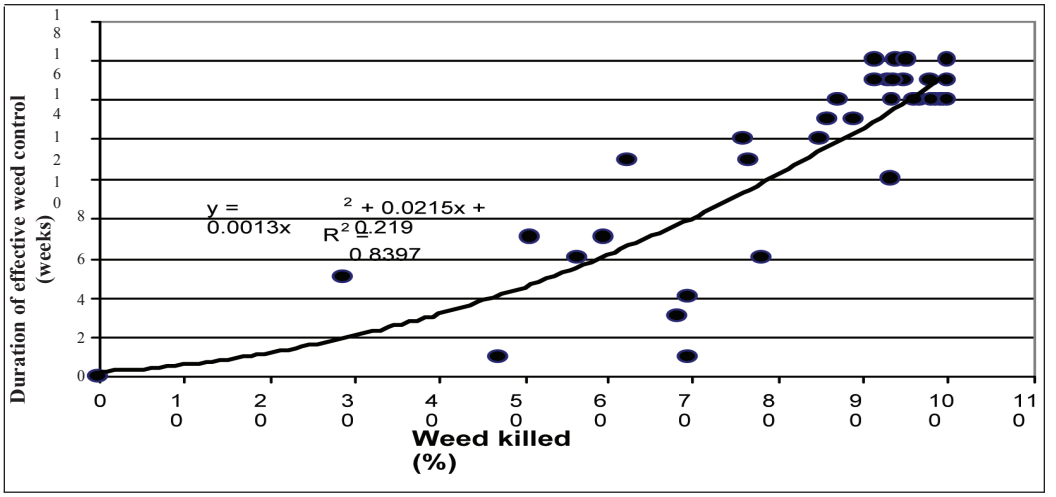

Fig. 2: Regression of the duration of effective weed control on the percentage of weed killed (same data were used in Rosli Mohamad et al., 2010. Pertanika J. Trop. Agric. Sc. 33(2): 193, same issue)

TABLE 2
The costs of paraquat, glufosinate ammonium and glyphosate in weed management (RM ha^{-1} $year^{-1}$)

Treatments (herbicides)	Herb. Dose (Lha^{-1})	Herb. price (RM/L)	Herb. Cost/ ha /round	Labour Cost/ ha /round	Duration (weeks)	Round /year	Cost/ ha/ round (RM)	Cost ha/ year (RM)
200 g paraquat./ha	1.0	13.8	13.8	15.0	4.0	13.0	28.8	374.7
400 g paraquat/ha	2.0	13.8	27.5	15.0	6.0	8.7	42.5	369.3
600 g paraquat/ha	3.0	13.8	41.3	15.0	8.8	6.0	56.3	335.1
800 g paraquat/ha	4.0	13.8	55.0	15.0	11.8	4.4	70.0	310.8
200 g gluf. Amm/ha	1.3	35.0	46.6	15.0	15.0	3.5	61.6	214.2
400 g gluf. Amm./ha	2.7	35.0	93.5	15.0	14.8	3.5	108.5	382.8
600 g gluf. Amm./ha	4.0	35.0	140.0	15.0	14.8	3.5	155.0	547.2
800 g gluf. Amm./ha	5.3	35.0	186.6	15.0	14.8	3.5	201.6	711.5
400 g glyphosate/ha	1.1	13.8	15.3	15.0	14.5	3.6	30.3	109.0
800 g glyphosate/ha	2.2	13.8	30.5	15.0	14.8	3.5	45.5	160.7
1200 g glyphosate/ha	3.3	13.8	45.8	15.0	14.8	3.5	60.8	214.6
1600 g glyphosate/ha	4.4	13.8	61.1	15.0	14.8	3.5	76.1	268.5

These are indicated by regression equations and their R-square values, namely 0.87, 0.81, and 0.66 at 8, 12, and 16 WAT, respectively. The independent variables were found to be significant at the probability levels of <0.05. These results prove that the treatments which produce poor efficacy can cause weeds to grow and recover in a short time.

Meanwhile, the percentage of weeds killed could also be used to predict the duration of effective weed control because it has significant positive correlation and regression (*Fig. 2*). In other words, a higher percentage of weeds killed leads to a longer duration of effective weed control, as indicated by the values of regression equation ($y = 0.0013x^2 + 0.0215x + 0.219$) and R-square (0.84). In this equation, the independent variables are also significant at the probability levels of < 0.05. The treatments with poor efficacy (i.e. low percentage of weeds killed) produce shorter effective weed control duration. On the contrary, longer weed control duration leads to lesser actual number of spraying rounds required per year. These findings indicate that the percentage of weeds killed plays an important role in evaluating the efficacy of herbicides applied because it has been found to affect the percentage of weed growth reduction, the duration of effective weed control, and spraying round/year.

Cost Effectiveness

The cost effectiveness of herbicides applied is not only affected by their efficacy but also by the dose and price of the herbicides applied. In this case, glufosinate ammonium was found to be the most effective herbicide and a longer duration

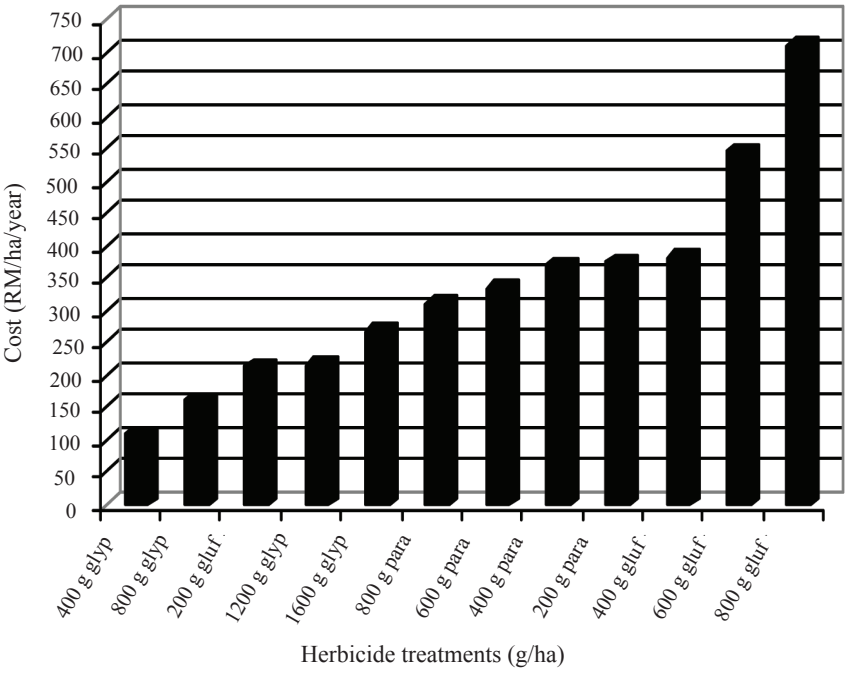

Fig. 3: Ranked cost of paraquat, glufosinate ammonium and glyphosate in controlling weed in immature oil palm (RM/ha/year)

of effective weed control, but it is not the most cost effective herbicide because the price is rather high (i.e. RM35.00 L^{-1}). On the other hand, paraquat is relatively cheap (RM13.75 L^{-1}) but not effective to control weeds, and its duration of control is also relatively shorter than that of glufosinate ammonium and glyphosate. The most cost effective herbicide with a good efficacy at a low dose used in the treatments should be cheap and produce a longer duration of weed control, as indicated by lesser spraying rounds required per year (Table 2 and *Fig. 3*). At 400g a.i. ha^{-1}, glyphosate was found to be the most cost effective pesticide for weed treatment (RM108.95 ha^{-1} $year^{-1}$), followed by glyphosate at 800g a.i. ha^{-1} (RM160.70 ha^{-1} $year^{-1}$), and glufosinate ammonium at 200g a.i. ha^{-1} (RM 214.19 ha^{-1} $year^{-1}$).

CONCLUSIONS

In short, glufosinate ammonium and glyposate produce better efficacies than paraquat, as indicated by the percentage of weeds killed, the percentage in the reduction of weed growth and the duration of effective weed control. Meanwhile, a similar efficacy does not always produce the same cost-effectiveness. The most cost effective treatment was produced by glyphosate at 400 g a.i. ha^{-1}, and was followed by glyphosate (at 800 g a.i. ha^{-1}) and glufosinate ammonium (at 200 g a.i. ha^{-1}) which cost RM108.95, RM160.70 and RM214.19 ha^{-1} $year^{-1}$, respectively.

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Dormancy and Cardinal Temperatures during Seed Germination of Five Weedy Rice (*Oryza* spp.) Strains

Adam B. Puteh*, Roziah Rosli and Rosli B. Mohamad
Department of Crop Science,
Faculty of Agriculture, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
**E-mail: aputeh@yahoo.com*

ABSTRACT

Temperature during seed imbibition has been found to influence germination rate and final percent germination. Seeds of one cultivated variety and five weedy rice strains, collected from different localities in Peninsular Malaysia, were used to determine their degree of dormancy and cardinal temperatures. Meanwhile, standard germination and tetrazolium chloride (TTC) tests were used to evaluate the percentage of seed viability and degree of dormancy. Seed germination test at six different constant temperatures (between 10 and 35°C) was applied to determine the cardinal temperatures estimated by linear regression models, base temperature, T_b, optimum temperature, T_o, and maximum temperature, T_c. The TTC test was found to be a simple and quick test to determine the degree of seed dormancy among different weedy rice strains, when used together with a standard germination test. Germination rate was found to be related to the degree of dormancy but it had no influence on the range of cardinal temperatures. The T_b among the five weedy rice strains was in the range of 2-7.3°C. The T_o varied between 28.1 and 37.5°C, with an average of 32.5°C. This temperature (T_o) was higher than that of the cultivated MR73 variety (24.3°C), whereas the range of T_c was 42.2-43.3°C. The study indicated that the non-dormant cultivated rice seed had lower T_b and T_o values than the dormant seed of weedy rice.

Keywords: Weedy rice, germination, seed dormancy, cardinal temperatures

INTRODUCTION

Weedy rice (*Oryza* spp.) infestation occurs in most major rice-growing areas of the world. The invasive nature of weedy rice has become a major concern, especially in direct seeded rice cultivation. In Malaysia, the presence of weedy rice in a direct-seeded field can reduce yield up to 74% (Bakar *et al.*, 2000). Early shattering has enabled them to escape harvest, while seed dormancy ensures their survival in the soil seed bank. The degree of infestation through soil seed bank germination varies between production years. Nonetheless, the reasons behind these observations are not known. Thus, determining the temperature range at which weedy rice seed germinates will help to predict seedling emergence.

Temperature is an important single factor affecting the capacity for germination by regulating dormancy, and it also critically determines the rate of progress toward completion of germination once a seed is stimulated (Alvarado and Bradford, 2002; Bradford, 2002). The degree of seed dormancy influences the temperature range, at which seed will germinate, with the range increasing as seeds loose dormancy (Benech-Arnold *et al.*, 2000; Vegis, 1964). These critical temperatures,

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*Corresponding Author

which are commonly referred to as cardinal temperatures, consist of the base and maximum temperatures, below or above which germination will not occur, while the optimum temperature is where germination is the most rapid (Bradford, 2002). This concept was initially proposed on the whole plant basis, but it is also applicable to seed during germination. Bewley and Black (1994) described cardinal temperatures as the range of temperatures over which seeds of a particular genotype could germinate. In cultivated rice varieties, a high percentage of germination is attained in two days at 27-37°C, while no germination is found to occur at 8°C and 45°C (Yoshida, 1981). However, the cardinal temperatures for seed germination have never been reported in weedy rice strains, which are helpful for predicting the degree of seed dormancy and infestation in the field.

Models which describe seed germination behaviour, in response to a range of temperatures during imbibition, have been proposed and developed (Covell *et al.*, 1986; Ellis and Butcher, 1988; Alvarado and Bradford, 2002; Hardegree, 2006). The model commonly known as the thermal time model has been extensively used and successfully applied to describe germination timing and seedling emergence in crops (Finch-Savage and Phelps, 1993) and weed species (Roman *et al.*, 2000). This particular thermal time model predicts germination rate at sub-optimal temperatures (from the minimum temperature to the optimum temperature) and supra-optimal temperature (i.e. from the optimum temperature to the maximum temperature) in a linear function (Hardegree, 2006). The thermal time model has also been successfully used to predict weeds seedling emergence in the field under temperate growing conditions (Forcella *et al.*, 2000; Vleeshouwers and Kropff, 2000).

Natural selection on germination responses to seasonal environmental cues in some species has been proposed as a significant determinant for the genotype to establish in a given seasonal environment (Donohue, 2005). However, little information is available on the germination responses of weedy rice strains to temperature during imbibition, and there has been no record of its cardinal temperatures to date. Thus, understanding the variation in temperature during seed imbibition may establish germination responses of weedy rice strains seedling emergence in the field. The aims of this study were to (1) determine the cardinal temperatures, base (T_b), optimum (T_o) and maximum (T_c) of the different weedy rice strains, and (2) determine the germination rate within these cardinal temperatures.

MATERIALS AND METHODS

Plant Material

For the purpose of this study, five weedy rice strains and one cultivated variety were used. Seeds of the cultivated variety MR 73 were obtained from the Malaysian Agriculture Research and Development Institute (MARDI). The seeds of five weedy rice strains were randomly collected from several locations in Malaysia, namely Seberang Perak, Kuala Pilah, Besut, Perlis, and Kemubu Agricultural Development Authority in Peninsular Malaysia. Hereafter, these five strains are termed as SP, KP, Besut, Perlis and KADA strains, respectively. The seeds were collected in January and February 2008 and stored at 0°C in double sealed plastic bags for two months before conducting the study. Seed moisture was kept in the range of 9-11% prior to storage and at the start of the experiments.

Seed Viability Tests

For standard germination test, the imbibing seeds were left at ambient temperature in the laboratory at 25±3°C. The seeds were germinated on double layered moistened (±10mL distilled water) filter papers (Whatman, no. 1, in 80 mm diameter by 10 mm deep disposable plastic Petri dishes. The seeds were soaked in 10% Clorox for surface sterilization for 3 min. prior to the testing.

Seedling evaluation on the number of germinated seeds was done daily, starting on day 2 after imbibition for 14 days. Seeds which did not germinate after 14 days were considered as

dead seeds. In contrast, seeds were considered as germinated when the radical emergence was >5 mm. The test was replicated twice with 50 seeds per replication.

For the tetrazolium chloride test, two replicates of 50 seeds per replicate were imbibed in distilled water at room temperature for 24 h. The middle portion of the seeds was pierced with a needle before soaking them in 0.1% of 2, 3, 5-triphenyl tetrazolium chloride (TTC) salt solution. The seeds imbibing in the TTC solution were exposed to 35°C in the oven for 2 h. Viable seed was evaluated based on the topographical staining pattern on the embryo, as described in the ISTA procedure (ISTA, 1993).

Seed Germination Test at Different Temperature

A germination test at different temperatures for all the seeds was conducted in the germination chamber in darkness. Nonetheless, the preliminary works did not show any seed sensitivity to light during germination (Rosli, 2008). Meanwhile, the preparation of seeds and imbibition media are similar to the procedures used for the standard germination test, as described above. The imbibing seeds were exposed to the constant temperatures of 10, 15, 20, 25, 30, or 35°C. This experiment was performed in two replications consisting of 50 seeds per replicate. Seeds were considered as germinated when the radicle was >5 mm. The evaluation was done daily for 20 days, beginning on Day 2 after sowing.

Statistical and Data Analysis

All the collected data were subjected to the analysis of variance using the Statistical Analysis System (SAS) Software, version 8.2. When ANOVA indicated a significant effect, the least significant difference (LSD) was performed to determine significant differences among the means of the treatments.

Meanwhile, the germination rates were calculated as the inverses of times to radicle emergence (Alvarado and Bradford, 2002). The reciprocals of the time to germination were plotted to estimate the optimum temperature, at which the rate of germination was maximum (T_o). The rates of germination were also subjected to the linear regression analysis to describe cumulative germination response of temperature (SAS Institute, 2005). The cumulative percentage germination (CGP), obtained from the germination tests at different temperatures, were used to calculate the cardinal temperatures. Intersected-line models were used as proposed by Garcia-Huidobro *et al*. (1982). The equation used to describe the rates of germination between base and up to optimum temperatures is as follows:

$$1/t = (T - T_b)/\theta_1 \qquad (1)$$

In order to describe the germination responses above T_o, but below the maximum temperature (T_c), equation (2) was used:

$$1/t = (T_c - T)/\theta_2 \qquad (2)$$

where t is the time taken in days for the CGP to reach a given percentage, T is the temperature, while T_b, T_o and T_c are the base, optimum and maximum temperatures, respectively. These models predict the germination rate for a given seed fraction (sub-optimal and supra-optimal range) in a linear function of temperature. The intercepts of the fitted linear regression lines on the temperature axes were used to estimate T_b and T_o. T_o, was calculated as the intercept of sub-optimal and supra-optimal temperature function (Hardegree, 2006).

RESULTS

Viability and Degree of Dormancy

Based on the standard germination test, the initial quality of the five weedy strains was in the range of 19-86% (Table 1). The germination percentage of the cultivated variety (MR73) was found to be the highest (98%). Meanwhile, the lowest germination percentage among all the weedy rice strains was observed in the KADA strain and the highest was in the SP strain, with 19% and 86%, respectively.

The TTC test used was to determine the percentage viability, as well as the degree of dormancy among the weedy strains and for MR 73. The highest percentage of viability was also recorded in MR73 with 99% (Table 1). This suggests that no seed dormancy is present in this particular cultivated variety. Based on the TTC test, a higher percentage of seed viability was observed in all the weedy rice strains, except for the SP strain, as compared to the percentage of viability based on the standard germination test. Based on the TTC test, the viability percentage was found to be 70-83%. Based on this test, the seed of SP strain was not dormant. As for Besut and Perlis strains, the seeds appeared to have a slight dormancy. The data indicate that there is a variation in the degree of seed dormancy among the weedy strains used in this study.

TABLE 1
Percentage viability of the cultivated rice variety and weedy rice strains based on the standard germination and tetrazolium chloride (TTC) tests

Strain/Variety	Cumulative germination (%)	TTC (%)
MR 73†	98	99
Seberang Perak	86	83
Kuala Pilah	51	76
KADA	19	74
Besut	65	71
Perlis	74	80
LSD	10	2

† cultivated rice variety

TABLE 2
The germination percentage of the cultivated rice variety and weedy rice strains at constant temperatures

Variety/strain	Percentage germination					
	10˚C	15˚C	20˚C	25˚C	30˚C	35˚C
MR 73†	58	78	94	98	94	29
Seberang Perak	8	10	54	62	82	54
Kuala Pilah	20	32	50	54	60	34
KADA	0	0	0	8	22	12
Besut	0	0	34	28	48	22
Perlis	6	8	20	24	50	36
LSD	26.2	16.6	17.0	14.1	20.2	19.6

† cultivated rice variety

Germination and Germination Rate at Constant Temperatures

The increase in the temperature (i.e. from 10°C to 30°C) during imbibition enhanced the germination percentage of both the cultivated rice variety and weedy strains (Table 2). All the weedy rice strains were found to have low germination percentages (≤82%) as compared to MR73 (>90%) within the range of constant temperatures. Increasing the imbibition temperature to >30°C was found to cause a rapid decline in the germination percentage of both the cultivated variety and weedy strains. Meanwhile, the maximum germination percentage of the cultivated variety was observed at 25°C, whereas this was observed at 30°C for all the weedy rice strains. No germination was observed in KADA weedy strain at temperature 20°C and below, indicating that this strain is highly dormant.

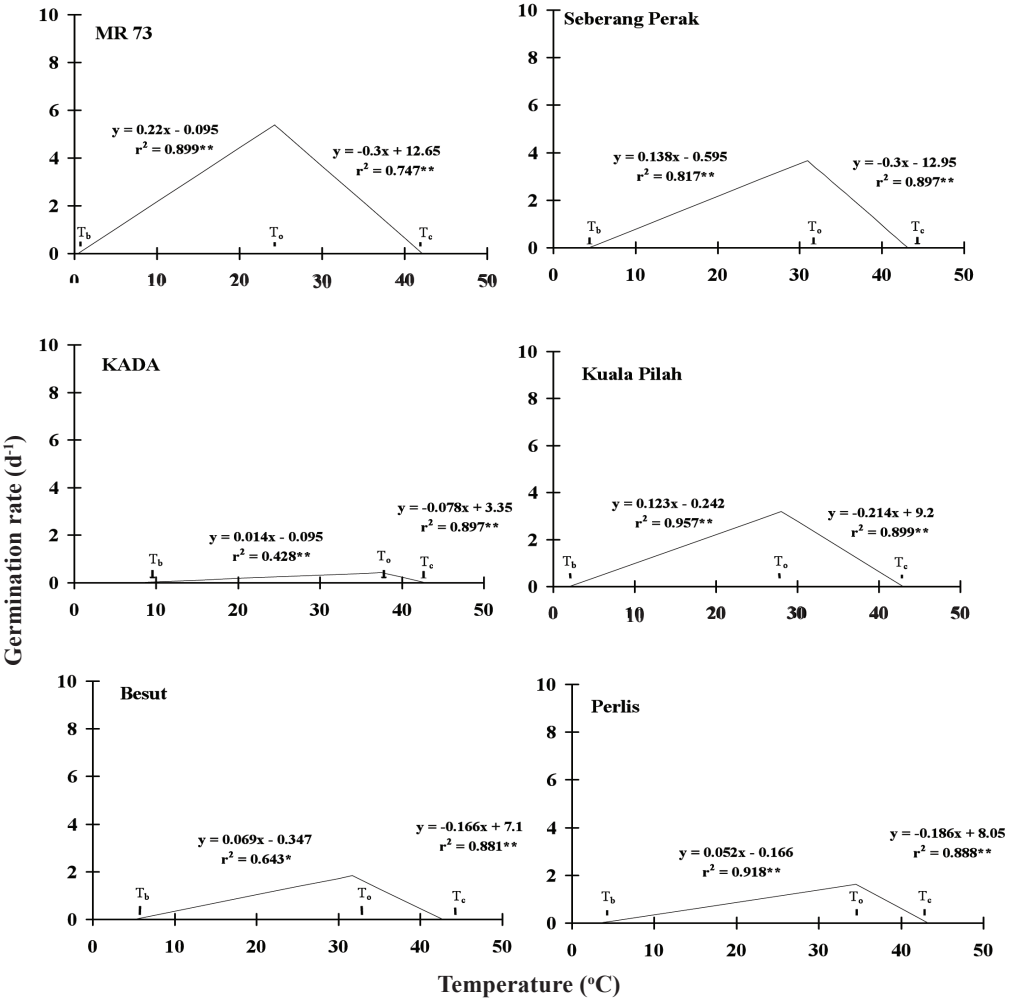

*Fig. 1: Germination rates at the suboptimal and supraoptimal temperature range in response to the different temperatures for MR73 cultivated rice variety, Seberang Perak, Kuala Pilah, KADA, Besut and Perlis weedy rice strains. T_b, T_o, and T_c indicate base, optimum and maximum temperature, respectively. * and ** , significant at P<0.05 and <0.01, respectively*

The higher germination percentage in the cultivated variety at different constant temperatures (10-30°C) could be attributed to the relatively higher germination rate (Table 3). Similarly, a lower germination percentage in weedy rice strains was due to the lower germination rate, particularly at 25°C and lower. The highest germination rate in the cultivated variety and the weedy strains was observed at 25 °C and 30 °C, respectively.

Germination Rate and Cardinal Temperatures Based on the Linear Model

The estimated germination rates, within the sub-optimal and supra-optimal range of temperatures, vary between the weedy rice strains. All the germination rates, which were calculated from the estimated germination time course, showed a significant correlation with temperature at both the sub-optimal and supra-optimal ranges of temperatures (*Fig. 1*). The highest estimated germination rate was recorded for MR73, which was 0.226 day^{-1} in the sub-optimal range. On the contrary, the lowest estimated germination rate was observed in the KADA strain (0.014 day^{-1}), while the highest was in SP strain with 0.128 day^{-1}, based on the linear regression model in the sub-optimal range.

The decline in the germination rate within the supra-optimal range for the weedy rice strains was between -0.078 day^{-1} to -0.33 day^{-1}. Meanwhile, the cultivated rice variety and SP weedy strains had similar germination rate (within the supra-optimal range of temperature), suggesting that the weedy rice strain has a similar germination characteristic with the cultivated rice variety (MR73) at higher temperature. Within this supra-optimal range of temperature, the KADA weedy strain was found to have the lowest estimated germination rate of -0.078 day^{-1}.

The germination rate for the weedy rice strains and the cultivated variety increased linearly with the increase in the germination temperature (*Fig. 1*). Meanwhile, the lowest estimated T_b for MR73 was 0.4°C (Table 4 and *Fig. 1*). The range of the estimated T_b for the weedy rice strains was between 2.0 - 7.3°C, while the KP strain had the lowest T_b. The T_o for the

TABLE 3
The germination rate at different imbibition temperatures of a cultivated rice variety and weedy rice strains

Variety/strain	Germination rate (d^{-1})					
	10˚C	15˚C	20˚C	25˚C	30˚C	35˚C
MR 73†	2.9	3.9	4.7	4.9	4.7	1.5
Seberang Perak	0.4	0.5	2.7	3.1	4.1	2.7
Kuala Pilah	1.0	1.6	2.5	2.7	3.0	3.0
KADA	0	0	0	0.4	1.1	0.6
Besut	0	0	1.7	1.4	2.4	1.7
Perlis	0.3	0.4	1.0	1.2	2.5	2.8
LSD	1.3	0.8	0.9	0.7	1.1	1.0

† cultivated rice variety

seed germination ranged from 28.1 to 37.5°C for the weedy rice strains (Table 4). The estimated T_o for MR73 was found to be the lowest (24.3°C) as compared to the weedy rice strains, whereas the highest T_o of 37.5°C was observed in the KADA strain. Nonetheless, T_c did not differ much between the weedy rice strains and MR73. The narrow range of T_c among the two varieties was between 42.2 – 43.3°C, suggesting that the non-dormant rice seed will not germinate above 43°C.

DISCUSSION

Under ideal germination environments, such as in the laboratory condition, seed dormancy is strongly imposed in some weedy rice strains. Weedy rice strains have often been associated with seed dormancy (Gu *et al.*, 2005). The results indicated that the seed of the KADA strain is highly dormant relative to the seeds of other strains. However, the data presented in this study are still insufficient to determine the type or class of dormancy involved in the tested weedy rice strains. Reducing the percentage of viability between the TTC test and the standard germination test will indicate the degree of dormancy of a seed lot. The SP strain does not have seed dormancy, suggesting that not all weedy rice strains have seed dormancy. It appears that the strain of SP is closely related to the cultivated variety. Therefore, those weedy rice strains producing non-dormant seeds will result in a more widespread infestation in the field throughout the year.

The degree of seed dormancy in weedy rice varies between the strains. Since the strains used in this study were collected from different locations, environmental conditions are therefore suggested to play important roles in determining the degree of dormancy. The variation in the degree of seed dormancy is not only influenced by the environment at the location where the plants are grown, but it is also influenced by genetic factors (Li and Foley, 1997; Gu *et al.*, 2005). The rate of germination appears to be related to the degree of dormancy, but it does not seem to be related to the range of cardinal temperatures. The seed of the KADA strain was found to be very dormant and thus had the lowest germination rate. However, the range of the cardinal temperatures, T_b to T_c, was quite similar to the non-dormant seed of the SP strain. The results indicated that for the non-dormant seed (e.g. in cultivated MR73 variety), T_b would be shifted to a much lower temperature relative to the weedy strains. Meanwhile, T_o for the weedy strains, except for the KP strain, was above 30°C. In this study, the increase in the degree of seed dormancy does not shift T_c in rice, but it changes T_o to a much higher temperature.

T_c was almost similar among the weedy rice strains and the cultivated variety. This suggests that the maximum temperature limit for seed germination is species specific, and it is not influenced by seed dormancy. It is interesting to note that the degree of seed dormancy will change, i.e. either shorten or widen, the sub-optimal and supra-optimal ranges of temperature. This study has clearly indicated that the sub-optimal range of the temperature of the dormant seed is widened by 26-31°C temperature points compared to the non-dormant cultivated variety and the SP strain. The supra-optimal range of temperature is concurrently shortened with the increase in seed dormancy. The results also provide evidence that there is an ecotypic variation in the base and optimum temperatures for seed germination in weedy rice.

Fluctuation in soil temperatures is commonly associated with weed seed emergence in several species (Foncella *et al.*, 2000; Vleeshouwers and Kropff, 2000). However, in tropical growing environments, fluctuation in soil temperature may be negligible, yet higher weedy rice infestation is commonly observed when temperature during preceding growing season is higher. Higher T_o requirement for the weedy rice strains observed in this study could possibly trigger the germination process and the reason for high weedy rice infestation when the temperature during preceding growing season was above normal.

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Characteristics of Pulp Produced from Refiner Mechanical Pulping of Tropical Bamboo (*Gigantochloa scortechinii*)

Zaidon Ashaari[1*], Sabiha Salim[1], Rasmina Halis[1], Mohd Nor Mohd Yusof[2] and Mohd Hamami Sahri[1]

[1]Faculty of Forestry, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

[2]Forest Research Institute Malaysia, 52109 Kepong, Kuala Lumpur, Malaysia

[*]E-mail: zaidon@putra.upm.edu.my

ABSTRACT

Bamboo properties are somewhat similar to certain timbers but it has an advantage of having longer fibres, making it suitable for the production of pulp for paper and hardboard. However, the pulping process is a very crucial stage to produce fibres with an optimum quality. This study was carried out to characterize the pulp of *Gigantochloa scortechinii* using refiner mechanical pulping (RMP). The parameters evaluated included the effects of pre-treatment soaking in NaOH or steaming of chips and effects of refiner plate gap on pulp quality. Pulp quality was assessed based on the properties, yield, and lignin content of fibres. The pre-treatment with NaOH at 60°C for 6 h was found to produce superior quality pulp and lesser lignin content compared to pre-treatment by steaming at 150°C for 3 h. Meanwhile, the refiner plate gap test showed that the two cycles of refining (2.5-mm followed by 0.5-mm plate gap) reduced the lumpiness of the fibre, but it had lower felting power and Runkel ratio. Two cycles of refining process also led to higher fibre yield, produced more unbroken and slender fibres as compared to when one cycle treatment using 2.5-mm plate gap was used.

Keywords: *Gigantochloa scortechinii*, refiner mechanical pulping, bamboo pulp

INTRODUCTION

Bamboo has gained a great attention as potential raw material for wood-based industry in Malaysia. The bamboo plant can be harvested from its natural habitat or grown in a large scale (Azmy and Abd. Razak, 2000). *Gigantochloa scortechinii*, which is locally known as *Buluh semantan* is one of the most common species harvested and its use is mostly associated with traditional uses. Today, bamboo has been explored and expanded for high value-added products such as composites and laminated products. For a number of years, work has been carried out in bamboo producing countries to enhance the utilisation and range of products that can be manufactured from bamboo (Ganapathy, 1999).

Bamboo has the properties which are somewhat similar to certain timbers (Azmy and Abd. Razak, 2000), but it has an advantage of having longer fibres which makes it suitable for the production of pulp for paper and hardboard. However, bamboo is very hard compared to wood (Ganapathy, 1999*)* and its pulping process, especially when done mechanically, will impose problems if the material is not initially softened. A pre-treatment of the material is thus required prior to refiner mechanical pulping to obtain smooth and unbroken fibres for making hardboard (Kollmann *et al*., 1975). One of the

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[*]Corresponding Author

common pre-treatments is the conventional steaming in which higher temperature and pressure are used to soften fibres. Nonetheless, this process will produce more brittles and higher amount of broken fibres after mechanical refining.

Another potential method is through mercerisation using sodium hydroxide solution. The alkali solution helps to degrade lignin and soften fibre physically and chemically (Sreekala *et al*., 1997). In particular, sodium hydroxide treatment functions as an irreversible mercerization effect to increase amorphous cellulose. This process is vitally important to yield high fibre recovery after refining and prevent fibre breakage or damage. In refiner mechanical pulping, besides pre-treatment process, the quality of pulp is also influenced by the gap of refiner disc plate. A high refiner plate gap will only produce loose fibres and the tendency to become lumpy is also greater, while smaller plate gap will lead to finer fibres, and hence reduce the yield.

This paper discusses the characteristics of pulp yielded from the pre-treatment of tropical bamboo (*Gigantochloa scortechinii*), either by steaming or by soaking in NaOH solution prior to refiner mechanical pulping (RMP). RMP was chosen in this study because it incurred lower cost as compared to chemical pulping and also prevented bamboo fibres from producing fines that could decrease the yield (Rowell *et al*., 2000) as well as hardboard strength (Beg and Pickering, 2004). The effects of refiner plate gap and the number of refining cycles on the properties of fibres are also reported.

MATERIALS AND METHODS

Materials

Fresh bamboo culms of *Gigantochloa scortechinii* Gamble (around 3-4 years old) were obtained from the Forest Research Institute Malaysia (FRIM) Research Plot at Chebar Besar Forest Reserve of Nami, in Kedah, Malaysia. A bamboo splitter that has eight fractions was used to split each culm into eight splits. The epidermis and nodal parts of the splits were removed using a single-faced planner. The bamboo strips produced had an approximate dimension of 20-mm (width), 4-mm (thickness), and 1000-mm (length), chipped to approximately 20 mm x 20 mm pieces and air-dried until equilibrium with the surrounding moisture content (MC). The air-dry MC of the chips was determined using the standard oven-drying method.

Mechanical Pulping of Bamboo Chips

The chips were divided into two batches. The first batch was steamed in a digester at 150°C with a pressure maintained at 5.95 $kgcm^{-2}$ for 3 h. For this process (during the first 1 h period), the temperature and pressure were gradually increased, while the final temperature and pressure were maintained for the next 2 h. The second batch was soaked in 2% NaOH solution and maintained at 60°C (Sreekala *et al*., 2002) for 4, 6 or 8 h. The optimum soaking time for this treatment was evaluated. This is very important to prevent the fibre from being over-treated which could reduce its properties (Beg and Pickering, 2004). After soaking, the chips were washed thoroughly with cold water to remove sugars which could affect refining of chips (Rowell *et al.*, 2000).

The pre-treated chips were mechanically defibrated using a single disc refiner (Andritz Sprout-Bauer Model). The effects of the plate gaps and the number of refining cycles on the properties of fibre were also investigated. Three stages of refining were conducted with the disc plate gaps set between the refining plates at 2.5 mm, 0.5 mm and 0.1 mm, respectively. The preliminary results showed that with the use of 2.5 mm plate gap, the fibre yield recovery was approximately 60%, whereas those produced after refining at 0.1 mm plate gap size had only 40% fibre yield recovery. Thus, only the fibre morphology of the refined fibres, using 2.5 mm and 0.5 mm plate gaps, was analyzed. The refining process was performed in two different cycles; first with a refiner plate gap of 2.5 mm only, and secondly, initial refining using 2.5 mm, followed by 0.5 mm plate gaps. The purpose of

the two-cycle refining was to reduce the adverse effect of harsh actions by the plates to the fibres. After refining, the wet fibres were manually squeezed to get rid of the water. Fibres from the untreated bamboo were macerated according to the standard laboratory manual and the data were used for comparison purposes.

Fibre Evaluation

Fibre yield recovery was determined by calculating the mean oven dry weight of the fibres yielded from the refiner per kilogramme of chips input. One g each of the wet fibres from different pre-treatments was stained in safranin 1%. They were then washed in alcohol series of 30%, 50%, 70%, and 95% alcohol each for 2 min, and finally with xylene. Several strands of fibres were placed on the slide, covered with a glass cover and labelled. The observations were made on a Leitz DMRB Image Analyzer which was attached to a digital camera. The length, diameter, and cell wall thickness of the fibres were determined by direct measurement of the magnified image of the fibres mounted on the slide. Fifty measurements were made from each of the five slides. Photographs of the sections (i.e. 20x magnification) were taken and printed.

The colour of the pulp was examined by comparing the pulp solution (10% w/v) from each process with Munsell Soil Colour Charts. Meanwhile, the lignin content of the pulp, together with the untreated bamboo, was determined according to the TAPPI Standard T222 OS-74 (Anonymous, 1974).

The analysis of variance (ANOVA) was performed on fibre property values to detect any differences between the pre-treatment processes and the numbers of refining cycles.

TABLE 1
Fibre properties of *G. scortechinii* yielded from different pre-treatments of chips followed by 2 cycles of RMP

Fibre characteristics	Pre-treatments				
	Steaming at 150°C and 5.95 $kgcm^{-2}$	Soaking in 2% NaOH at 60°C			Laboratory processed fiber
		4 h	6 h	8 h	
Fibre yield (%)	50.7c	65.2b	77.2a	74.6a	-
Length, *L* (mm)	1.45d	1.66c	1.96b	1.71c	3.20a
Width, *D* (μm)	26.84a	26.77a	26.77a	26.55a	20.64b
Cell wall thickness, *w* (μm)	5.51c	10.32a	10.76a	10.74a	6.90b
Lumen width, *l* (μm)	17.82a	6.14c	5.25d	5.07d	14.04b
Felting power (*L*/*D*)	54	62	73	64	156
Runkel ratio (2*w*/*l*)	0.62	3.36	4.10	4.24	4.17
Lignin content (%)	25.67a		19.90b		26.94a
Colour of fibre solution	Dark brown		Yellow		Pale yellow

Means followed by the same letter are not significantly different at $p < 0.05$ using LSD

RESULTS AND DISCUSSION

The results for the fibrous properties of *G. scortechinii* from different pre-treatment processes, followed by 2 cycles (2.5-mm plate gap followed by 0.5-mm plate gap) of the mechanical pulping, are shown in Table 1. The properties of fibres resulted from refining using different plate gaps are given in Table 2.

Effect of Pre-treatments on Fibre Properties

In this study, the fibre recovery from the steam pre-treatment of bamboo (50.7%) was lower than NaOH pre-treatment (65.2- 77.2%). In particular, soaking in NaOH for 6 h yielded the highest recovery. A higher percentage of broken fibres was found in the steam-treated bamboo and the fibre produced was rather rigid (*Fig. 1a*). This was probably attributed to the lignin which was still present in a large quantity (25.67%) in the loose fibre. The lignin content in the untreated bamboo was found to be around 26.94%, while the lignin content in NaOH-treated fibre was 19.9% (Table 1). The results reflected that the steaming temperature used in this study (150°C) was insufficient to degrade the lignin from the fibre (Suchsland and Woodson, 1991; Sjostrom, 1993). Both the thermal and mechanical actions involved in the pulping process could also lead to the brittleness of the fibre, causing it to collapse easily and form fines. Steaming process has also been found to produce lumpy fibres. This is a result of flocculation where water content in the fibres is relatively higher, and thus making them more absorbent or hydrophilic. Unlike the steam pre-treated fibre, fibres which were pre-treated with NaOH were hydrophobic. The treatment had partially removed lignin and hence produced microfibril with greater crystallinity.

The high fibre recovery from the NaOH pre-treatment is partly due to the effectiveness of this particular process in changing cellulose I to cellulose II, resulting in increment of crystalline fibre chain and reduction of amorphous line. Natural cellulose has cellulose I crystalline structure, but on alkalisation, it changes to cellulose II, in which the parallel polymer chains of cellulose I was aligned anti-parallel and higher exposition of OH^- (Vilaseca *et al*., 2006). The high content of hemicellulose, coupled with the reduction of lignin during alkalisation, would contribute to more fibres being easily extracted from the treated chips. Pickering *et al*. (2006) found that chemically processed fibre was 32% richer in cellulose as compared to the non-chemically processed fibres. NaOH pre-treatment helps swelling the fibrils and also

TABLE 2
Fibre properties of *G. scortechinii* chips produced after refining at different cycles

Fibre morphology	Refiner plate gap size (mm)			
	Steaming at 150°C and 5.95 $kgcm^{-2}$		Soaking in 2% NaOH at 60°C for 6 h	
	1 cycle	2 cycles	1 cycle	2 cycles
Length, *L* (mm)	2.39a	1.45b	2.18a	1.96b
Width, *D* (micron)	26.58a	26.84a	26.63a	26.77a
Cell wall thickness, *w* (μm)	4.80b	5.51b	10.72a	10.76a
Lumen width, *l* (μm)	18.98b	17.82b	5.19a	5.25a
Felting power (*L*/*D*)	90	54	82	73
Runkel ratio (2*w*/*l*)	0.51	0.62	4.13	4.10

Means within the pre-treatment followed by the same letter are not significantly different at $p < 0.05$ using LSD

cleaning the fibre bundle surface (*Fig. 1b* and *c*), and as a result increased the tensile strength of the fibre. As the fibre strength improved and became more plastic, it would not be easily damaged by mechanical pulping and more fibre could be extracted (Pickering *et al*., 2006; Mwaikambo and Ansell, 2003).

The results also showed that the quality of fibre from NaOH pre-treatment was dependent on the soaking time. The longest fibre was recorded from 6 h soaking (1.96 mm) and the shortest was in 4 h (1.66 mm). Nonetheless, prolonging the soaking time to 8 h did not significantly affect the length of fibre (1.71 mm). The steam treatment was found to produce shorter fibre (1.45 mm) as compared to the NaOH treatment. The high variation of fibre length in the steam treatment (71.1%) indicated that the fibres were broken during pulping (*Fig. 1a*), as a result of embrittlement of the fibres caused by the application of high temperature (150°C) and pressure (5.95 $kgcm^{-2}$). Generally, the mechanical pulp had fibres shorter than the actual fibres produced using the laboratory mercerisation process (i.e. 3.20 mm).

Regardless of the pre-treatment process, the width of fibre produced by RMP was relatively similar, i.e. between 26.55-26.84 µm. These values were relatively higher than the fibres extracted in the laboratory. Similarly, the thickness of the cell wall for the NaOH-treated fibre (10.32-10.74 µm) was significantly higher than that of the fibre which was produced in the laboratory (6.90 µm). A similar observation was reported by Mwaikambo and Ansell (2003). The NaOH treatment was found to help the cell wall to swollen and to produce fibre with small lumen size known as closed lumen (*Figs. 1b* and *1c*). An internal fibrillation of the cell wall was also noticed in the NaOH-treated fibres (*Fig. 1d*). Lignocellulosic fibre is usually packed with microfibrils but it was split after the alkali treatment (Cao *et al*., 2006). This phenomenon is termed as fibrillation that breaks the treated fibre bundle down into smaller ones by the dissolution of the hemicellulose. Fibrillation

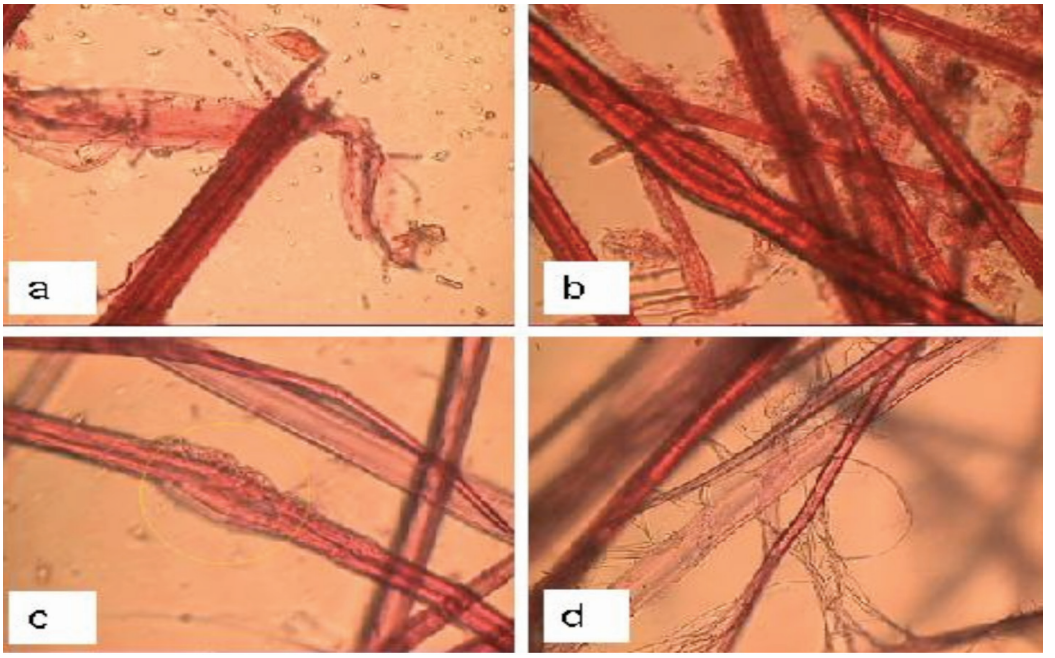

Fig. 1 (a): Broken fibre from steam-treated chips, (b) Swollen unclean surface fibre from 4 h-NaOH-treated chips, (c) Swollen clean surface fibre from 8 h-NaOH chips, and (d) Fibrillation of fibres from NaOH-treated chips

has the advantage of increasing the surface area available for contact with the matrix and hence improving the interfacial adhesion (Bisanda and Ansell, 1992).

Higher felting power results in a better fibre-matrix adhesion (Gassan and Bledzki, 1999; Mwaikambo and Ansell, 2003; Cao *et al*., 2006). Fibre with high felting power indicated that the fibres produced are very slender (long and thin fibres) and with good pulp quality (Britt, 1970). The felting power of the steam-treated fibres was 54 and this was 73 for the NaOH-treated fibre. The values are very much lower as compared to the laboratory produced fibre (146), but it is similar with the range values for the southern pine (28-440) (Sjostrom, 1993). The results also suggested that the felting power for the mechanical pulping bamboo fibre would give a good interfibre bonding in the production of paper or hardboard. The NaOH-treated fibres had high Runkel ratio (i.e. with 4.10), while steam-treated fibres had 0.62. Meanwhile, the Runkel ratio of the untreated fibres was 4.17.

As for colour, the solution of steam-treated fibre is darker (dark brown) than NaOH-treated fibre (yellow), while the solution of the untreated fibre has a yellowish colour when the Munsel Soil Colour Chart was used as a reference. The darker colour found in the steam-treated fibre is partly due to the heat and pressurised system in the process which decolourises the fibres, and this may probably be due to the high amount of lignin retained in the fibres (25.67%). Fengel and Shao (1985) reported that lignin softens and becomes thermoplastic at 90°C, while degrades and dissolves when the temperature reaches 170°C. However, the temperature used in this study was only 150°C which maintained the lignin in a plastic form in the fibre structure (Hsu *et al*., 1986). In this study, the NaOH treatment was found to remove only 7.04% lignin from the bamboo. Therefore, the low concentration of NaOH solution (2%) used in this study might not be sufficient enough to degrade all the lignin. Nonetheless, the treatment with 5% NaOH successfully removed a great amount of lignin from palm fibres (Geethamma *et al.*, 1995), while the treatment with 6% NaOH removed lignin of hemp, jute, sisal, and kapok fibres (Mwaikambo and Ansell, 2003).

Effect of Refiner Plate Gap on the Properties of Fibre

Adjustment of refiner plate gap would vary the size of the fibre bundles of wood (Blomquist *et al*., 1981). The effects of refiner plate gap on the fibre properties are shown in Table 2. In general, chips which underwent one cycle of refining had a higher fibre length than those refined for two cycles, regardless of the pre-treatment. The pulps produced, however, were lumpy (*Fig. 2*). For the steam-treated chips, the length of fibre was reduced from 2.39 mm to 1.45 mm, when they were refined from one cycle to another. Those treated with NaOH for 6 h produced fibre with the mean lengths of 2.18 mm and 1.96 mm when refined for one and two cycles, respectively. Wood fibre has been reported to be easily damaged by the rotating knife when it was first treated with steam (Das *et al*., 2000).

The diameters of fibre from both the pre-treatments were not significantly different from each other. The values ranged from 26.22 to 26.84 µm. Meanwhile, the thickness of the cell wall of the NaOH-treated fibre which underwent one cycle and two cycles of refining was similar (10.72-10.76 µm). However, steam-treated fibres with two cycles of refining had a thicker wall (5.51 µm) as compared to a single cycle of refining (4.80 µm). The increment in the thickness of the cell wall after the second refining was attributed to the flattening or collapse of fibre due to the narrow gap of plate action. Clark (1985) revealed that the high temperature steaming, coupled with the harsh action of refiner plate gap, would easily cause fibre to collapse. Regardless of the pre-treatment, the felting power of the fibre was adversely affected by the number of refining cycles. A higher felting power (90) was found on the steam-treated fibre which had undergone 1 cycle of refining. It was decreased to 54 when refined with a smaller plate gap. The same result was

Fig. 2. Fibres from two cycles (2.5-mm plate gap followed by 0.5-mm) of refining (left) and Lumpy fibres from one cycle (0.5-mm plate gap) of refining

observed for the NaOH-treated fibres, where two cycles of refining produced a higher felting power fibre (82) than that of one cycle (73), but with insignificant variation as compared to the steam-treated fibre. The Runkel ratios of the NaOH-treated fibre, with one and two cycles of refining, are similar (i.e. between 4.10-4.13) and these values were markedly higher than the steam-treated fibre where the values are 0.51 and 0.62 for one and two cycles, respectively.

CONCLUSIONS

The pre-treatment of *G. scortechinii*, prior to mechanical pulping, was found to significantly affect the pulp properties. The results showed that soaking bamboo in 2% NaOH solution maintained at 60°C produced higher fibre recovery, superior quality, and lighter pulp colour than those which were pre-treated by steaming at 150°C for 3 h. Within the NaOH treatments, bamboo soaked for 6 h produced an optimum pulp quality compared to 4 and 8 h soakings. In the refiner plate gap test, two cycles of refining (2.5 mm followed by 0.5 mm plate gap) reduced the lumpiness of the fibre, but it had a lower felting power and Runkel ratio compared to the one-cycle refining (2.5 mm plate gap). This treatment also resulted in higher fibre yield, produced more unbroken and more slender fibres than the one-cycle treatment. The results also revealed that an optimum quality of mechanical bamboo pulps for hardboard production could be obtained through pre-treatment of chips by soaking in 2% NaOH for 6 h, followed by 2 cycles of refining (first with 2.5-mm and followed by 0.5-mm plate gaps).

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Genetic Variation of Selected *Camellia sinensis* (Cultivated Tea) Varieties in Malaysia Based on Random Amplified Microsatellite (RAMs) Markers

Latip, S.N.H.[1], Muhamad, R.[1*], Manjeri, G.[1] and Tan, S.G.[2]
[1]Department of Plant Protection, Faculty of Agriculture,
[2]Department of Cell and Molecular Biology,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 UPM,
Serdang, Selangor, Malaysia
[*]E-mail: ritamuhamad@yahoo.com

ABSTRACT

Studies on the genetic variation among *Camellia sinensis* L. varieties (cultivated tea) in Malaysia were conducted by using RAMs markers. Six varieties were selected from Sungai Palas Boh Estate, Cameron Highlands and nine varieties were selected from Bukit Cheeding Boh Estate, Banting. These tea varieties were classified as resistant, intermediate, or susceptible varieties based on the level of infestation by the mosquito bug, *Helopeltis theivora*. DNA was extracted from the leaves of 225 individuals belonging to different varieties from the two populations. Four RAMs primers were used to evaluate the genetic variation in 15 varieties of tea. Distances were calculated based on Nei and Li's (1979) similarity coefficients using the data from the RAMs markers. A cluster analysis employing UPGMA was done and the dendrogram grouped the tea varieties into two clusters with intermediate variety grouping and the resistant (the first cluster) or susceptible (the second cluster) varieties. The first cluster consisted of all the varieties from Cameron Highlands, except for BC223 (resistant) and 63/14 (resistant) from the Banting population, while the other clusters consisted of all the varieties from the Banting population, except for BC196 (resistant) from the Cameron Highlands population. The dendrogram showed that the genetic differences were based on the populations' geographical distributions and partially based on their resistance towards attack by *H. theivora*.

Keywords: Genetic variation, tea, RAMs, resistant, susceptible varieties

INTRODUCTION

Tea, *Camellia sinensis,* is a beverage crop native to South East Asia, and has been introduced into many other countries (Wachira *et al*., 2000). The genus *Camellia* is composed of over 80 taxa (Sealy, 1958), of which only one, *C. sinensis* L. (O. Kuntz), is frequently used commercially as a source of beverage tea (Wachira *et al*., 1997). Two varieties of tea, the *assamica* and the *sinensis*, which differ in their morpho-anatomical, chemical, and genetic points of view are now fully recognized. Despite its agronomic importance, tea is still characterized and selected using environmentally and ontogenetically dependant morpho-anatomical traits (Green, 1971). However, it has been argued that these may not reflect the true level of genetic differentiation as most are subjected to large environmental effects (Wachira *et al*., 1997).

Although the mosquito bug, *Helopeltis theivora*, is recognized as a leaf-destroying

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*Corresponding Author

pest of tea in Malaysia, little is known about the genetic background of this particular insect and its host plant, tea. The lack of information has led to the loss of genetic variability and allelic differences of the tea varieties planted in Malaysia. Moreover, the number of molecular work done on *H. theivora* is still limited; however, some information regarding molecular study on tea is available. Knowledge of genetic diversity among the available tea varieties is important as it will have to be preserved and characterized for future breeding and crop improvement programmes that constitute the fundamental support structure for the tea industry (Balasaravanan *et al*., 2003). Studies have also been carried out to examine the genetic diversity of tea as its diversity has suffered severe erosion over the years because of intensive selection and breeding for desirable agronomic traits. Balasaravanan *et al*. (2003) studied the genetic diversity among South Indian tea germplasm (*C. sinensis, C. assamica, C. assamica* spp. Lasiocalyx) using polymorphic microsatellite markers of *C. sinensis*, but the study focused only on the allelic differences of clones from different regions. RAPD and AFLP markers have intensively been used to develop genetic linkage maps of *C. sinensis* (Christine *et al*., 2000). Meanwhile, studies using Random Amplified Polymorphic DNA (RAPD) and organelle specific polymerase chain reactions were used to establish the affinities for the cultivated tea and its wild relatives (Francis *et al*., 1997). In this study, the RAMs primers were used. These markers are alternatively known as ISSR (Inter Simple Sequence Repeats). It uses simple sequence repeats anchored at the 5' end by a short arbitrary sequence. The RAMs primers are highly polymorphic and dominant in inheritance (Wang *et al*., 2009). The goals of this study were to amplify bands which were used to investigate the level of genetic variation present in the tea variety in Malaysia and the relationships among the resistant and susceptible varieties of tea to the mosquito bug (*H. theivora*) in the highlands and lowlands of Malaysia using random amplified microsatellites (RAMs) as molecular genetic markers.

MATERIALS AND METHODS

Materials

The leaf samples of different tea *C. sinensis* (L) varieties were collected from two populations, namely Bukit Cheeding Boh Estate, Banting, Selangor, Malaysia (lowland tea) and Sungai Palas Boh Estate, Cameron Highlands, Pahang, Malaysia (highland tea). Six varieties were selected from the Cameron Highlands population and there were nine varieties from the Banting population. They were differentiated into being resistant, intermediate, and susceptible varieties of tea (Table 1), based on the infestation values of *H. theivora* on them (unpublished data). The means for all the resistant scores were analyzed using the Duncan LSD. Consequently, fifteen individuals from each of the varieties from the Banting and Cameron Highlands populations were analyzed.

DNA Extraction and Amplification

DNA of individuals belonging to the different varieties of tea was isolated using the protocol of Doyle and Doyle (1987) with some modifications. The PCR was carried out in a 10µl total reaction mixture volume consisting of 20 ng of genomic DNA, 1X PCR buffer (Promega), 250 µM of each dNTPs (Promega), 0.5 µM of primer, 1.5 units of Taq polymerase (Promega), 2.0mM of $MgCl_2$ and topped up with deionized distilled water to 10 µl. The mixture was overlaid with 15 µl mineral oil. The PCR amplifications were carried out in a thermocycler (T3 Biometra) following the method used by Williams *et al*. (1990) with minor modifications done to the thermal cycles, as follows: 96ºC for 3 min; 40 cycles of 96ºC for 10 s, an optimal annealing temperature of each primer for 10 s, 72ºC for 30 s. A final extension step of 72ºC for 5 minutes was included after 40 cycles of amplification. Twenty RAMs primers, designed by Kumar (2003) for mungbeans and Hoh *et al*. (2004) for river catfish, were screened to test for the amplifications using the protocol as stated above. For the primers that produced too many bands or complex banding patterns, the annealing

TABLE 1
Sampling sites, and list of tea varieties and their types of resistance level to mosquito bug *H. theivora's* attack

Sampling sites	Resistant	Intermediate	Susceptible
Cameron Highlands	BC 196	AT 53	BC 1248a
	BC 664	TRI 2024	TV 9
	BC 223	66/3	65/6
Banting	63/14	65/4	BC 1248
	63/12	65/16	63/4

temperature was increased by 5°C. Nonetheless, the primers that did not produce distinct banding patterns were eliminated from the study. With some combinations of primers and genomic DNA template, a non-discrete range of amplification products that appeared as a 'smear' visualized on a gel could be converted to discretely sized bands by reducing the concentration of either the polymerase or the genomic DNA (Williams *et al*., 1990). The amplification products were analyzed by electrophoresis on a 2.0% (w/v) horizontal agarose gel in 1 x TBE buffer at 78 V for 1 to 1½ hours, depending on the size of the amplified fragments from each primer. A 100bp ladder (Promega) was used as a molecular size standard. The PCR products were detected by staining the agarose gel in ethidium bromide (10 μg/μl) and subsequently visualizing the gel over UV light. The gel was photographed and documented using an Alpha®Imager 2200 (Alpha Innotech, USA) system.

Statistical Analysis

The RAMs banding profiles were visually scored for all the DNA samples and for each primer. The recording of the data was according to the presence/absence criterion (1= presence of band; 0 = no band). It is worth highlighting that faintly stained bands that were not clearly resolved were not considered in the data collection. Similarity coefficients were calculated across all the possible pairwise comparisons of individuals, both within and among populations, using the following formula:

$$S_{xy}=2n_{xy} / n_x + n_y$$

where n_{xy} is the number of common bands shown in both individuals x and y, and n_x and n_y are the total numbers of bands observed in individuals x and y respectively (Nei and Li, 1979). The data obtained were used to compile pairwise distance matrices based on the similarity coefficient of Nei and Li (1979), using the RAPDistance version 1.04 software (Armstrong *et al*., 1995). As a means of providing a visual representation of the genetic relationships, a dendrogram was constructed based on the distance values derived from $1\text{-}S_{xy}$ between pairs of individuals within and between populations. Then the dendrogram was constructed using the unweighted pair group method with arithmetic averaging (UPGMA) employing the SAHN (Sequential, agglomerative, hierarchical, and nested clustering) programme from NTSYS-pc version 1.6 (Numerical Taxonomy and Multivariate Analysis System) (Rohlf, 1993). The UPGMA method defines the intercluster distance as the average of all the pairwise distances for members of different clusters.

RESULTS

After initially screening 20 RAMs primers, four primers (Table 2) were identified to be informative for the purpose of resolving genetic marker differences, within and among the varieties of tea. An example of the polymorphisms detected among some test samples by primer BP-05 is shown in *Fig. 1*. From the 15 varieties of tea studied, a total of 63 bands or DNA markers were generated using the four primers. The overall data showed that the largest fragment was 1,000bp, while the

TABLE 2
The optimized conditions for each of the RAMs primers

Primer code	Sequence (5' to 3')	$MgCl_2$ concentration	Annealing temperature	DNA volume
BP-05	NNN YYB MBM $B(AG)_6$	3.0mM	51ºC	20ng
BP-08	KKY HYH YHY $(GTT)_5$	2.5mM	51ºC	20ng
BP-11	KKY HYH Y $(CAG)_5$	2.0mM	48ºC	20ng
BP-13	KKB SBS BSB $(CT)_6$	2.0mM	51ºC	20ng

Note: K=G/T; N=A/C/G/T; H=A/C/T; Y=T/C; B=C/G/T; M=A/C; S=C/G

smallest fragment was 200bp in size. Table 3 shows the number of RAMs bands generated by the four primers in each of the 15 varieties of tea studied. The total number of polymorphic bands was 57 out of the 63 reproducible bands. In particular, primers BP-05 and BP-08 showed the highest percentage of polymorphic bands (100.0%), followed by BP-13 and BP-11 with 89.5% and 63.6%, respectively. Meanwhile, BC 196 (85.7% of polymorphic bands) had the highest number of polymorphic bands as compared to the other varieties, variety 65/4 showed the lowest number of polymorphic bands with 68.3% of polymorphic bands.

The matrixes of distances between the 15 varieties of teas are shown in Table 4. The highest distance value between varieties was found to be between variety TRI 2024 (D) and variety 66/3 (J), which were from Cameron Highlands and Banting, respectively, (0.7092) while the lowest distance value was found to be between variety 65/6 (M) and variety BC 1248 (N), which were from the same population in Banting (0.5496). This finding showed that the genetic differences were based on the populations' geographical distributions. In the clustering analysis, two major groups were formed; however, these were not conclusively

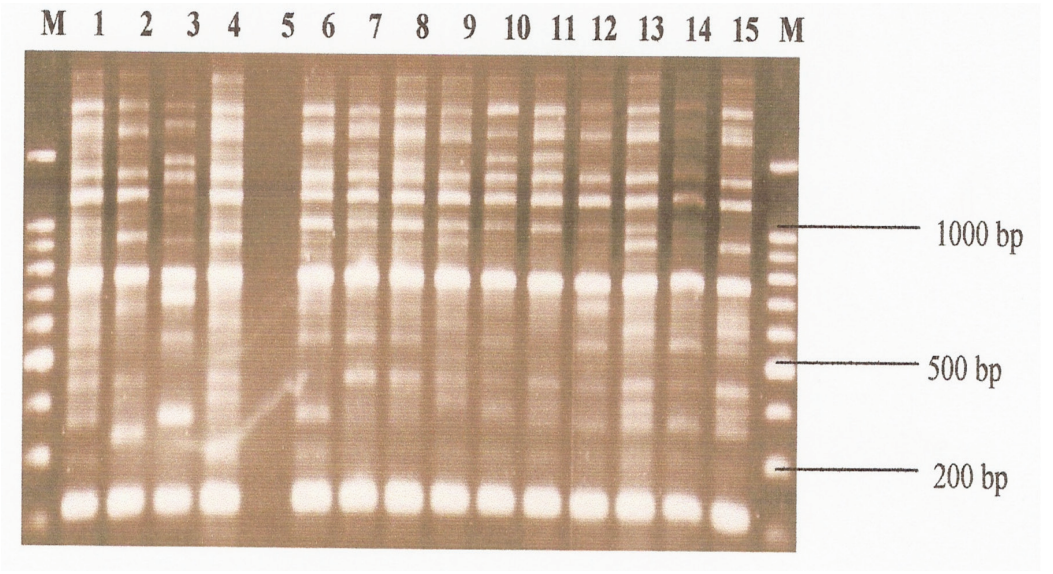

Fig. 1: RAMs profile of C. sinensis *variety AT 53 generated using primer BP-05. PCR product was run on 2% agarose gel using 1X TBE buffer. Lane M: 100bp size marker (Promega); Lane number 1-15: Different individuals of variety AT 53*

TABLE 3
The number of RAMs bands generated by four primers in each of the 15 tea varieties

Primer	Total reproducible bands	Total polymorphic bands	Number of polymorphic bands according to varieties of tea														% of polymorphic bands
			A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
BP-05	18	18	18	18	17	18	18	18	18	18	17	18	18	18	18	18	17	100.0
BP-08	15	15	14	14	14	15	13	15	13	14	14	14	12	15	15	14	14	100.0
BP-11	11	7	5	2	5	3	4	5	6	4	5	3	2	4	7	4	4	63.6
BP-13	19	17	17	15	16	14	13	12	10	11	15	13	11	11	13	14	14	89.5
Total	63	57	54	49	52	50	48	50	47	47	51	48	43	48	53	50	49	88.3
% polymorphism			85.7	77.8	82.5	79.4	76.2	79.4	74.6	74.6	81.0	76.2	68.3	76.2	84.1	79.4	77.8	

Varieties:

A=BC 196
B=BC 664
C=AT 53
D=TRI 2024
E=BC 1248a
F=TV 9
G=BC 223
H=63/14
I=63/12
J=66/3
K=65/4
L=65/16
M=65/6
N=BC 1248
O=63/4

TABLE 4
Distances based on Nei and Li's similarity coefficients among 15 varieties of tea

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A	-														
B	0.5728	-													
C	0.6346	0.6032	-												
D	0.6715	0.6370	0.6244	-											
E	0.6615	0.6322	0.6305	0.6388	-										
F	0.6387	0.6106	0.6169	0.6385	0.6018	-									
G	0.6700	0.6244	0.6246	0.6441	0.6304	0.6086	-								
H	0.6213	0.5763	0.5816	0.6171	0.5975	0.5716	0.5754	-							
I	0.6210	0.5900	0.6196	0.6459	0.6155	0.5968	0.6142	0.5735	-						
J	0.6621	0.6645	0.6932	0.7092	0.6993	0.6565	0.6618	0.6379	0.6207	-					
K	0.6670	0.6620	0.6887	0.7068	0.6667	0.6571	0.6721	0.6604	0.6101	0.6610	-				
L	0.6450	0.6372	0.6613	0.6741	0.6786	0.6595	0.6549	0.6444	0.6212	0.6537	0.6441	-			
M	0.6269	0.6174	0.6557	0.6747	0.6627	0.6570	0.6543	0.6278	0.5967	0.6421	0.6528	0.6125	-		
N	0.6369	0.6390	0.6754	0.6972	0.6755	0.6634	0.6712	0.6510	0.6081	0.6308	0.6283	0.6264	0.5496	-	
O	0.5912	0.5919	0.6125	0.6369	0.6210	0.6116	0.6185	0.5848	0.5569	0.6075	0.6082	0.5712	0.5608	0.5687	-

Cameron Highlands population:

A=BC 196	D=TRI 2024	
B=BC 664	E=BC 1248a	
C=AT 53	F=TV 9	

Banting population:

G=BC 223	J=66/3	M=65/6
H=63/14	K=65/4	N=BC 1248
I=63/12	L=65/16	O=63/4

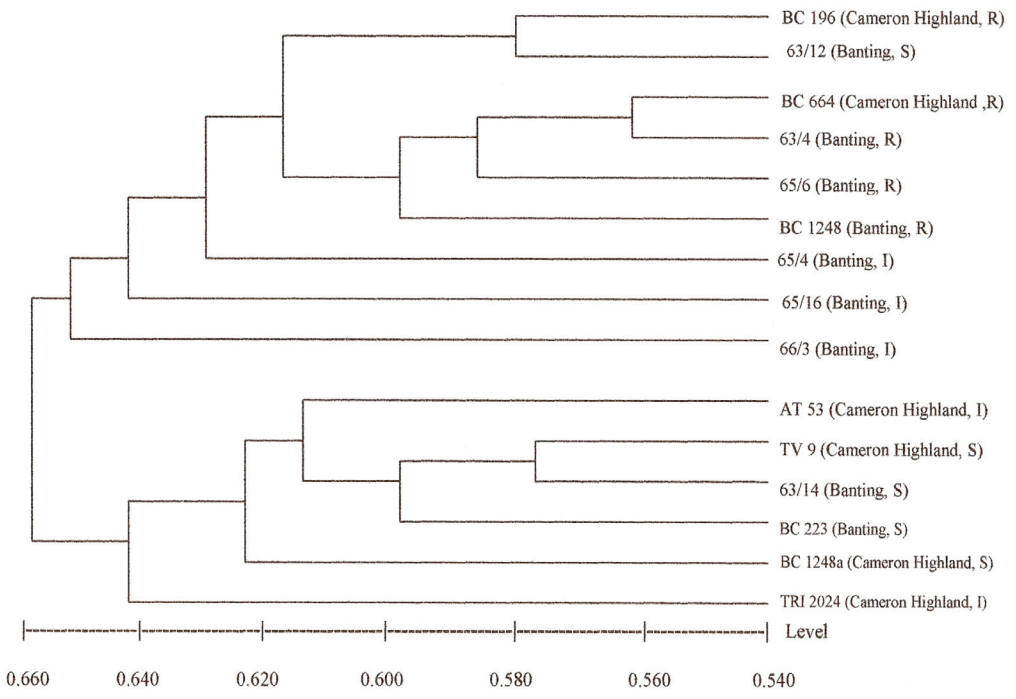

Fig. 2: Dendrogram showing the relationships between the varieties of C. sinensis *derived from UPGMA cluster analysis using distances derived from the Nei and Li (1979) similarity coefficients based on RAMs markers *R=Resistant, I=Intermediate, S=Susceptible*

grouped according to their insect resistance types (i.e. resistant, intermediate, and susceptible varieties of tea), as this was mainly based on their geographical populations (*Fig. 2*). The first cluster consisted of the tea varieties from the Banting population, except for BC 196 (resistant) and BC 664 (resistant) which were from the Cameron Highlands population. The tea varieties found in this cluster were all of the resistant type, except for variety 63/12, which was a susceptible variety and varieties 66/3, 65/4 and 65/16, which were intermediate varieties. The second cluster consisted of mainly the varieties from the Cameron Highlands population, except for BC 223 and 63/14 which were from Banting. The tea varieties in this cluster were mainly of the susceptible type, except for AT 53 and TRI 2024, which were included in the intermediate varieties. The resistant, susceptible, and intermediate varieties were clustered within their own sub-clusters in each of the two major clusters.

DISCUSSION

The dendrogram showed that the differences were mainly based on the populations' geographical distributions and partially based on their resistance towards insect (*H. theivora*) attack. The cluster analysis had mainly grouped the fifteen varieties based on the areas from which the samples were collected (except for BC 196 and BC 664 which were from the Cameron Highlands population that were clustered in the first cluster, and grouped most of the Banting varieties together), while BC 223 and 63/14 (from the Banting population) were clustered in the second cluster that grouped most of the Cameron Highlands varieties together. A possible reason for this could be that some of

the varieties at Cameron Highlands were brought from Banting to be planted there and vice-versa, since both plantations are owned by the same company.

If the genetic differences were fully based on the tea varieties' resistance towards *H. theivora* attack, the dendrogram would have shown three main clusters. The first cluster would consist of the varieties that showed resistance, the second cluster would consist of the intermediate strains, and the third cluster would consist of varieties that showed susceptibility. The reason why they were not clustered could be that the resistance levels of these strains were also dependant on the environment and the developmental stages of the plants (Kaundun and Park, 2002). The visual insect resistance scorings (data not shown), which were done in the present study, might not be as accurate as compared to the DNA markers, especially for the intermediate varieties. The results gathered in this study on the same tea varieties, but based on 153 dominant RAPD markers (data not shown) produced by the ten primers from Kit A of Operon Technology Inc., USA also gave similar clustering patterns as those based on the RAMs markers presented here. This finding suggests that both DNA marker methods could potentially be used for the determination of genetic variations among the tea varieties.

CONCLUSIONS

The results from the RAMs markers complemented the observation that feeding damage on tea leaves could be used to indicate the resistance levels of the plants to insect attacks. The varieties which were of the resistant, susceptible and intermediate types tended to be clustered within their own sub-clusters in each of the two major clusters. In this study, the RAMs markers were found to be capable of detecting high levels of polymorphisms in the various tea varieties that had enabled the researchers to determine their genetic diversity. Further studies could be done using codominant genetic markers such as single locus DNA microsatellites markers and allozymes to obtain a better understanding of their genetic relationships. This will help in preserving and characterizing the existing tea varieties for future breeding and crop improvement programmes that constitute the fundamental support structure for the Malaysian tea industry.

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Maturing Pattern for Body Weight, Body Length and Height at Withers of Jamnapari and Boer Goats

Ariff, O.M.[1*], R.M. Hifzan[2], A.B.M. Zuki[1], A.J. Jiken[3] and S.M. Lehan[4]
[1]Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[2]Strategic Livestock Centre MARDI, Kluang, Malaysia
[3]Strategic Livestock Centre MARDI, Serdang, Malaysia
[4]GK Farm Air Hitam, Johor, Malaysia
[*]E:mail: ariff@vet.upm.edu.my

ABSTRACT

The objective of this study was to examine the growth pattern of three size measurements, namely body weight, body length, and height at withers of Jamnapari and Boer goats reared under common semi-intensive environment in Johor, Malaysia. Cross-sectional data of the above measurements of 234 Jamnapari and 312 Boer female goats (age ranged from 6 to 54 months) were used to fit Gompertz and von Bertalanffy growth curve models for the estimation of mature size, constant of integration, and maturing rate for the three size measurements. Jamnapari and Boer female goats were found to be significantly different ($p < 0.05$) in term of their mature weight (52.19 and 58.23 kg, respectively, for the Gompertz model and 53.89 and 59.31 kg, respectively, for the von Bertalanffy model). The estimates of height at maturity were significantly larger ($p < 0.05$) for the Jamnapari than Boer females. Although the maturing rate for body weight of the Jamnapari and Boer goats was not different, but their rates of maturing for body length and height at withers were found to be significantly different ($p < 0.05$). The correlation coefficients between the mature size and rate of maturing for their body weight, body length, and height at withers were negative, implying that goats of larger size measurements tended to have a slower growth rate in relation to their mature size.

Keywords: Goats, Boer, Jamnapari, body weight, growth models, mature size

INTRODUCTION

The goat population in Malaysia comprises mainly of the Kajang breed and Kajang crossbreds. In an attempt to improve the productivity of the local goats, many goat breeds have been introduced into the country by the government and private enterprises for the purpose of upgrading the indigenous Kajang goats. Two major goat breeds (namely, Jamnapari goats from Java, Indonesia and Boer goats from South Africa and Australia) were imported in substantial numbers in the past several years. These two goat breeds have been observed to acclimatize well to the Malaysian environment which has the temperature range of 26 – 32°C, relative humidity of 80 – 90%, and an average total annual rainfall of 2500 mm. Many of these goats are reared semi-intensively in raised floor-houses and allowed to graze on native and cultivated pastures from late morning until early afternoon, and are fed with supplementary concentrate feed during the rest of the day.

Jamnapari is a dual purpose milk- and meat-type goat breed found mainly in the Etawah district of Uttar Pradesh in India (Acharya, 1982). The breed is described as predominantly

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[*]Corresponding Author

white in hair coat colour with a thick hair growth on its back, a large body, short and flat horns, and long and pendulous ears. Boer goats have their origin in South Africa. They are known for excellent growth, fertility, and meat quality (Malan, 2000). It is a breed that has been able to adapt to a wide range of climatic and feeding environments and is highly fertile with a kidding rate exceeding 189%.

Information regarding the growth pattern of different breeds of livestock is useful in developing a genetic improvement programme to produce the most efficient biological type for a particular feeding environment in a specific market situation (Stobart *et al*., 1986). Altering the growth pattern (e.g. by reducing mature weight) provides an opportunity to breed females of the parental population which costs less to maintain (Cartwright, 1979). This will markedly reduce maintenance cost of does, and hence contribute to a reduction in the total cost of kid production. Thus, the characterization for major traits related to the growth of available goat breeds will lead to a more efficient utilization of these important genetic resources, as has been shown in cattle by Brown *et al*. (1976).

The objective of this study was to examine the growth pattern of three size measurements, namely body weight, body length and height at withers of two breeds of goats, i.e. Jamnapari and Boer, which are semi-intensively reared at two farms in the Johor, Malaysia. In this study, growth pattern was described by two parameters, namely A (an asymptotic measure of size) and k (the rate of maturing), which were obtained from two growth curve models, Gompertz and von Bertalanffy (Brown *et al*., 1976).

MATERIALS AND METHODS

Data Source and Animal Management

The cross-sectional data for the body weight, body length, and height at withers belonging to Jamnapari and Boer female goats were used in this study. The data of the above parameters were taken from 234 Jamnapari and 312 Boer female goats, distributed over 5 age groups, as shown in Table 1. The age of goats with birth records was determined as the difference in months between the date of birth and the date of measurement. Those goats with no records of birth date had their age estimated by the number of permanent incisors present (<12 months of age = presence of 4 pairs of milk teeth, 13-18 months of age = 1 pair of permanent incisors, 19-24 months of age = 2 pairs of permanent incisors, 25-36 months of age = 3 pairs of permanent incisors, 37-48 months of age = 4 pairs of permanent incisors and >49 months of age = teeth spreading apart).

The Jamnapari goats were located at Ladang GK Air Hitam, Johor, Malaysia. They comprised of young and primiparous does from an original importation of 400 breeding females from Java, Indonesia in November 2007 and kids born in 2008. The imported goats were

TABLE 1
The number of female goats of the Jamnapari and Boer breeds by age groups

Breed	Age group (months)					
	6-12	13-24	25-36	37-48	≥49	Total
Jamnapari	64	79	38	18	37	234
Boer	31	12	247	16	6	312

of purebred Jamnapari and high graded or crossbred Jamnapari stock, possessing common features of predominantly white hair coat, roman nose, thick growth of hair on the posterior end of the body, and long pendulous ears. Mating was carried out throughout the year in separate mating groups, each with a buck assigned to about 20 does. Every day, from around 10:00 a.m. to 1:00 p.m., the goats, except does with pre-weaned kids, were allowed to graze native pasture comprising mainly of grasses, *Axonopus compresses, Paspalum conjugatum* and *Imperata cylindrica*, and broad-leafed plants, *Asystasia intrusa*. For the remaining period of the day, the goats were confined in-door on slatted floors in raised houses. While in-door, the goats were fed with concentrate feed at a rate of 300 g/doe/day (14.9% crude protein and 8.9 MJ/kg ME). The ingredients such as palm kernel cake, rice bran, maize, soy bean meal, palm oil mill effluent, and oil palm frond were used in the goat concentrate, which was provided together with green chopped Napier grass (*Pennisetium purperum*) fed *ad libitum*. Water was supplied through piped-water nipples at all times of the day.

The Boer goats used in this study were imported from three registered farms in Australia in April 2007. They were kept in raised houses on slatted floors at MARDI Research Station, in Kluang, Johor. The animal management of the Boer goats was similar to that of the Jamnapari goats mentioned above, except the grazing pasture was entirely of the cultivated Guinea grass (*Panicum maximum)*.

Parameters

In this study, three parameters of size measurement (body weight, body length, and height at withers) were taken on the female goats sampled in the two farms on two different periods. Data for the Jamnapari goats were collected from 22 to 24 December 2008 and this was done from 12 to 14 October 2008 for the Boer goats. Body weight without overnight fasting was obtained using an electronic weighing scale for all the goats. Meanwhile, measurement of the body length was done as the horizontal distance from the point of the withers to the ischium or pin bone, and height at withers was measured as the vertical distance from the top of the withers to the ground. Using a measuring tape, the measurements of the body length and height at withers were recorded in centimetres. The animals were in the average body condition of 3 (i.e. the 5-point body condition scores of 1= being emaciated, 3= being in moderate fat cover, and 5= being in excess fat cover) when their weight and body measurements were taken.

Growth Functions

Two growth curve models, namely Gompertz and von Bertalanffy (Brown *et al*., 1976), were chosen to be fitted to individual records for the body weight, body length, and height at withers of the Jamnapari and Boer goat datasets. The SAS package PROC NLIN (SAS, 1998) was utilized to estimate the growth parameters of the two growth curve models below:

$$\text{Gompertz model: } Wt = Ae^{-Be^{-kt}}$$

$$\text{von Bertalanffy model: } Wt = A(1-Be^{-kt})^3$$

where Wt is the observed measurement of size (body weight, body length, and height at withers) at age t in months, A is the asymptote for measure of size, B is a constant of integration and k is the rate of maturing per day. The parameter A is the asymptotic limit for the size measurement and it is not an estimate of the largest size measurement reached by an animal. The constant of integration, B, has no biological interpretation, and the rate of maturing, k, is the growth rate after birth, which is relative to the mature measure of size.

Degree of maturity (U) for the body weight was calculated as a ratio of weight at age t in months divided by the asymptotic weight A obtained from the growth curve models (Fitzhugh and Taylor, 1971). The parameter U was calculated based on the parameter A for each breed.

An analysis of variance using PROC GLM (SAS, 1998) was carried out to determine the difference between the breeds for the three

parameters of growth curves obtained for the Jamnapari and Boer goats. Similarly, PROC GLM (SAS, 1998) was conducted for the degrees of maturity for body weight (UW), body length (UL), and height at withers (UH) as dependent variables, whereas breed and age group as the independent variables.

RESULTS AND DISCUSSION

Mature Size and Rate of Maturing

The asymptotic values for the body weight, as represented by the parameter A of the Gompertz and von Bertalanffy growth curve models, were significantly higher for Boer compared to Jamnapari does (Table 2). The estimates for the mature weight from the von Bertalanffy model were higher than those of the Gompertz model, but not for the maturing rate in both the Jamnapari and Boer goats. Boer does were 10.8% heavier at maturity than Jamnapari does based on the estimates of the mature weight from the Gompertz and von Bertalanffy models. However, the rates of maturing of both breeds of goats were not different, although the heavier Boer goats were associated with a slightly lower rate of maturing in both the Gompertz and von Bertalanffy models. Tsukahara *et al*. (2008) reported a lower mature weight of Katjang and crosses of Katjang-German Fawn, which ranged from 28.8 to 33.3 kg (estimated using the Bertalanffy model) and from 28.1 to 32.9 kg (estimated using the Gompertz model). The estimates for the maturing rate for the body weight in this study were generally lower in the Bertalanffy model as compared to the Gompertz model, as was also found by Tsukahara *et al*. (2008).

The correlation coefficient between the mature weight and maturing rate was large and negative, i.e. -0.96 and -0.84 for the Jamnapari and Boer goats, respectively (for the Gompertz model) and -0.97 and -0.87 for the Jamnapari and Boer goats, respectively (for the von Bertalanffy model), as shown in Table 3. The correlation coefficients were higher ($p > 0.05$) in Jamnapari compared to Boer breeds for both the growth curve models.

The body length at maturity derived from the Gompertz and von Bertalanffy models did not differ significantly between the Jamnapari and Boer does (Table 2). In particular, Boer does had slightly longer body length at maturity as compared to Jamnapari does. The rate of maturing for the Boer does' body length was significantly higher ($p < 0.05$) than that of Jamnapari goats. The correlation between the mature size for the body length was large and negative in both the Jamnapari and Boer does (Table 3) – a similar finding as that for the body weight.

The estimates of height at withers at maturity, derived from the Gompertz and von Bertalanffy models, were larger ($p < 0.05$) for the Jamnapari than Boer does (Table 2). For the height at withers, the Jamnapari does were 8.8% taller at maturity than the Boer goats. Unlike the rate of maturing for the body length, the Jamnapari does were shown to mature much faster ($p < 0.05$) for height at withers than the Boer goats. Similarly, the correlation coefficients between the Jamnapari and Boer goats were high and negative, thus, a strong negative correlation was found between the maturity size and rate of maturing for height at withers in both the Jamnapari and Boer females. The correlation between the mature size and rate of maturing for height at withers was higher ($p > 0.05$) in Jamnapari than Boer does.

Degree of Maturity

As for the degree of maturity for the body weight, no significant difference was detected between the Jamnapari and Boer goats during the phase of growth for ages prior to 12 months to maturity above 49 months (Table 4). Jamnapari and Boer goats attained a similar degree of maturity at the same phases of growth. However, the degree of maturity for body length showed a significant difference ($p < 0.05$) between the Jamnapari

TABLE 2
Least square means for the growth curve parameters[1] and coefficient of determination (R^2) for the Gompertz and von Bertalanffy models fitted to body weight, length of body and height at withers for the Jamnapari and Boer female goats

	Breed	
	Jamnapari	Boer
Number	213	234
Body weight (kg)		
Gompertz:		
A[1]	$52.19^{a}\pm3.66$	$58.23^{b}\pm3.08$
B[1]	1.451 ± 0.080	1.434 ± 0.126
k[1]	0.045 ± 0.007	0.043 ± 0.007
R^2	0.97	0.96
von Bertalanffy		
A	$53.89^{a}\pm4.44$	$59.31^{b}\pm3.44$
B	0.397 ± 0.028	0.425 ± 0.036
K	0.036 ± 0.007	0.030 ± 0.006
R^2	0.97	0.96
Length of body (cm)		
Gompertz:		
A	83.63 ± 4.14	85.20 ± 1.73
B	0.557 ± 0.403	0.544 ± 0.044
K	$0.035^{a}\pm0.008$	$0.045^{b}\pm0.006$
R^2	0.99	0.99
von Bertalanffy		
A	84.28 ± 4.51	85.39 ± 1.79
B	0.172 ± 0.012	0.169 ± 0.013
K	$0.033^{a}\pm0.008$	$0.043^{b}\pm0.006$
R^2	0.99	0.99
Height at withers (cm)		
Gompertz:		
A	$74.66^{a}\pm0.90$	$68.12^{b}\pm1.19$
B	0.497 ± 0.027	0.351 ± 0.035
K	$0.078^{a}\pm0.012$	$0.045^{b}\pm0.008$
R^2	0.99	0.99
von Bertalanffy		
A	$74.72^{a}\pm0.92$	$68.17^{b}\pm1.21$
B	0.155 ± 0.008	0.112 ± 0.011
K	$0.075^{a}\pm0.012$	$0.044^{b}\pm0.008$
R^2	0.99	0.99

Means with different superscripts in the same row differ significantly at $p<0.05$
[1]A, asymptotic measure of size (mature size); B, constant of integration; k, rate of maturing

TABLE 3
The correlation coefficients between the mature size and rate of maturing derived from the Gompertz and von Bertalanffy growth models for the body weight, body length and height at withers in the female Jamnapari and Boer goats

Parameter	Gompertz		von Bertalanffy	
	Jamnapari	Boer	Jamnapari	Boer
Body weight	-0.9599	-0.8405	-0.9726	-0.8701
Body length	-0.9784	-0.8490	-0.9819	-0.8609
Height at withers	-0.8830	-0.8557	-0.8899	-0.8632

and Boer goats throughout all the phases of growth, except from 37 to 48 months of age for both the Gompertz and von Bertalanffy models. Meanwhile, the degree of maturity for height at withers, the difference between Jamnapari and Boer goats was only significant for the pre-yearling period. The results derived from the analysis of variance for UW, UL, and UH revealed that the effect of breed was significant ($p< 0.05$) for UW and UL and breed x age group interaction effect was significant ($p < 0.05$) for UW and UH (result not shown).

The estimates for the mature weight for the Jamnapari and Boer goats of 52.19 and 58.23 kg, respectively (for the Gompertz model) and 53.89 and 59.31 kg, respectively (for the von Bertalanffy model), were higher than those reported for the Katjang and Katjang-German Fawn crossbred goats (Tsukahara *et al*., 2008). Similarly, the estimates for the mature weight derived from the von Bertalanffy model were higher than those obtained from the Gompertz model.

Beside body weight, body length, and height at withers are the other two size measurements of interest in this study. The estimates of parameter A for the body weight, body length, and height at withers indicated that the Boer goats were longer and heavier but shorter in height at maturity than the Jamnapari goats. Animals with longer bodies were associated with heavier weight as was also found in cattle (Brown *et al*., 1974). In an earlier study by Brown *et al*. (1973), Hereford bulls were reported to have shapes of varying descriptive categories, such as large framed and heavy, wide at shoulders, loins and hips but with shorter body and height, deep in flank, wide hips and tall, but narrow loin and shoulders and long body, short in stature, and narrow at the loin. The Boer goats could be categorized as having long body and are heavy, while the Jamnapari goats are tall but with shorter body. Genes, which are simultaneously responsible for increasing body length and decreasing height at withers, could also be postulated to have positive effect on body weight.

This study revealed that a negative correlation was observed between the mature weight and rate of maturing in the two breeds (Jamnapari and Boer goats), which ranged from -0.84 (the Gompertz model) for the Boer goats to -0.97 (the von Bertalanffy model) for the Jamnapari goats. The Jamnapari goats with a lower mature weight showed a faster rate of maturing whereas the Boer goats had a higher mature weight and a slower maturing rate. This finding is in agreement with the negative relationship found between the mature weight and the rate of maturing reported by Bathaei and Leroy (1998) in sheep and DeNise and Brinks (1985) in cattle. Similarly, Tsukahara *et al*. (2008) also showed negative correlation coefficients between the parameters A and k, which ranged from -0.13 to -0.81 (von Bertalanffy) and -0.07 to -0.81 (Gompertz) for the Katjang and Katjang crossbred goats. Fitzhugh (1976) postulated that cows with higher mature weight would take a longer time

TABLE 4
Least square means and standard errors for the degree of maturity for the body weight (UW) and length of body, (UL) and height at withers (UH) by age groups for the female Jamnapari and Boer goats using the parameters derived from the Gompertz and von Bertalanffy models

Age	UW		UL		UH	
	Jamnapari	Boer	Jamnapari	Boer	Jamnapari	Boer
Gompertz model						
6-12 mo	23.70 ± 1.84	31.77± 5.31	57.35[a]± 0.96	65.34[b]± 2.76	65.74[a]± 0.88	72.42[b]± 2.61
13-24 mo	50.40± 3.07	47.76± 3.25	73.07[a]± 1.60	79.89[b]± 1.69	84.53± 1.47	87.99± 1.55
25-36 mo	63.28± 2.11	59.49± 0.83	79.14[a]± 1.10	84.41[b]± 0.43	91.86± 1.01	89.46± 0.39
37-48 mo	78.31± 1.40	71.27± 3.76	86.89± 0.73	89.98± 1.95	98.07± 0.68	94.56± 1.80
≥49 mo	84.41± 1.75	83.73± 2.08	90.57[a]± 0.92	95.04[b]± 1.08	98.95± 0.84	97.42± 0.99
Von Bertalanffy model						
6-12 mo	22.95 ± 1.80	31.19± 5.19	56.91[a]± 0.95	65.18[b]± 2.75	65.69[a]± 0.88	72.37[b]± 2.53
13-24 mo	48.81± 2.99	46.89± 3.18	72.51[a]± 1.59	79.70[b]± 1.68	84.46± 1.46	87.92± 1.55
25-36 mo	61.28± 2.06	58.40± 0.81	78.53[a]± 1.09	84.21[b]± 0.43	91.78± 1.01	89.39± 0.39
37-48 mo	75.84± 1.37	69.97± 3.67	86.22± 0.73	89.77± 1.94	97.99± 0.67	94.49± 1.80
≥49 mo	81.75± 1.71	82.21± 2.04	89.87[a]± 0.91	94.82[b]± 1.08	98.87± 0.84	97.34± 0.99

[a, b] Means with different superscripts in the same row between two breeds for UW, UL and UH of each age groups are different at $p < 0.05$

to mature, and are therefore older when reaching a constant degree of maturity as compared to cows with lower mature weight, as increase in the mature size is associated with longer time taken to mature.

Body weight at a certain age is an economically important trait because of its value in the sale of slaughter animals. Market price of slaughter animals is related to age, which is often quoted based on a per unit weight. Animals differ in age when a targeted market weight is reached, as indicated by the differences in the degree of maturity at specific age; the same finding was also reported by Fitzhugh and Taylor (1971). Variation in age at a constant degree of maturity exists between and within breeds, thus emphasizing the potential of selecting animals within a genetic group based on the rate of maturing or mature weight.

CONCLUSIONS

As shown by the measurements of the body weight, body length, and height at withers in this study, breeds of goats differ in their mature size. In particular, Boer goats with heavier mature weight are associated with a slower rate of growth relative to their mature weight and they have been shown to reach a constant degree of maturity much later as compared to the Jamnapari goats. Breeds of goats that originated from the Asiatic Tropics would have evolved as a faster maturing genotype to maintain a relatively smaller mature weight in adjusting to the less than optimum feeding environment of the local habitat, as shown by the Jamnapari breed undertaken in this study.

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Temporal Changes in Chemical Properties of Acid Soil Profiles Treated with Magnesium Limestone and Gypsum

Shamshuddin, J.[1*], Sharifuddin, H.A.H.[1], Che Fauziah, I.[1], Edwards, D.G.[2] and Bell, L.C.[2]

[1]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

[2]School of Land and Food Sciences, University of Queensland, Qld 4072, Australia

[*]E-mail: samsudin@agri.upm.edu.my

ABSTRACT

Effects of ground magnesium limestone (GML) and gypsum on the properties of Ultisols at two sites, involving a corn groundnut rotation, were studied over 24 months. GML or gypsum was incorporated into the soils of the Bungor and Rengam Series (Typic Paleudults), at the rates of 0, 0.5, 1.0, 2.0, 4.0, and 8.0 t ha^{-1} to depths of 15 cm (GML and gypsum) and 30 cm (GML). Increases in soil pH and exchangeable Ca and Mg arising from GML application were confined mainly to the zone of incorporation. However, there was a decrease of exchangeable Al at deeper depths. After 15 months, there was a tendency for the pH and exchangeable Ca and Mg on the zone of incorporation to decrease and for the exchangeable Al to increase, effects being the least at the high rates of GML application. There was no significant change in pH or exchangeable Al as a result of gypsum application, but there were increases in exchangeable Ca and extractable SO_4^{2-} in both the zone of incorporation and the subsoil. With time, the exchangeable Ca and extractable SO_4^{2-} in the sub-soils were found to increase, but their concentration in the zone of incorporation decreased. The pH of the soil solution of the control treatment was about 4.0, while Al and Mn concentrations were 150 and 50 μM, respectively. At the GML rate of 2.0 t ha^{-1} or less, Al in the soil solution was found to exist in the inorganic monomeric form. Meanwhile, the GML application at the rates > 4 t ha^{-1} could have resulted in complexation of some of Al. In particular, Al^{3+} was the dominant Al species at low pH and liming resulted in a decrease of Al^{3+} species and increase of hydroxyl-Al monomers. GML needed to raise the pH of the soil solution to about 5 was 2.0 t ha^{-1}; this consequently decreased Al and Mn concentrations to a low level. The application of gypsum resulted in a decrease and an increase of Al^{3+} and $AlSO_4^+$ activities, respectively.

Keywords: Acid soil, ground magnesium limestone, gypsum, Ultisol

INTRODUCTION

Acid soils (Ultisols and Oxisols) are widespread in Southeast Asia, occupying about 72% of Malaysia, 82% of Thailand, and 43% of Indonesia (IBSRAM, 1985). By far, the most dominant highly weathered soils in Malaysia are the Ultisols. In this country, Ultisols and Oxisols are characterized by high acidity, low effective cation exchange capacity (ECEC), and high aluminium saturation throughout the soil profiles (Tessens and Shamshuddin, 1983).

Foster *et al*. (1980) reported that many of these soils are sufficiently acid that good crops of maize and groundnut could be produced only after application of lime. Liming trials on typical Ultisols (Shamshuddin *et al*., 1991; Ismail *et al*., 1993; Sharifuddin *et al*., 1995) and Oxisols (Shamshuddin *et al*., 1992) in Malaysia indicate the need of liming for annual crop production.

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[*]Corresponding Author

Preliminary information indicates that crop production is not good on Ultisols; this limitation may be due to aluminium and manganese toxicities, as well as calcium and magnesium deficiencies in addition to other factors such as deficiencies of phosphorus and micronutrients and water stress arising from subsoil acidity. Therefore, it is important to develop measures to ameliorate the soil acidity to enable sustainable food crop production due to relatively large area of Ultisols in Malaysia and the high potential for food crop production.

Determining the reasons for the amelioration of acid soil infertility by such materials as limestone and gypsum is commonly undertaken by assessment of changes in the solid phase properties of soils following amendment. Additionally, there is the opportunity to understand the process of amelioration through the study of the solution phase properties of soil, particularly in relation to the phytotoxicity of Al, and the availability of Ca and Mg.

Inorganic Al is phytotoxic to roots, and the intensity of toxicity is highly correlated with Al^{3+} activity in the soil solution (Alva *et al*., 1986a). On the other hand, it is believed that organically-bound Al is non-toxic (Hue *et al*., 1986). Adams and Hathcock (1984) could not explain observed Al toxicity in the presence of Al species in soil solution, and this was probably because the method used for determination did not discriminate between inorganic monomeric and polymeric Al. Similarly, Wright *et al*. (1987) were unable to estimate phytotoxic Al in the soil solution from the surface using the spectrophotometric method. Kerven *et al*. (1989), however, were able to separate inorganic monomeric from organically-complexed Al using a short-term pyrocatechol violet method.

The field experiments were conducted in Malaysia on two Ultisols to assess the effects of GML and gypsum applications on soil properties and growth of three crops of sweet corn and groundnut grown in rotation. The specific objectives of this study were: (1) to investigate the chemical changes in the solid and solution phase properties of the soils following GML and gypsum application; and (2) to determine the longevity of liming effects.

MATERIALS AND METHODS

Site Selection and Characterization

Puchong and Chembong were the sites in Peninsular Malaysia selected for the field trials. The soil at Puchong was of the Bungor Series (loamy, siliceous, isohyperthermic, family of Typic Paleudult), whereas the soil at Chembong was of the Rengam Series (clayey, kaolinitic, family of Typic Paleudult). Relevant physico-chemical data of the soil profiles at the two experimental sites are given in Table 1.

The annual rainfall in the vicinity of Puchong is 2369 mm, while evaporation is 1735 mm. At Chembong, the annual rainfall and evaporation are 2314 and 1718 mm, respectively. Monthly evaporation varies little throughout the year at both sites. Such a climate results in the soils at Puchong and Chembong being exposed to strongly leaching environments, especially during the wet months of March, April, May, and October. However, water deficit is experienced in June and July.

Experimental Design

At Puchong, three separate experiments using GML or gypsum were carried out, viz. Experiment 1, in which GML was incorporated to 15 cm depth, Experiment 2 in which GML was incorporated to 30 cm depth and Experiment 3 in which gypsum was incorporated to 15 cm depth. The slope at the experimental area was around 1-2%. The same set of experiments was conducted at Chembong, where the area is flat with a slope of <1%.

The limestone selected for the field experiment was the most common source available in Malaysia and is locally known as ground magnesium limestone (GML) because of its high magnesium content (Table 2). X-ray diffraction analysis (data not shown) showed

TABLE 1
Selected physical and chemical properties of the soils at the Puchong (Bungor Series) and Chembong sites (Rengam series)

Series	Horizon	Depth (cm)	pH (1:1)		Exchangeable cations							Organic C (%)	Clay (%)	Mineral**	
			H_2O	1M KCl	Ca	Mg	Na	K	Al	ECEC	CEC*			Major	Minor
					.....cmol(+)/kg.....										
Bungor	Ap	0-27	4.91	3.79	1.05	0.30	0.02	0.22	4.02	5.61	13.90	1.95	25	Kn, Gib	Gt, Qtz, Mica
	B21t	27-75	4.76	3.88	0.83	0.18	0.02	0.06	3.98	5.07	9.08	0.80	30		
	B22t	75-125	4.92	3.97	0.81	0.16	< 0.01	0.04	3.07	4.08	7.15	0.33	28	Kn, Gib	Gt, Qtz, Mica
	B23t	125-150	5.01	3.98	0.77	0.16	0.02	0.04	3.24	4.23	6.43	0.26	25		
Rengam	Ap	0-20/24	4.83	3.51	1.05	0.17	0.02	0.08	2.68	4.00	8.80	2.13	40	Kn	Gib, Gt, Qtz.
	B1t	20/24-60	4.43	3.49	0.72	0.14	< 0.01	0.05	2.83	3.74	7.98	1.21	45		
	B2t	60-98	4.44	3.54	0.69	0.14	< 0.01	0.04	2.3	3.17	7.22	0.82	44	Kn	Gib, Gt, Qtz.
	BC	98-150	4.44	3.53	0.79	0.15	0.02	0.03	2.45	3.44	5.75	0.41	35		

* 1 M NH_4OAc
** Kn = kaolinite, Gib = gibbsite, Gt = goethite, Qtz = quartz

that this GML was dominated by dolomite with traces of kaolinite, mica, chlorite, quartz, and goethite. Meanwhile, the contents of calcium and other nutrients in the GML and gypsum are given in Table 2.

At each site, the treatments consisted of 0, 0.5, 1.0, 2.0, 4.0, and 8.0 t ha^{-1} GML or gypsum in a Randomized Complete Block Design (RCBD), with four replications. There was also an absolute control where no basal fertilizer was applied. The size of each experimental plot was 6.5 m x 4.5 m. The first crop of sweet corn (*Zea mays*) was planted 1 month after the application of GML or gypsum, and this crop was immediately followed by groundnut (*Arachis hypogaea*). This cropping sequence was continued for six cropping seasons, with a total period of 24 months. Basal fertilizers (Table 3) were applied on the basis of past experience and leaf analysis of each crop (data not shown).

TABLE 2
Nutrient concentrations in ground magnesium limestone and gypsum

Element	GML	Gypsum
Ca (%)	18.5	25.1
Mg (%)	6.7	< 0.01
Cu (mg/kg)	17.6	7.2
Fe (mg/kg)	2819	103
Mn (mg/kg)	97	27
Zn (mg/kg)	29	8
P (mg/kg)	1.73	< 0.01

TABLE 3
Nutrient rates for sweet corn and groundnut

Crop sequence	N*	P**	K***	Ca**	S**
			(kg/ha)		
Corn 1	120	100	150	68	7
Groundnut 1	0	0	0	0	0
Corn 2	100	30	50	20	2
Groundnut 2	22	29	56	20	2
Corn 3	120	100	50	68	7
Groundnut 3	22	29	56	20	2

* As urea
** As triple superphosphate
*** As muriate of potash

Sampling and Soil Analysis

At each experimental site, a soil pit was dug and the profile was described and subsequently classified using the soil taxonomy (Soil Survey Staff, 1999). The soils from the pits were sampled according to genetic horizons and the data obtained were used for site characterization (Table 1).

At harvest, corn and groundnut yields were recorded and leaves were sampled. The soil samples in the experimental plots were taken using an auger at 0-15, 15-30, 30-45, and 45-60 cm depths at 1 month after the application of GML or gypsum and subsequently to each corn or groundnut harvest. Five cores were sampled from each plot and bulked, while the soil was air-dried, ground, and passed through a 2 mm sieve.

The pH of the soils from the soil pits was determined in water (1:1) and in 1 M KCl (1:1) for classification purposes, while the pH of the amended soils was determined in water (1:2.5) and in 0.002 M $CaCl_2$ (1:1) after 1 h of intermittent shaking and 1 day of equilibration. Electrical conductivity (1:5) was determined after 1 h of intermittent shaking and 1 day of equilibration in water. Exchangeable aluminium was extracted by 1 M KCl and determined colorimetrically (Barnhisel and Bertsch, 1982). Organic carbon was estimated using the Walkley and Black method (Nelson and Sommers, 1982). Meanwhile, the cation exchange capacity (CEC) was determined by 1 M NH_4OAc buffered at pH 7 (Chapman, 1965) and by the summation of exchangeable bases and exchangeable aluminium (henceforth referred as ECEC). Charge curves for anions and cations at various pH values were determined using the method of Gillman and Sumner (1987). Sulphate was extracted by 0.008 M $Ca(H_2PO_4)_2$ (Farina and Channon, 1988) and determined using the turbidimetric method proposed by Freney (1986). The basic exchangeable cations were extracted by 1 M NH_4OAc, calcium and magnesium were determined by atomic absorption spectrophotometer, whereas potassium was determined by flame photometer. Exchangeable sodium was not determined in all the samples as its amount was found to be negligible (<0.02 $cmol_c kg^{-1}$), both for the soil pits and for selected amended soils. Texture analysis was carried out using the method of Day (1965). The mineralogical analysis of the clay fraction was determined using the X-ray diffraction analysis.

Soil Solution Extraction and Analysis

Soil samples (topsoil) from Experiment 1 at Puchong at month 1 were rewetted to matrix suction of 10 kPa. After 1 day of incubation, the soil solutions were extracted by centrifuge for 1 h at 2000 rpm. Both the calcium and Mg in the soil solution were determined using the atomic absorption spectrophotometer, while K was determined with the use of flame photometer.

Additional soil samples (topsoil) taken at month 7 were incubated for 1 day at matrix suction for 10 kPa, and the solutions were subsequently extracted by centrifuge for 30 minutes, using fluorocarbon trichlorofluoroethane (Menzies and Bell, 1988). Soil solutions recovered were filtered through 0.22 µm Melipore filters, following which pH and electrical conductivity (EC) were immediately determined on 2 ml sub-samples. Calcium, Al, Mg, Si, Fe, Mn, and S were determined in the remaining solution by inductively coupled plasma atomic emission spectroscopy (ICPAES). Aluminium determined by ICPAES was regarded as the total aluminium (Al_T) in the soil solution.

Inorganic monomeric and organically-complexed Al (at month 7) was separated using the short-term pyrocatechol method proposed by Kerven *et al*. (1989). The activities of the Al species were calculated using the ALMONO computer programme from the University of Queensland, as described by Blamey *et al*. (1983). The soil solution pH, EC, inorganic monomeric Al, Al_T and S were used as inputs from EC.

RESULTS

Physico-Chemical Properties of Untreated Soils

Both the Bungor and Rengam soils were strongly acidic, low in basic cations, but high in exchangeable Al and Al saturation (Table 1), reflecting their occurrence in a strongly leaching environment. The soils were highly weathered, with kaolinite being the dominant mineral in the clay fraction, whereas smaller amounts of gibbsite, goethite, quartz, and mica were also present.

The soils from the experimental sites exhibited pH-dependent charge, reflecting their mineralogy (*Fig. 1*). For both the surface (0-15 cm) and subsoil (30-45 cm) horizons, CEC_T (Al and Ca absorption) was more than twice CEC_B (Ca absorption) at pH values < 3.5. CEC_B increased rapidly as soil pH increased to about pH 5.5 where CEC_T was equalled to CEC_B. A small anion exchange capacity (AEC) developed at pH values below 5.5.

Chemical Properties of Amended Soils

The trends in pH measured in water and $CaCl_2$ were similar, and only the data for $CaCl_2$ are presented. In general, the pH values measured in water were 0.3 unit greater than those measured in $CaCl_2$. Increases in pH resulting from GML addition were primarily confined to the 0-15 cm depth (*Fig. 2*). At this depth and at month 3, addition of 2 t ha^{-1} of GML caused an increase in pH from 4.05 to 4.69, with pH 5.2 being reached at the highest rate of 8 t ha^{-1} in the Bungor soil. With increasing time beyond month 3, the pH values in the surface tended to decline at the 2 t ha^{-1} but remained steady at 4 and 8 t ha^{-1}. Similar trends were also observed for the Rengam soil. In Experiment 2, the pH in the topsoil (0-15 cm depth) of Bungor Series applied with 4 and 8

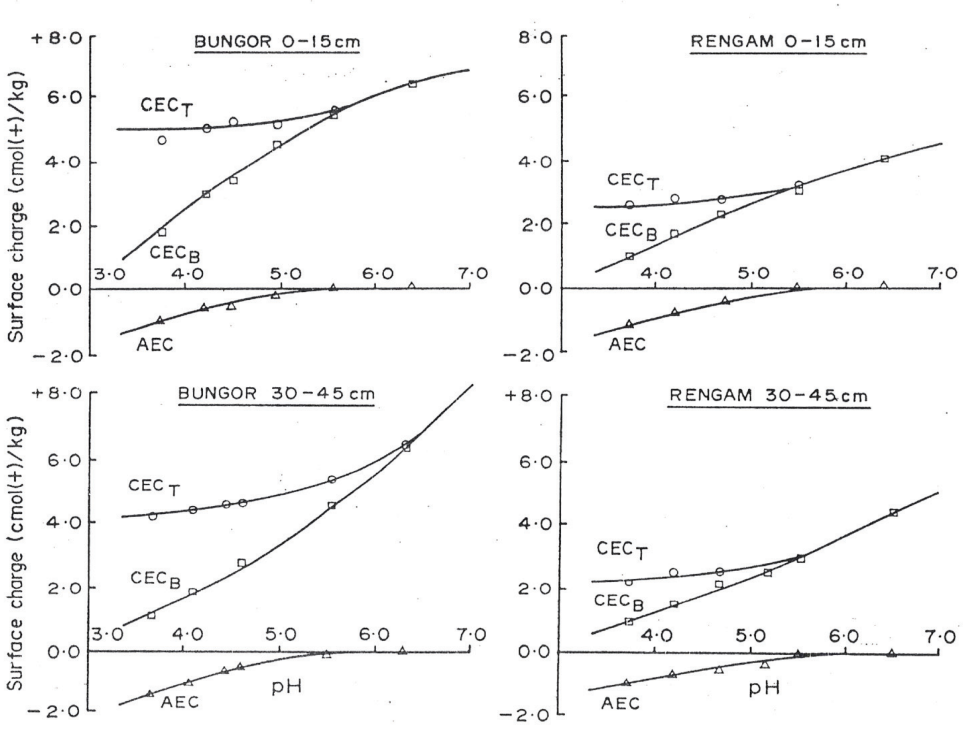

Fig. 1: Changes in CEC and AEC with pH for Bungor and Rengam Series soils

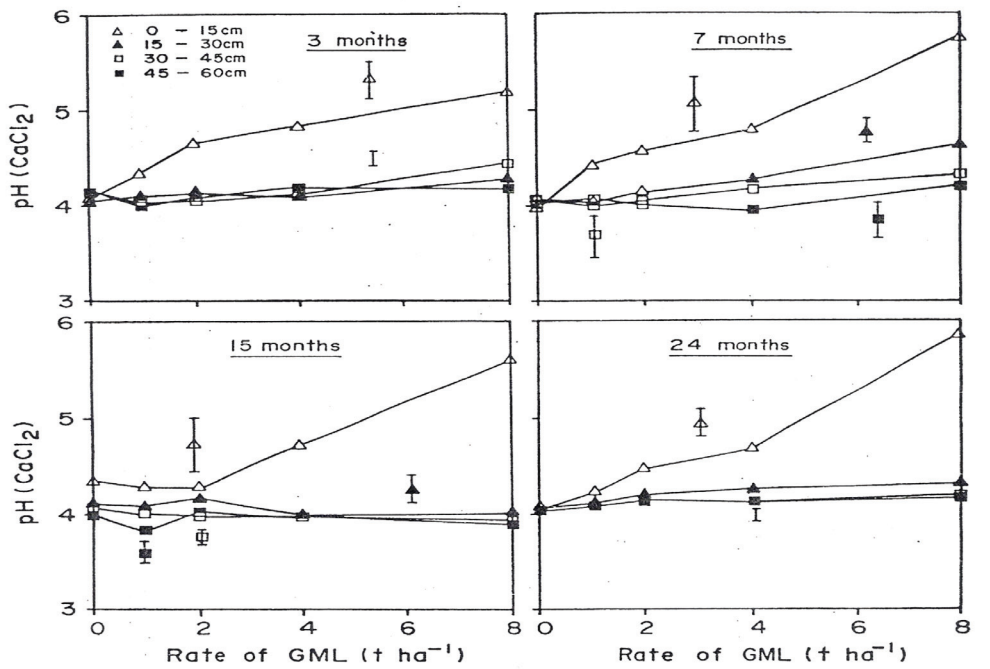

Fig. 2: Soil pH for different horizons of the Bungor soil at months 3, 7, 15 and 24 after application of GML to a depth of 15 cm (Experiment 1). LSD values are for ≤ 0.05

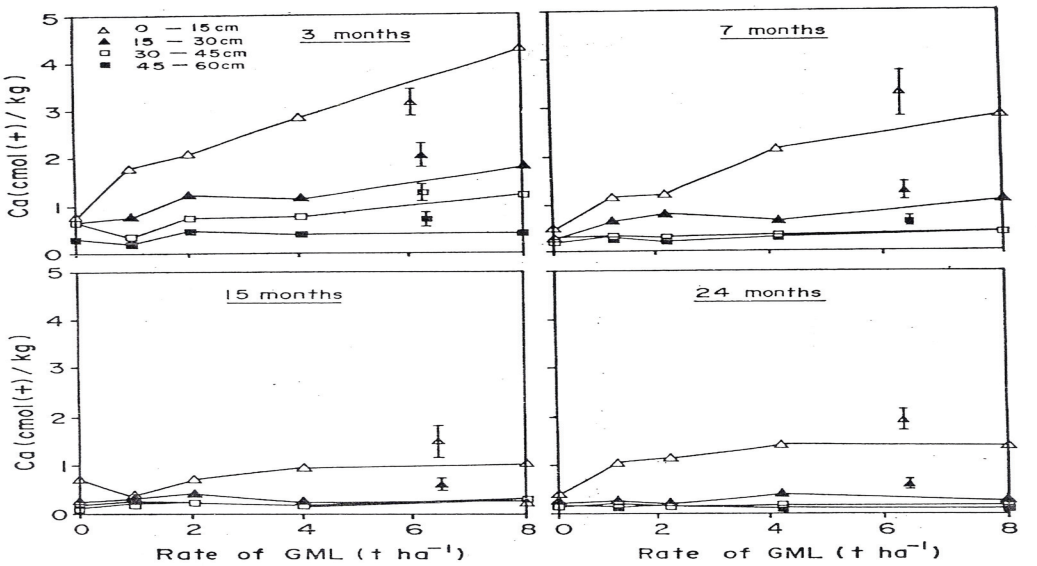

Fig. 3: Exchangeable Ca in different horizons of the Bungor soil at months 3, 7, 15 and 24 after incorporation of GML to a depth of 15 cm (Experiment 1). LSD values are for P ≤ 0.05

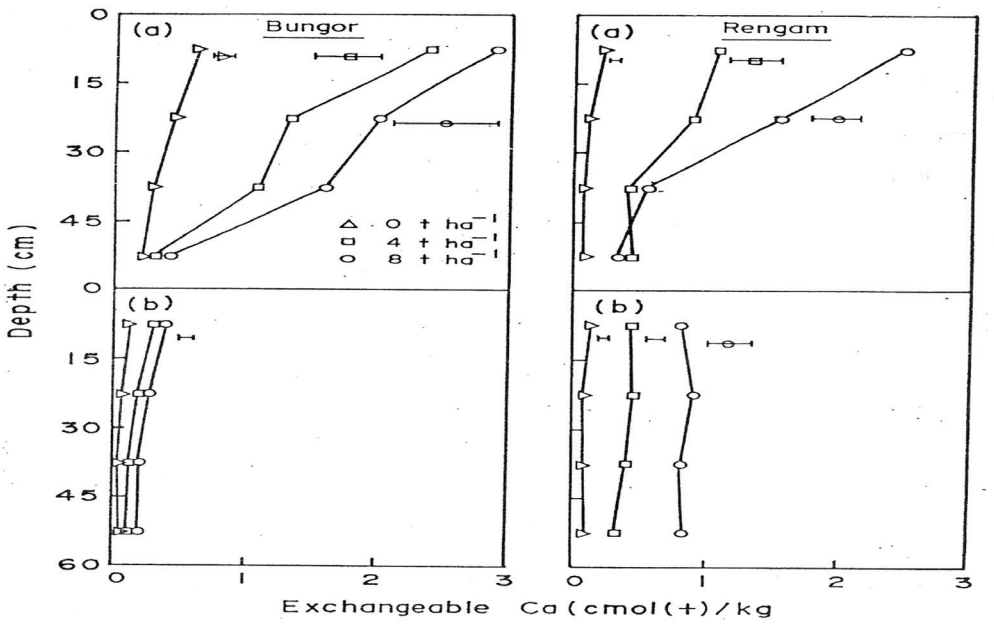

Fig. 4: Exchangeable Ca in different horizons of the Bungor and Rengam soils at (a) 7 months and (b) 24 months after incorporation of gypsum to 15 cm. LSD values are for $P \leq 0.05$

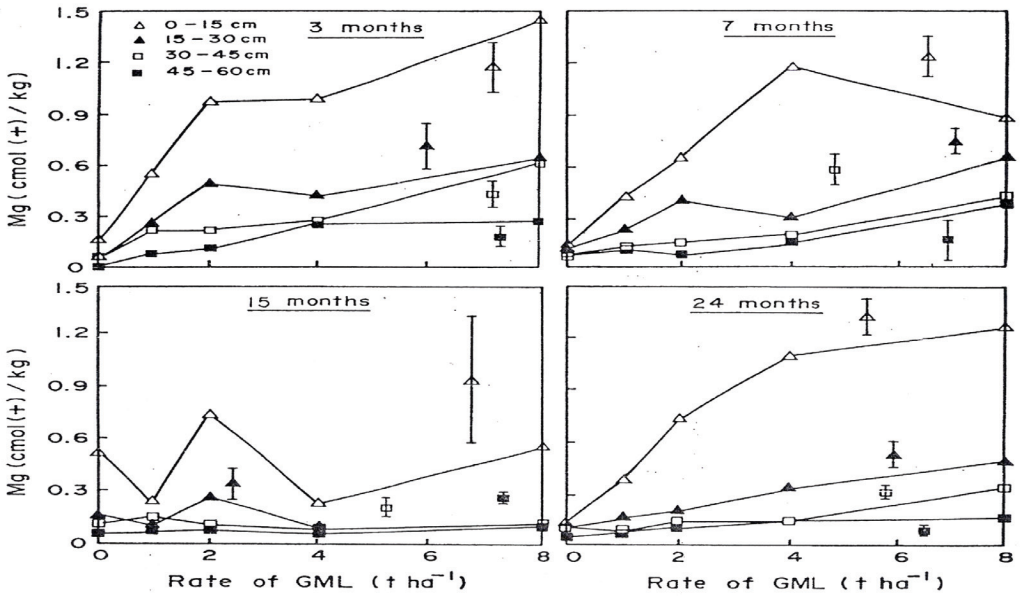

Fig. 5: Exchangeable Mg in different horizons of the Bungor soil 3, 7, 15 and 24 months after incorporation of GML to a depth of 15 cm (Experiment 1). LSD values are for $P \leq 0.05$

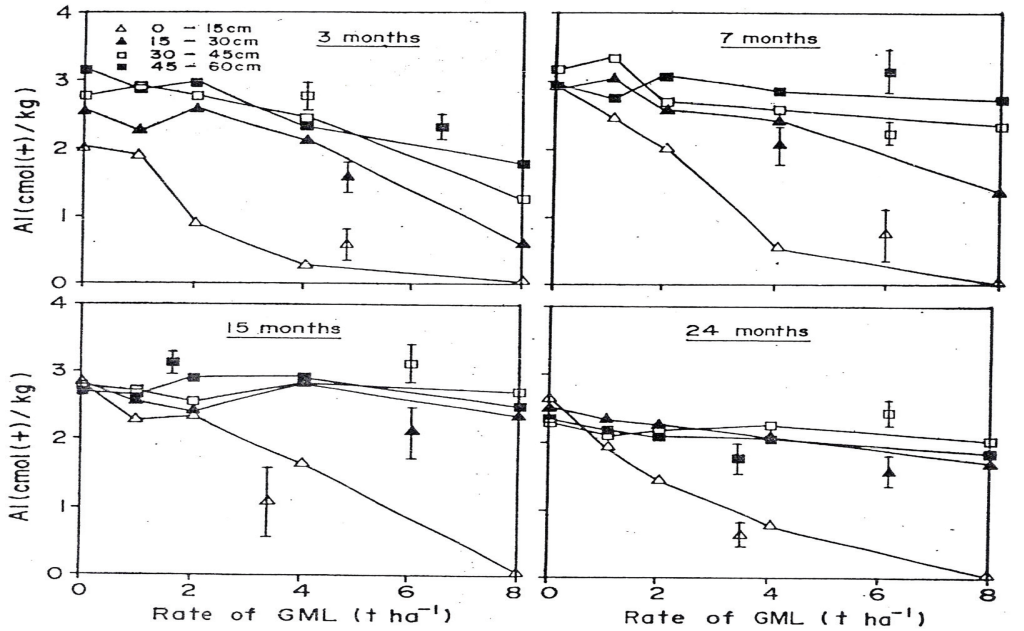

Fig. 6: Exchangeable Al in different horizons of the Bungor soil 3, 7, 15 and 24 months after incorporation of GML to a depth of 15 cm (Experiment 1). LSD values are for $P \leq 0.05$

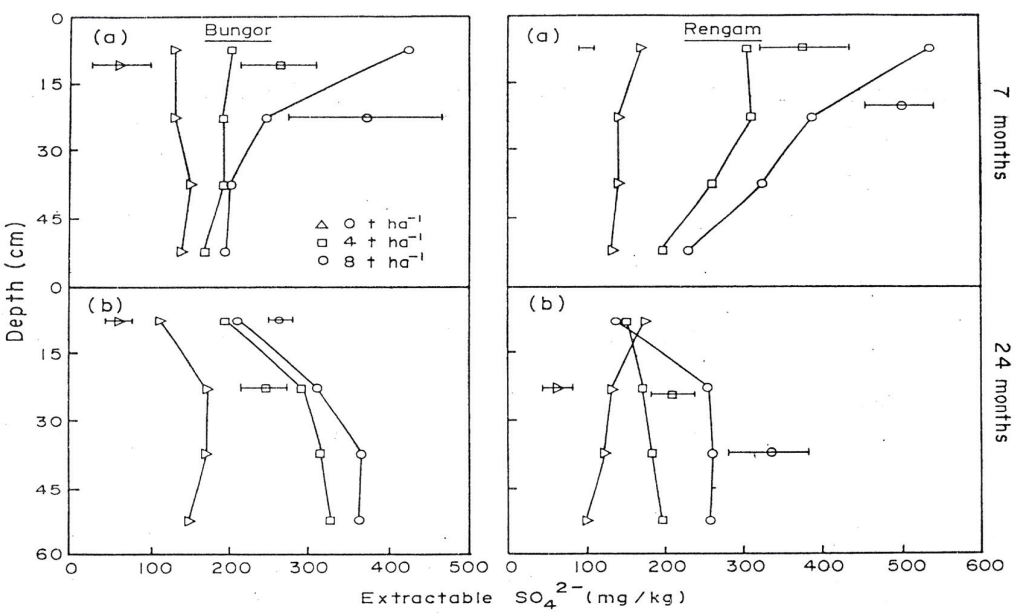

Fig. 7: Extractable SO_4^{2-} in different horizons of the Bungor and Rengam soils at (a) 7 months and (b) 24 months after incorporation of gypsum to 15 cm. LSD values are for $P \leq 0.05$.

t GML ha^{-1} was 5.4 and 6.3, respectively. The corresponding pH in the Rengam soil was 5.8 and 6.2. As for both the experimental sites, the application of gypsum had little or no effect on soil pH, either with depth or over time.

When GML was applied, the changes in EC were small (data not presented). For the Bungor and Rengam soils, the application of the highest rate of GML (8 t ha^{-1}) to the 0-15 cm layer increased EC from 0.12 to 0.20 dS m^{-1} and 0.11 to 0.17 dS m^{-1}, respectively after 3 months. Meanwhile, after 15 months, the EC values dropped to 0.05 and 0.09 dS m^{-1}, respectively. There was an increase in EC for both soils as a result of gypsum application, especially at the high rates of application.

Increases in exchangeable Ca in the GML experiments in Bungor soil at 3 and 7 months after application were largely confined to the zone of incorporation; the data for Experiment 1 (0-15 cm incorporation) are shown in *Fig. 3*. At the end of the third crop (15 months after the application of GML), the amount of exchangeable Ca in the treated soils was found to decline to a level similar to that of the control (*Fig. 3*). At Chembong, exchangeable Ca was high in the plots supplied with 4 and 8 t GML ha^{-1}. Generally, Ca in the topsoil at GML of 2 t ha^{-1} rate decreased to the level of the control at month 24. In both soils, however, there was no evidence of Ca movement in horizons beyond the depth of incorporation. The incorporation of gypsum in the surface 15 cm of Bungor and Rengam soils increased exchangeable Ca in the short-term, and this effect extended to the 15 to 30 cm layer in the Rengam soil and to the 30 to 45 cm layer in the Bungor soil (*Fig. 4*). After 24 months, however, there was little difference observed in the levels of exchangeable Ca in the horizons, but the levels at the 4 and 8 t ha^{-1} rates of gypsum were higher than in the untreated soil, particularly in the case of the Rengam soil.

In the Bungor soil, exchangeable Mg significantly increased in the 0-15 cm layer, following the incorporation of GML to that depth; this effect, first recorded at month 3, persisted at month 24 (*Fig. 5*). There was a trend towards a slight increase in exchangeable Mg in the sub-soils layers, particularly at the highest rates of GML application. Similar results were also observed for the Rengam soil.

Exchangeable K in the topsoil (Experiment 1) of both the Rengam and Bungor soils at month 3 ranged from 0.1 to 0.5 $cmol_c kg^{-1}$, with an average value of 0.2 $cmol_c kg^{-1}$. In the sub-soils of both soils, exchangeable K

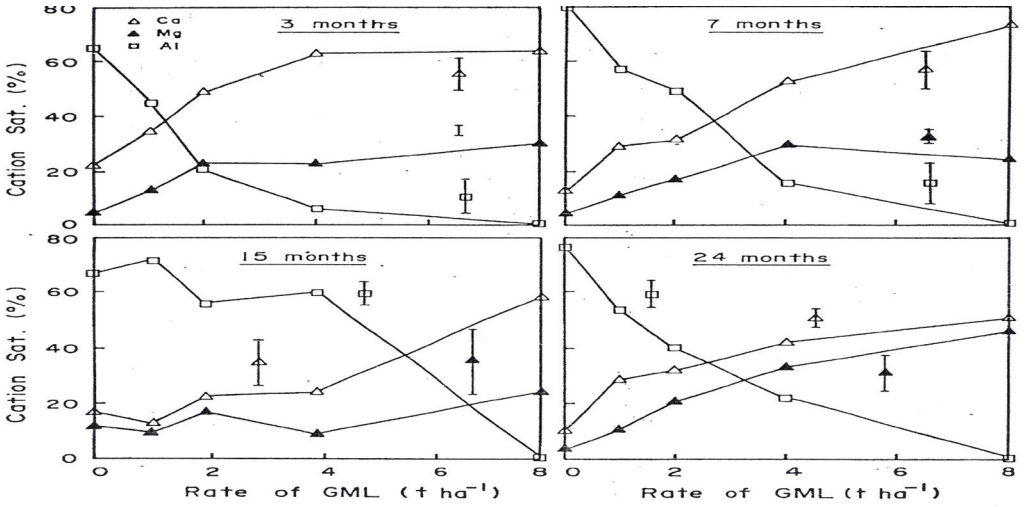

Fig. 8: Ca, Mg and Al saturations in the surface 15 cm of the Bungor soil at 3, 7, 15 and 24 months after incorporation of GML to a depth of 15 cm (Experiment 1). LSD values are for P ≤ 0.05

was < 0.1 $cmol_c kg^{-1}$. Exchangeable K in the topsoil of both soils at month 7 was < 0.1 $cmol_c kg^{-1}$. Meanwhile, exchangeable K at month 7 (groundnut) was lower than at month 3 (corn) because the soils were not fertilized for groundnut (Table 3). At months 15 and 24, exchangeable K in the topsoil was 0.1-0.2 $cmol_c kg^{-1}$. Generally, it was observed that neither GML nor gypsum significantly affected the amount of exchangeable K in the soils.

The trends in exchangeable Al following GML incorporation were similar for the soils at both sites and both depths of incorporation, whereas only data for the 15 cm incorporation for the Bungor soil at Puchong are presented (*Fig. 6*). At month 3, the exchangeable Al in the zone of incorporation was reduced to one-half of its original value after the application of 2 t ha^{-1} of GML and to zero at the highest application rate. There was also a trend towards a reduction in exchangeable Al in the sub-surface layers at month 3, this effect being significant ($P<0.05$) at the 8 t ha^{-1} application rate (*Fig. 6*). Beyond month 3, the exchangeable Al levels began to rise, but at month 24, a significant depression in exchangeable Al below the untreated soil was still evident for the GML rates beyond 2 t ha^{-1}. In the topsoil of Rengam Series (Experiment 1), at month 3, exchangeable Al became zero due to the application of 4 t GML ha^{-1}, and the values started to increase after 15 months. In Experiment 2, similar trends were observed in both the Bungor and Rengam soil series. In particular, there was no significant change in exchangeable Al in soils at both Puchong and Chembong sites with the addition of gypsum, even at the rate of 8 t ha^{-1}. However, Al saturation in the soils was decreased due to the increase in exchangeable Ca supplied by gypsum.

A progressive downward movement of SO_4^{2-} in the soils amended with gypsum was observed (*Fig. 7)*. The increase in SO_4^{2-} at month 7 was mostly marked in the 0-30 cm zone. At month 24, the SO_4^{2-} levels in the surface layers of the amended soils were shown to decline, whereas the downward movement of this nutrient resulted in an increase in its levels right through the deepest layer sampled (45-60 cm) (*Fig. 7*).

Changes in Al saturation in the surface of 15 cm for the Bungor soil, when GML was incorporated to 15 cm, are given in *Fig. 8* Similar trends were also observed for the Rengam soil. The application of GML at 2 t ha^{-1} was sufficient to decrease Al saturation from >60% to <20% at month 3. After 7 months, however, the Al saturation was found to increase to >40%. The buffering effect of the soil also resulted in increases in Al saturation at other GML rates, although the 8 t ha^{-1} rate was sufficient to keep the level close to zero, even after month 24.

Calcium saturation in the surface of the untreated Bungor soil was 22% at month 3, and this was increased to > 60% at the GML application of 4 t ha^{-1} (*Fig. 8*). With increasing length of time, the saturation of Ca in the surface of the amended soil tended to decrease with time. Magnesium saturation followed similar trends to those recorded for the Ca saturation (*Fig. 8)*.

Chemical Properties of Soil Solution

The concentration of calcium in the topsoil of Bungor Series (Experiment 1) at month 1 was increased from 1247 to 4077 µM due to the application of 8 t GML ha^{-1} (*Fig. 9*). Meanwhile, the concentration of calcium in the sample applied with 2 t GML ha^{-1} was 2705 µM. There was a significant drop in the concentration of Ca in the soil solution with depth (*Fig. 10*). The concentration of calcium in the soil solution of the nil treatment below 45 cm depth was only 309 µM, while the concentration of Ca in the subsoil slightly increased with the increasing rate of GML application. At month 7, the concentration of Ca in the soil solution ranged from 2900 to 4758 µM, without any significant difference between the different treatments.

Fig. 9 depicts the changes in the concentration of Mg in the soil solution of Bungor topsoil (Experiment 1) with the increasing rate of GML application. The concentration of magnesium was found to increase from 655 to 1396 µM, and this resulted from the application of 2 t GML ha^{-1}. Meanwhile, the concentration of magnesium in the sample treated with 8 t GML ha^{-1} was 3300 µM. It was observed that the concentration of

Mg was higher in the top than in the subsoil, even in the nil treatment. The progressive increase in the GML rate was followed by a concomitant increase in the concentration of Mg in the subsoil (Figure 10). At month 7, a three-fold increase in the concentration of Mg was due to application of 8 t GML ha^{-1} was observed.

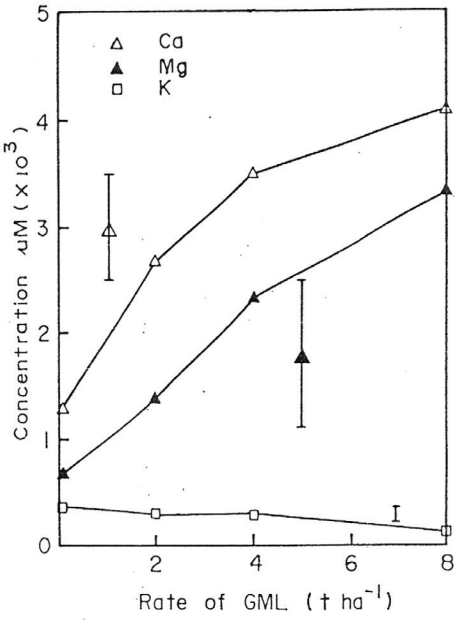

Fig. 9: Ca, Mg and K concentrations in the soil solution from topsoil of Bungor soil in Experiment 1 at month 1 after GML application. LSD values are for P ≤ 0.05

Some of the Ca and Mg released from the dissolution of GML replaced K in the exchange complex. Most of the replaced K might have remained in the soil solution, while some found their ways into the soil profile via leaching. As such, the concentration of K in the soil solution decreased with the increasing rate of GML application (*Fig. 9*). It was observed that the K concentration in the soil solution decreased from 345 to 121 µM due to the application of 8 t GML ha^{-1}.

Other elements determined in the soil solutions from Experiment 1 at Puchong and Chembong sites at month 7 were Al, Fe, Mn and Si. Mitscherlich equations were fitted to the regressions of Al, Fe and Mn concentrations in both soils with pH data. It was found that the concentrations of Al, Fe and Mn were highly correlated with pH of the soil solution. This implies that the availability of Al, Fe and Mn to plant decreases with an increase in soil solution pH. Soil solution pH was raised to about 5 due to the application of GML at 2 t ha^{-1} (Table 4). At pH5, the concentration of Al was reduced to < 25 µM, while the concentration of Mn was decreased to < 15 µM. Soluble Mn decreased much more slowly as pH increased than soluble Al, similar to what had been reported by Hue *et al*. (1987). Apparently, the concentration of iron was also reduced by the application of 2 t GML ha^{-1}. There was a general decrease of Si concentration with an increase in pH. Curtin and Smilie (1983) reported a similar reduction in the concentration of Si by liming.

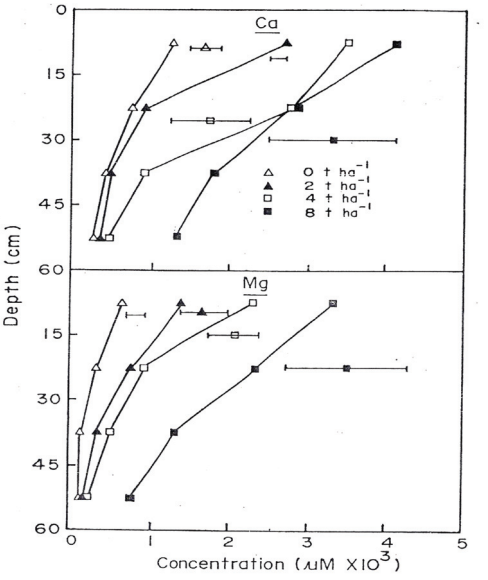

Fig. 10: Changes in the soil solution Ca and Mg with depth in Bungor soil 1 month after GML application. LSD values are at P ≤ 0.05

At month 7, the concentration of sulphate (Experiment 1) in the soil solution from the topsoil of both Bungor and Rengam soils increased with the increasing rate of GML

TABLE 4
Effects of GML application on pH and the amount of inorganic Al in the soil solution of Bungor and Rengam soils in Experiment 1 (0-15 cm depth)

Rate of GML (t ha^{-1})	pH	Al_{inorg}	Al_{org}	Al_{inorg}/Al_T
		----------%	----------	
		Bungor Series		
0	3.91	134	0	100
0.5	4.22	96	0	100
1.0	4.75	34	0	100
2.0	4.93	17	0	100
4.0	5.37	5.4	2.1	72
8.0	6.11	0.5	3.2	13
		Rengam Series		
0	4.01	199	0	100
0.5	4.22	110	0	100
1.0	4.33	112	0	100
2.0	4.51	76	0	100
4.0	5.90	5.5	3.1	62
8.0	6.15	1.6	2.4	40

application. The concentration of sulphate in the soil solutions of Bungor and Rengam soils, treated with 8 t GML ha^{-1}, was 1604 and 878 µM, respectively. The increase in the concentration of sulphate was probably due to the exchange of adsorbed sulphate by hydroxyl on the exchange sites and the reduction in AEC with increasing pH.

At month 7, the soil solutions extracted from the soils of Bungor and Rengam Series were very clear, suggesting that little or no carboxylic and/or fulvic acids were present. Hence, Al in the soil solution was expected to be present mostly in the inorganic monomeric form. The amounts of inorganic and organically-complexed Al, as determined by the short-term (60 second) pyrocatechol violet method of Kerven *et al*. (1989), are given in Table 4.

Total aluminium (Al_T) was also measured using the pyrocatechol violet method after 20 minutes reaction, which could be used as a check. It was found that if Al measured by PCV, 20 minutes reaction was less than that measured by ICPAES, whereas some Al in the solution was found to be complexed with organic materials. These were the cases for the samples treated with 4.0 t GML ha^{-1} or more (Table 4). It was also noted that the samples treated with 2 t GML ha^{-1} or less contained 100% inorganic monomeric Al. The samples treated with 4 t GML ha^{-1} or more contained low concentration of Al, and as such Al was no longer present at the level toxic to crop growth.

The ratio of Al reacted in 60 s (PCV) to the Al reacted in 20 minutes was determined. It was found that whenever this ratio exceeded

0.85, the Al in the soil solution was estimated to be 100% inorganic monomeric. This simple procedure can be used as a test to determine the soil solution which contained organically-complexed Al.

The percentage of Al complexation increased with the increase in pH, and this finding is in agreement with that reported by Curtin and Smillie (1983). The organically-complexed Al is known to be non-phytotoxic (Hue *et al*., 1986). This, thus, limits alleviates Al toxicity in three ways: 1) it decreases Al concentration in the soil solution; 2) it reduces Al toxicity by increasing Ca and Mg in the solution (Besso and Bell, 1992); and 3) it promotes complexation of Al by organic matter, thereby reducing the concentration of inorganic monomeric Al. Curtin and Smillie (1986) believe that an increase in the organically-complexed Al after liming was associated to the solubilization of organic matter.

Major Al species in the control or nil treatment was Al^{3+} (Table 5). When soil solution pH was increased due to GML application, both Al^{3+} and $AlSO_4^+$ activities were found to decrease, while hydroxyl-Al monomer activity increased. At the GML rate of 1.0 t ha^{-1} or higher, $Al(OH)_2^+$ became the major Al species. A similar observation had already been made by Pavan *et al*. (1982). At the GML rate of 4.0 t ha^{-1} or higher, Al^{3+} and $AlSO_4^+$ species were either absent or very small.

Al^{3+} and $AlSO_4^+$ ions were the major Al species in the gypsum treated samples at month 7 (Table 5). The next major species was $Al(OH)_2^+$. The soil solutions which contained very high activities of $AlSO_4^+$ were also found to contain high concentrations of SO_4^{2-} and Ca, suggesting the presence of high amounts of gypsum in the soil samples. Therefore, the application of gypsum was observed to have brought about a reduction of Al^{3+} and an increase of $AlSO_4^+$ activities.

DISCUSSION

Solid Phase Properties

Both soils at Puchong (Bungor Series) and Chembong (Rengam Series) were strongly acidic and highly weathered with kaolinite being the dominant mineral, although the Bungor Series soil contained some mica (Table 1). The presence of the latter resulted in the Bungor soil having a higher CEC than the Rengam soil; additionally, it contained higher exchangeable Al in spite of having a higher pH than the Rengam soil. The buffering capacity of highly weathered soils depends upon the amount of exchangeable Al and proportion of kaolinite in the clay fraction (Shamshuddin and Tessens, 1983); this, in turn, would be expected to affect the longevity of liming effects.

The presence of high amounts of kaolinite and some goethite in the clay fraction resulted in an increase in negative charge on the clay surfaces when soil pH was raised by the GML application. This is similar to the finding reported by Shamshuddin and Ismail (1995). Meanwhile, negative charge was increased by two-fold when soil pH was raised to 6.0 (*Fig. 1*). The progressive increase in the soil pH, due to increasing rate of liming, was mainly observed in the surface soil (*Fig. 2*). This observation helps to explain the retention of Ca released by GML in 0-15 cm zone (*Fig. 3*). On the other hand, the application of gypsum resulted in insignificant increase of pH and negative charge on the clay surfaces. Some of the Ca in the gypsum experiment were in excess of the soil exchange capacity and thus moved deeper into the soil profile, as shown in *Fig. 4*.

At month 24, most of the extractable SO_4^{2-} in the gypsum experiment was found below 45 cm depth. Exchangeable Ca in the surface and subsoil was reduced to the level of the control (*Fig. 4*), while most of the Ca provided by gypsum had probably moved deeper into the profile or lost via leaching. There was, therefore,

a greater mobility of gypsum compared with GML. Thus, gypsum is a good source of Ca to alleviate Ca deficiency.

Bruce *et al*. (1989) reported that critical Ca saturation for soybean was 11%. It is evident that Ca is sufficient in the topsoil of the two soil series undertaken in the present study. Ca saturation was 22% in the nil treatment at Puchong site at month 3 (*Fig. 8*). Nonetheless, this value became lower at the subsequent sampling time. Generally, Ca saturation exceeded 20% in the amended topsoil with > 1 t GML ha^{-1}.

Magnesium saturation of 10-15% is regarded as sufficient for crop growth (Eckert, 1987). In the nil treatment of Experiment 1 (Puchong), Mg saturation was <10% (*Fig. 8*). Meanwhile, exchangeable Mg was <0.15 $cmol_c kg^{-1}$ in the subsoil, a level considered as critical for the growth of corn and groundnut (Tropsoils, 1984). Data presented in *Fig. 5* and *9* suggest that at least 1 t GML ha^{-1} is needed to increase exchangeable Mg and Mg saturation to >0.15 $cmol_c kg^{-1}$ and >10%, respectively in these soils.

Meanwhile, the application of gypsum resulted in an increase of EC. The highest EC was obtained for the topsoil of Bungor soil (Experiment 1) at month 3 due to the application of 8 t gypsum ha^{-1}, with a value of 0.89 dS m^{-1}. The calculated ionic strength (Griffin and Jurinak, 1973) was 11.6 mM and, at this value, the activity of Al^{3+} predicted by the equation of Bruce *et al*. (1989) was about 1.3 μM.

Friesen *et al*. (1980) reported that corn shoot yield declined at 28% Al saturation, while an expert system, Tropsoils (1984) believed

TABLE 5
Changes in activities of Al species affected by GML and gypsum application in Bungor soil in Experiment 1(0-15 cm depth)

Rate of GML (t ha^{-1})	pH	Al^{3+}	$Al(OH)^{2+}$	$Al(OH)_2^+$	$Al(OH)_3$	$AlSO_4^+$
		----------μM----------				
			GML			
0	3.91	55.4	3.3	4.8	0	3.9
0.5	4.22	28.6	4.6	15.7	0.1	5.4
1.0	4.75	11.2	2.0	7.4	0	1.9
2.0	4.93	1.2	0.7	11.3	0.5	0.5
4.0	5.37	0.5	0.4	7.5	0.2	0.2
8.0	6.11	0	0	0.3	0.1	0
			Gypsum			
0	4.30		4.7	19.0	0.1	14.5
0.5	4.22		3.1	10.7	0.1	251.6
1.0	4.25		2.7	9.8	0.1	8.1
2.0	4.66		3.3	31.0	0.5	105.7
4.0	4.26		6.6	24.6	0.1	105.9
8.0	4.55		5.1	36.6	0.4	43.8

that respective Al saturations for corn and groundnut grown on acid tropical soils were 30 and 40%. Data presented in *Fig. 8* suggest that > 2 t GML ha^{-1} is needed to reduce topsoil Al saturation to < 40%. In the untreated soils or those amended with gypsum, Al saturation was > 30%. Sharifuddin *et al*. (1995) reported that maximal corn yield was obtained on an Ultisol > 2 years after 2 t GML ha^{-1} had been applied.

Solution Phase Properties

The pH of the soil solution was found to increase with the rising rate of GML application, whereby the application of 8 t GML ha^{-1} resulted in an increase of pH from 4.0 to about 6 (Table 4). The pH of the soil solution was linearly correlated (R^2 = 0.848) with pH ($CaCl_2$), implying that pH (1:1) 0.002 M $CaCl_2$ could be used as an estimate of soil pH under field conditions. This is possible because the ionic strength of a 0.002 M $CaCl_2$ solution was near to the ionic strength of the soil solution extracted from a common tropical soil. Therefore, the application of gypsum did not affect the pH of the soil solution significantly (Table 5). Meanwhile, no significant decrease in pH was observed, even at the gypsum rate of 8 t ha^{-1}.

The pH of the soil solution in the nil treatment was about 4.0 (Table 4). At this pH value, the concentrations of Al and Mn in the soil solution were 150 and 50 μM, respectively. In a solution culture study, Blamey *et al*. (1983) found that soybean root elongation was reduced severely when the Al concentration exceeded 10 µM. Therefore, the pH of the Bungor and Rengam soils has to be raised by liming in order to bring down the concentrations of Al and Mn to the level suitable for crop production.

Increasing soil pH resulted in an increase of CEC_T (or CEC_B) of the Bungor and Rengam soils, and that the CEC_T was higher in the Bungor than in the Rengam soil. At the GML application of 8 t ha^{-1}, the pH was raised to 6.11 in the soil solution of the Bungor Series (Table 4). As such, more Ca would be retained than at lower pH. In addition, Ca is more tightly held to the mineral surfaces than either Mg or K. Thus, there would have been more Ca in the soil solution of the Bungor soil at month 7 than that presented in *Fig. 3* had it not been for this reaction.

In a study using Ultisols of Nigeria, Friesen *et al*. (1980) found that corn shoot yield declined at a soil solution Al concentration of 148 μM. Referring to data presented in Table 4, it was observed that the soil solution pH of Bungor and Rengam in the control treatment was 3.9 and 4.0, respectively. The available data indicated that at the soil solution of about 4, the concentration of Al was about 150 μM. Thus, corn grown on these soils without liming would be subjected to Al toxicity. Al^{3+} activity in the soil solution from the Bungor soil treated with 1 t GML ha^{-1} or less was > 10 μM (Table 5). In the gypsum experiment, Al^{3+} activity was generally >10 μM. The growth of soybean is affected by the presence of Al^{3+} activity at 4 μM (Bruce *et al*., 1988). Al toxicity is alleviated by the presence of SO_4^{2-}, as shown by the improvement of barley seedlings root elongation (Cameron *et al*., 1986). Release of SO_4^{2-} into the soil solution by the GML application would be expected to partly reduce Al toxicity by this mechanism.

An increase in the concentration of Ca, in the soil solution of soil applied with GML (*Fig. 9* and *10*) and/or gypsum, would have some effects on Al toxicity. Mitigation of Al toxicity by Ca had been reported by Alva *et al*. (1986a, b). $AlSO_4^+$ is known to be less toxic than Al^{3+} and in an increase of $AlSO_4^+$, and this, to a limited extent, would alleviate Al toxicity in the soil. The alleviation of Al toxicity by gypsum application in the acid soils of United States had been reported by Summer *et al*. (1986).

CONCLUSIONS

The data presented in this study provide a clear evidence of the benefits of liming acid soils. Ameliorative effects shown by high pH and low exchangeable Al in the zone applied with GML lasted more than 2 years at the rate of 2 t GML ha^{-1} or higher. Meanwhile, the increases in pH and exchangeable Ca, arising from the GML application, were confined mainly to the zone of incorporation (0-15 cm). However, a

reduction in exchangeable Al was observed at 15-30 cm depth. The application of gypsum did not result in a significant change in soil pH and exchangeable Al, but Ca and extractable SO_4^{2-} moved deeper into the profile. The application of gypsum was found to increase EC, which in turn, increased ionic strength that might increase Al^{3+} activity in the soil solution.

Under natural conditions, soil solution pH was about 4.0, at which the concentrations of Al and Mn in the soil solution were about 150 µM and 50µM, respectively. Thus, liming is needed to bring down the concentrations of Al and Mn to an acceptable level for crop production. The application of ground magnesium limestone at the rate of 2 t ha^{-1} increased soil solution pH to about 5, which would precipitate out most Al^{3+} ions. The corresponding concentrations of Al and Mn at that pH were < 25 µM and < 15 µM. Meanwhile, Al^{3+} was the major Al species at low pH. Therefore, increasing the pH by applying GML resulted in an increase of hydroxyl-Al monomers, especially $Al(OH)^{2+}$. The application of gypsum decreased Al^{3+} and increased $AlSO_4^+$, and hence, gypsum application, to a limited extent, would be expected to alleviate Al toxicity.

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Accuracy of the Saxton-Rawls Method for Estimating the Soil Water Characteristics for Mineral Soils of Malaysia

Christopher Teh Boon Sung* **and Jane Iba**
Department of Land Management, Faculty of Agriculture
Universiti Putra Malaysia, 43400 UPM, Serdang,
Selangor, Malaysia
**E-mail: cbsteh@yahoo.com*

ABSTRACT

The purpose of this study was to determine the accuracy of the Saxton-Rawls method in estimating the soil water characteristics of a wide range of mineral soils of Malaysia. This study found that it was necessary to calibrate the Saxton-Rawls method for the soils of Malaysia. The developed equation for calibration was $\hat{P}_i = a \cdot P_i(1 - P_i)$, where P_i and $\hat{P}_i$ are the uncalibrated and calibrated estimated values, respectively, for the soil sample no. i, and the parameter values of a were 2.225, 1.605, and 1.528 (for saturation, field capacity, and permanent wilting point) respectively. The calibrated method was validated against three independent soil data sets. The validation tests showed that the calibrated method remained stable and was more accurate than that without any calibration, by an average between 8 to 49%.

Keywords: Organic matter, Saxton-Rawls, soil water characteristics, soil water retention, texture

INTRODUCTION

Soil water characteristic describes the relationship between the soil matric potential and soil volumetric or gravimetric water content (Jury and Horton, 2004). It is a vital soil physical property because it describes how strongly a soil holds onto water. It also reveals the maximum amount of water could be stored by soil (i.e., water content at saturation point) and the maximum amount of water potentially available to plants (i.e., the difference in the water content between the field capacity point and permanent wilting point).

The measurement of soil water characteristics, however, is both time-consuming and expensive (Janik *et al*., 2007). Consequently, many methods or equations exist to estimate the soil water characteristics from multiple soil properties, typically soil texture, bulk density, and organic matter, as described in Saxton and Rawls (2006). Gijsman *et al*. (2002) compared the accuracy of eight modern estimation methods against the field data across many regions in USA. They concluded that the texture-based method by Saxton *et al*. (1986) was the most accurate. The Root Mean Square Error (RMSE) for the method proposed by Saxton *et al*. (1986), for example, was lower by 64% as compared to the average RMSE for the other methods used. One main advantage of the method by Saxton *et al*. (1986) is that it requires only information on the soil texture (sand and clay fractions) and organic matter (in per cent) to predict the characteristics of soil water. Recently, Saxton and Rawls (2006) improved the method by calibrating it against over 1700 different soil

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*Corresponding Author

types stored in the USDA/NRCS National Soil Characterization database (Soil Survey Staff, 2004).

Consequently, the objective of this study was to determine the accuracy of the Saxton-Rawls method to estimate the soil water characteristics of mineral soils of Malaysia. The soil water characteristics of interest in this study were the estimation of the volumetric soil water content at saturation, field capacity, and permanent wilting point.

MATERIALS AND METHODS

The equations used to estimate the characteristics of soil water are listed in Saxton and Rawls (2006), whereby the equation for the soil water content at permanent wilting point is:

$$PWP = \theta_{1500t} + (0.14\theta_{1500t} - 0.02) \quad [1]$$

with

$$\theta_{1500t} = -0.024S + 0.487C + 0.006OM + 0.005(S \times OM) - 0.013(C \times OM) + 0.068(S \times C) + 0.031 \quad [2]$$

where PWP is the soil water content at permanent wilting point ($m^3\ m^{-3}$), S and C are the sand and clay contents, respectively (fraction), and OM is the organic matter content (%). For the soil water content at field capacity, the equation is:

$$FC = \theta_{33t} + (1.283\theta_{33t}^2 - 0.374\theta_{33t} - 0.015) \quad [3]$$

with

$$\theta_{33t} = -0.251S + 0.195C + 0.011OM + 0.006(S \times OM) - 0.027(C \times OM) + 0.452(S \times C) + 0.299 \quad [4]$$

where FC is the soil water content at the field capacity ($m^3\ m^{-3}$). Finally, the equation used to estimate the water content of soil at saturation is:

$$SAT = \theta_{33} + \theta_{(S-33)} - 0.097S + 0.043 \quad [5]$$

with

$$\theta_{(S-33)} = \theta_{(S-33)t} + 0.636\theta_{(S-33)t} - 0.107 \quad [6]$$

and

$$\theta_{(S-33)t} = 0.278S + 0.034C + 0.022OM - 0.018(S \times OM) - 0.027(C \times OM) - 0.584(S \times C) + 0.078 \quad [7]$$

where SAT is the soil water content at saturation ($m^3\ m^{-3}$).

Four soil data sets, obtained from the literature, were compiled for this study. These data sets comprised only the mineral soils of Malaysia, with a wide range of particle size distribution. The first data set was used to determine the accuracy of the Saxton-Rawls method for the mineral soils of Malaysia. This data set was also used to calibrate the Saxton-Rawls method to improve its estimation accuracy. In order to validate the accuracy and stability of the calibrated Saxton-Rawls method, three more independent data sets were used.

The first data set was from Maene *et al*. (1983), who did extensive compilation and measurement on several physical properties of Malaysian soils, comprised 503 samples from 113 soil types from 61 soil series that cover six soil orders, namely Entisols, Inceptisols, Spodosols, Alfisols, Ultisols, and Oxisols. The measurements for each soil include texture, pH, organic carbon, bulk density, and soil water characteristics. These properties were typically measured for several consecutive soil layers from the surface down to 1.0 m depth. For some soils, however, these properties were measured to depths reaching 2.5 m and below. It is important to note that not all soil samples in the register were used in this study. The samples discarded include the ones with missing required data or, in rare cases, those with unusually high volumetric water content (≥1.0 $m^3\ m^{-3}$). This reduced the total number of soil samples selected to only 270. Meanwhile, the sand content for these final samples ranged from 1.5 to 94.0% (standard deviation = 23.7%), whereas the clay content ranged from 2.2 to 89.6% (standard deviation = 20.9%), and the organic matter

content that ranged from 0.0 (trace) to 5.3% (standard deviation = 0.7%).

The second data set comprised 192 soil samples from Maesschalck *et al*. (1983). These soil samples were collected from 16 experimental plots, covering approximately 0.12 ha of the Puchong farm at Universiti Pertanian Malaysia (now known as Universiti Putra Malaysia). In each plot, the soil samples were collected at every successive 0.1 m soil layer from the soil surface to 1.2 m depth. Although these samples were collected from an area classified as Bungor soil series (Typic Paleudult), the textures of these samples differed widely from sandy loam to clay. The content of sand ranged from 35.8 to 66.9% (standard deviation = 7.1%), the clay content ranged from 16.1 to 43.5% (standard deviation = 5.9%), and the organic matter content that ranged from 0.9 to 3.3% (standard deviation = 0.6%).

The third data set, taken from Teh (1996), comprised nine soil types with a wide range of textural classes collected from 0-150 mm of soil depth. The nine soil series were Munchong (Typic Hapluodox), Melaka (Xanthic Hapludox), Rengam (Typic Paleudult), Bungor (Typic Paleudult), Serdang (Typic Paleudult), Holyrood (Typic Kandiudult), and Sg. Buloh (Spodic Quartzipsamment). These soil series were collected from various locations in UPM campus, as well as from Sg. Buloh (for Holyrood and Sg. Buloh soil series). The sand content of the nine soil series ranged from 18.0 to 87.1% (standard deviation = 26.3%), the clay content that ranged from 10.0 to 72.7% (standard deviation = 20.8%), and the organic matter content that ranged from 0.9 to 3.2% (standard deviation = 0.8%).

The fourth data set, taken from Hamdan *et al*. (1999), comprised 12 A-horizons (typically the first 100 mm soil depth) and 12 B-horizons (typically from 100 to 450 mm soil depth). The soils were from 12 soil series, namely Bukit Termiang (Typic Hapludult), Musang (Typic Paleudult), Ulu Dong (Typic Paleudult), Durian (Plinthaquic Paleudult), Kerait (Aquic Paleudult), Nyalau (Typic Dystrochrept), Bekenu (Typic Paleudult), Tarat (Typic Hapludox), and the last four soil series were Rengam, Munchong, Serdang, and Bungor (their soil taxonomic classifications are as before). These soils were sampled from various locations in Peninsular Malaysia (seven soils) and West Sarawak (five soils) in Malaysia. One soil sample, however, was discarded (Tarat series, B-horizon sample) due to its unusually high volumetric water content (≥ 1.0 m^3 m^{-3}). The sand content of the 23 samples ranged from 5 to 75% (standard deviation = 24.3%), the clay content that ranged from 17 to 74% (standard deviation = 16.4%), and the organic matter content which ranged from 0.2 to 4.6% (standard deviation = 1.0%).

For all the four data sets, determination of the soil water characteristics was done using the pressure plate method (Richards, 1947). Meanwhile, the determination of organic carbon was carried out using the Walkley-Black dichromate titration method (Walkley and Black, 1934), with organic matter content taken as 1.72 × organic carbon.

An error index, Mean Absolute Error (MAE), was used to determine the mean error of estimation by the Saxton-Rawls method. Thus, MAE (in percentage) was calculated as:

$$MAE\ (\%) = \frac{100}{N} \cdot \sum_{i=1}^{N} \frac{\left| O_i - P_i \right|}{O_i} \qquad [8]$$

where i is the sample number (i = 1 to N), N is the total number of sample; and O_i and P_i are the observed (measured) and predicted (estimated) values, respectively, for sample i. A large MAE denotes a large mean error in the estimates.

The data analysis was done using Microsoft Excel 2003 (Microsoft Inc., Washington), whereas the calibration of the Saxton-Rawls method was done using Excel's Solver add-in, which uses a non-linear optimization algorithm known as Generalized Reduced Gradient (Lasdon *et al*., 1978).

RESULTS AND DISCUSSION

The mean estimation error (represented by the MAE index) by the Saxton-Rawls method ranged from 17.6 to 21.3% (with a mean of 19.2%) for the first data set (*Fig. 1*). Although the Saxton-

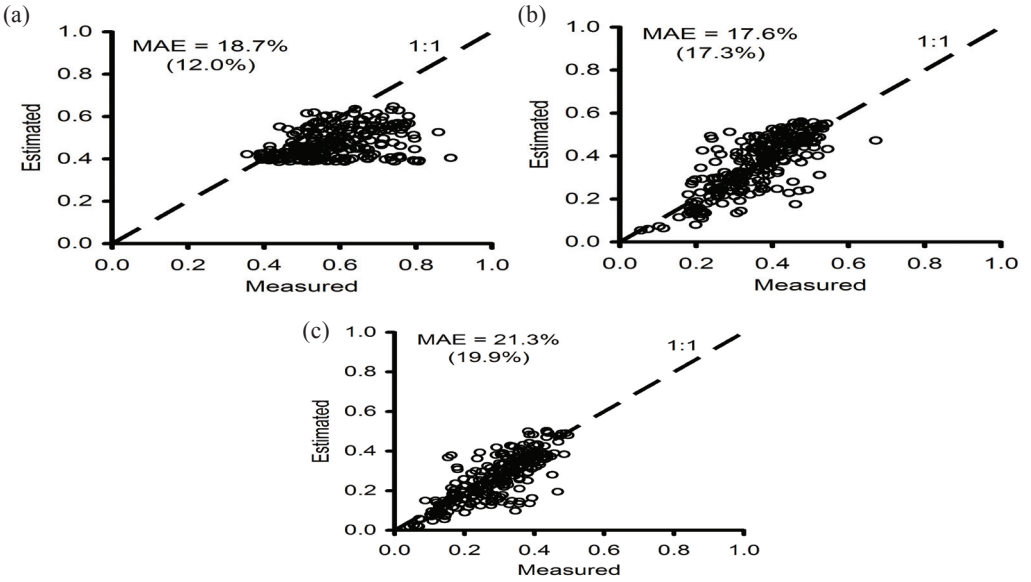

Fig.1: Estimation accuracy by the Saxton-Rawls method (uncalibrated) for the first data set from Maene et al., (1983). The plots are for volumetric soil water content at: a) saturation, (b) field capacity, and (c) permanent wilting point. Values in brackets are the standard deviation for the estimation error index, MAE (Mean Absolute Error), and the dashed diagonal line is the line of agreement

Rawls method tended to underestimate the soil water content at saturation (*Fig. 1a*), it showed none to little bias in the estimation of soil water content at field capacity and permanent wilting point (*Figs. 1b* and *1c*).

In an attempt to increase estimation accuracy, the following quadratic equation was used to calibrate the estimates to agree more closely with the measured values:

$$\hat{P}_i = a \cdot P_i(1 - P_i) \qquad [9]$$

where P_i and $\hat{P}_i$ are the uncalibrated and calibrated estimated values, respectively, for soil sample i, and a is a parameter where its value is such that the mean difference between the estimated and measured values are minimized. In other words, the value for a is determined in such a way that the MAE is minimized. The Solver tool included in Microsoft Excel was used to determine the respective a values for estimating the soil water content at saturation, field capacity, and permanent wilting point (Table 1). Meanwhile, the calibration of the Saxton-Rawls method using Eq. [9] (with appropriate a values from Table 1) reduced the mean MAE to 16% in the first data set, i.e., a reduction by 17% as compared to the uncalibrated Saxton-Rawls method (Table 2). After the calibration, MAE was found ranged from 14.4 to 18.1% as compared to 17.6 to 21.3% for the uncalibrated method.

TABLE 1
The value of the parameter a in Eq. [2] for calibrating the Saxton-Rawls method to estimate the volumetric soil water content at saturation, field capacity, and permanent wilting point

Soil water characteristics	a
Saturation	2.225
Field capacity	1.605
Permanent wilting point	1.528

Eq. [9] and the best-fitted a values (Table 1) were used again, unchanged, in the estimation of the soil water characteristics in the second,

TABLE 2
The Mean Absolute Error (MAE) (standard deviation in brackets) for the uncalibrated and calibrated Saxton-Rawls method for estimating the volumetric soil water content at saturation (SAT), field capacity (FC), and permanent wilting point (PWP) for the four soil data sets. Values are in per cent

Data set*	Estimates	SAT	FC	PWP	Mean
1	Uncalibrated	18.7 (12.0)	17.6 (17.3)	21.3 (19.9)	19.2
	Calibrated	14.4 (10.3)	15.3 (14.3)	18.1 (18.9)	16.0
2	Uncalibrated	28.1 (7.6)	20.6 (7.8)	34.9 (11.7)	27.9
	Calibrated	10.3 (6.6)	11.4 (7.0)	21.0 (11.3)	14.2
3	Uncalibrated	16.4 (9.8)	16.8 (20.2)	23.3 (18.3)	18.8
	Calibrated	11.0 (7.8)	15.5 (13.1)	18.4 (14.0)	15.0
4	Uncalibrated	20.6 (14.5)	29.5 (20.1)	43.3 (44.3)	31.1
	Calibrated	15.8 (10.4)	22.4 (14.6)	47.2 (45.0)	28.5

* Data set no. 1, 2, 3, and 4 are from Maene *et al.* (1983), Maesschalck *et al*. (1983), Teh (1996), and Hamdan *et al*. (1999), respectively

third, and fourth data sets. The validation tests revealed that the calibrated method remained stable and was still more accurate than the uncalibrated method (Table 2). The calibrated method, as compared to the uncalibrated method, had lower MAEs for estimating the soil water content at saturation, field capacity, and permanent wilting point. Additionally, as compared to the uncalibrated method, the calibrated method had lower standard deviations for MAE, denoting a smaller spread or variability of the estimation errors for the calibrated method. The calibration also reduced the mean MAE by 49, 20, and 8% in the second, third, and fourth data sets, respectively, as compared to that without any calibration.

All the results so far indicated that the calibrated method was more accurate than the uncalibrated method. One exception to this trend was the lower accuracy of the calibrated method (than the uncalibrated method) to estimate the soil water content at the permanent wilting point in the fourth data set (Table 2). Nonetheless, the overall accuracy of the calibrated method was still higher than the uncalibrated method in the fourth data set, albeit to a lesser extent of 8% as compared to 16, 20, and 49% in the other three data sets.

Soil water characteristic is a function of soil texture and structure (Gardner, 1973), particularly bulk density, particle size, mineral and organic composition, as well as pore-space density and distribution (Janik *et al.*, 2007). Despite requiring information only on the soil texture and organic matter, the Saxton-Rawls method has been shown to be reasonably accurate for over 1700 different soil types found in USA (Saxton and Rawls, 2006). Nevertheless, the present study revealed that it was necessary to calibrate the Saxton-Rawls

method to improve its estimation accuracy for mineral soils of Malaysia. Without calibration, the mean estimation error (i.e., the mean MAE for all the four data sets) by the Saxton-Rawls method was 24%. With calibration, however, its mean error was reduced to 18%.

CONCLUSIONS

Based on the findings of the study, calibrating the Saxton-Rawls method was found to be necessary to increase its accuracy in estimating the soil water characteristics for the mineral soils of Malaysia. The Saxton-Rawls method was tested on a wide range of particle size distribution of the Malaysian mineral soils. The results showed that without calibration, the Saxton-Rawls method had a higher mean estimation error of 24%, as compared to only 18% with calibration. When compared to that without calibration, the calibrated Saxton-Rawls method estimated the soil water content at saturation, field capacity, and permanent wilting point, with a higher accuracy, and an average between 8 to 49%.

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Distribution of Arabinogalactan Protein (AGP) Epitopes on the Anther-derived Embryoid Cultures of *Brassica napus*

Parameswari Namasivayam[1*], Jeremy N. Skepper[2] and David Hanke[3]
[1,3]Department of Cell and Molecular Biology,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[2]Multi Imaging Centre, Department of Physiology Development and Neuroscience,
Anatomy Building, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK
[3]Department of Plant Sciences, University of Cambridge, Downing Street,
Cambridge CB2 3EA, UK
[*]E-mail: parameswari@biotech.upm.edu.my

ABSTRACT

The anther-derived embryoid cultures of *Brassica napus* is stably embryogenic and has an extracellular matrix (ECM) layer covering the surface of the developing embryoids. In this study, the distribution of arabinogalactan protein (AGP) epitopes in the ECM layer and the embryogenic tissue of winter oilseed rape were investigated by immuno-labelling with anti-AGP monoclonal antibodies (mAb JIM4, JIM8, and JIM13). There was no labelling by the JIM4 and JIM8 mAbs in the ECM layer, unlike what was reported in other plant species. JIM13 epitope is developmentally regulated because it was only present in the ECM layer of the mature embryogenic tissue. These observations indicate a possible variability in the AGP epitopes present in the ECM layer among the different plant species. JIM8 and JIM13 epitopes were found in some epidermal cells of embryogenic tissue, but not in the non-embryogenic tissue, implying that AGPs might have a specific role in embryogenic competency or determining the cell fate of the *B. napus* embryogenic cells.

Keywords: Arabinogalactan protein, AGP, somatic embryogenesis, immunolocalisation, *Brassica napus*, embryogenic tissue, extracellular matrix layer

INTRODUCTION

Arabinogalactan proteins (AGPs) are proteoglycans containing over 90% (w/w) carbohydrate and can be found membrane bound, cell wall associated, in the intercellular spaces of the tissue or secreted into the medium in cell cultures (Fincher *et al.*, 1983; Knox, 1996; Nothnagel, 1997). The carbohydrate moiety of AGPs consists mainly of arabinose and galactose with minor amounts of uronic acids, while polysaccharides are O-linked to the protein core (Seifert and Roberts, 2007). AGPs have been shown to contribute to various aspects of plant development (Gao and Showalter, 2000; Rumyantseva, 2005) including cell division (Serpe and Northnagel, 1994), cell expansion (Willats and Knox, 1996; Ding and Zhu, 1997), programmed cell death (Chaves *et al*., 2002; Guan and Nothnagel, 2004), sexual reproduction (Coimbra *et al.*, 2007; Pereira *et al.*, 2006) and somatic embryogenesis (Pennell *et al.*, 1992; Kreuger and van Holst, 1993; van Hengel *et al.*, 2001; Samaj *et al.*, 2008).

The use of monoclonal antibodies that bind specific/individual epitopes of arabinogalactan has made it possible to analyse the occurrence

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[*]Corresponding Author

of AGPs localised in membranes, cell walls and of those secreted to the culture medium (Malinowski and Filipecki, 2002). Previous immunolocalisation studies have shown specific epitopes of AGPs as a marker for transitional states during carrot somatic embryogenesis (Stacey *et al*., 1990; Pennell *et al.*, 1992; McCabe *et al*., 1997; Toonen *et al*., 1997; Schultz *et al*., 1998). In *Picea abies*, it was reported that the lack of staining for the arabinogalactan protein epitope recognised by the monoclonal antibody JIM13 was an efficient marker for distinguishing proembryogenic masses (PEMs) from somatic embryos (Filonova *et al*., 2000). The expression of the JIM4 epitope was studied during the formation of embryos in continuous cultures of callus cells from hypocotyl explants of carrot (Stacey *et al*., 1990). Meanwhile, differential expression of the JIM4 epitope by cells at all stages, from PEMs to the mature embryo, indicated that plasma membrane AGPs are correlated with the position of cells in emerging plant forms.

Following that, AGPs have been shown to be released in the growth medium by cells in an embryogenic suspension culture that can stimulate somatic embryo development in a non-embryogenic culture (Kreuger and van Holst, 1993; Egertsdotter and von Arnold, 1995). This suggests that certain AGPs can be signalling molecules. Some researchers have suggested that a special class of AGPs, containing N-acetylglucosamine (the chitin monomer), can serve as a substrate for the activity of chitinase. Therefore, a pre-treatment of AGPs with EP3 endochitinase was found to have resulted in an optimal somatic embryo-forming activity (van Hengel *et al.*, 2001).

All these studies reinforce the message that AGPs, associated with the plasma membrane and cell wall of plant cells, contain information and can act as signalling molecules to neighbouring cells (cell-cell communication) in the form of surface epitopes produced by distinct tissues or groups of cells during the early stages of somatic embryogenesis. In their previous study, Namasivayam *et al.* (2006) reported the presence of an ECM layer in *Brassica napus* embryogenic cultures. This layer is composed of a fibrillar network that covers the surface of embryoids of a winter oilseed rape embryogenic culture from the pre-embryogenic stage until the early globular stage. Nonetheless, this ECM layer was not present in the non-embryogenic tissue grown under the same experimental conditions. The composition of this layer needs to be elucidated as the first step towards investigating its role during the early events of secondary embryogenesis. Although there have been several attempts to characterise the molecular components of the ECM layer in other plants, including maize (Samaj *et al.*, 1995), coconut (Verdeil *et al.*, 2001), Cichorium (Chapman *et al.*, 2000a: 2000b), wheat (Konieczny *et al.*, 2007) and hybrid fir (Samaj *et al.*, 2008), its composition is still poorly understood even now. It is also not clear if the same components that make up the ECM layer of all the other plant species are also present in the ECM layer of *Brassica napus*.

Therefore, the aim of this study was to find out if AGPs are amongst the ECM components, as well as if and how their epitopes are developmentally regulated during winter oilseed rape secondary embryogenesis. Therefore, an attempt to detect the presence of AGP epitopes in the ECM layer, embryogenic cell clusters in the hypocotyls of winter oilseed rape embryoids, and to compare with non-embryogenic tissue, was carried out by immuno-labelling with anti-AGP monoclonal antibodies (JIM4, JIM8, and JIM13).

MATERIALS AND METHODS

Antibodies

The monoclonal antibodies anti-AGPs (JIM4, JIM8, and JIM13) were kindly provided by Dr. Maureen McCann from the Department of Cell Biology, John Innes Centre, Norwich, UK.

Plant Materials

Sources and preparation of the plant materials for the pre-embryogenic (PEC), mature embryogenic (MEC), and non-embryogenic

(NEC) of *Brassica napus* ssp. *oleifera* cv. Primor tissue were generated using a method identical to the procedure described in Namasivayam *et al.* (2006).

Fluorescence Immunochemistry

All the tissue samples were fixed in 4% (w/v) formaldehyde in 0.1M PIPES buffer, pH 7.2 at room temperature for 4 hr. The tissues were rinsed in the same buffer, dehydrated in a graded series of ethanol solutions and embedded in LR White resin (Agar Scientific Ltd., Essex, UK). Sections were cut at 1 micrometre using a Leica Ultracut UCT (Viennna, Austria) and mounted in groups of 5 on the SuperFrost plus slides (VWR International, Strasbourg, France). Labelling with each antibody was carried out on duplicate slides.

The sections were incubated with 100 µl of blocking buffer (1% (w/v) Bovine Serum Albumin (BSA), 5% (v/v) normal goat serum, 0.01% (v/v) TritonX-100, 0.01% (v/v) Tween-20) in Tris buffered saline (TBS) pH 7.4 for 30 min at room temperature. The blocking buffer was replaced with primary antibody diluted in TBS containing 0.01% (v/v) Tween-20 and 0.01% (v/v) TritonX-100 (TBSTT), with a dilution factor of 1:20. A control without any primary antibody was incubated with blocking buffer. The sections were given 5 X 15 min washes with TBSTT on the next day and then incubated in the secondary antibody; goat anti-rat IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma, UK; 1:100 dilution) with TBSTT, and left for an hr in the dark. The sections were given 6 X 15 min washes with TBSTT, followed by 2 final washes in distilled water. The dried slides were mounted in CITIFLUOR glycerol solution and sealed with clear nail varnish. After that, the slides were viewed under a Leica DM RXA confocal microscope. FITC was excited by the 488 nm wavelength of an argon laser, and emission was collected at 515-530 nm. Photographs were taken using a digital camera (Nikon Cool Pix 950, Japan).

RESULTS

Immunolocalisation with Anti-AGPs

Immunolocalisation of AGP epitopes was carried out with three different monoclonal antibodies (mAbs), JIM4, JIM8, and JIM13 on three different tissues, namely pre-embryogenic tissue (PEC), mature embryogenic tissue (MEC), and non-embryogenic tissue (NEC). Concurrently, negative control labelling experiments with the goat anti-rat FITC conjugate only (no primary antibodies), and without the secondary antibodies, were included. These negative controls did not show any specific bright green FITC immunofluorescence signal but a greenish yellow (*Fig. 1A*) or yellow fluorescent background signal was observed (*Fig. 1B*). This was attributed to the phenolic compounds present in the cell walls, and was mainly found

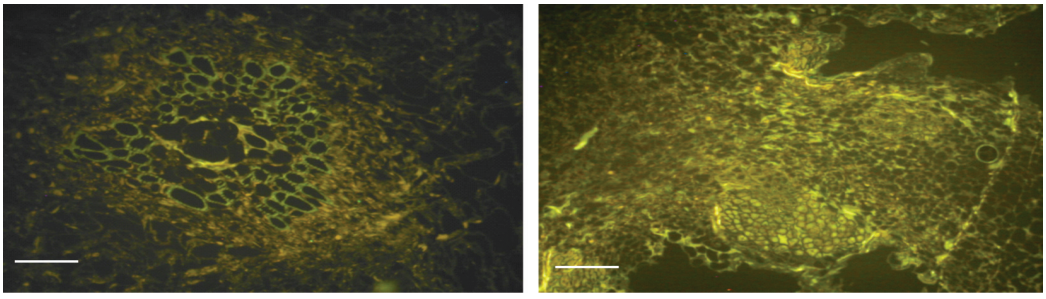

Fig. 1: Negative controls of the immunocytochmical reactions without primary antibodies. Negative controls for both non-embryogenic (A) and m ature embryogenic tissues (B) showed only greenish yellow autofluorescence in the xylem tissues and yellow fluorescence overall. Bars = 100 µm and 150µm respectively. MEC, mature embryogenic tissue; NEC, non-embryogenic tissue; C, cortex; P,pith; e, epidermis

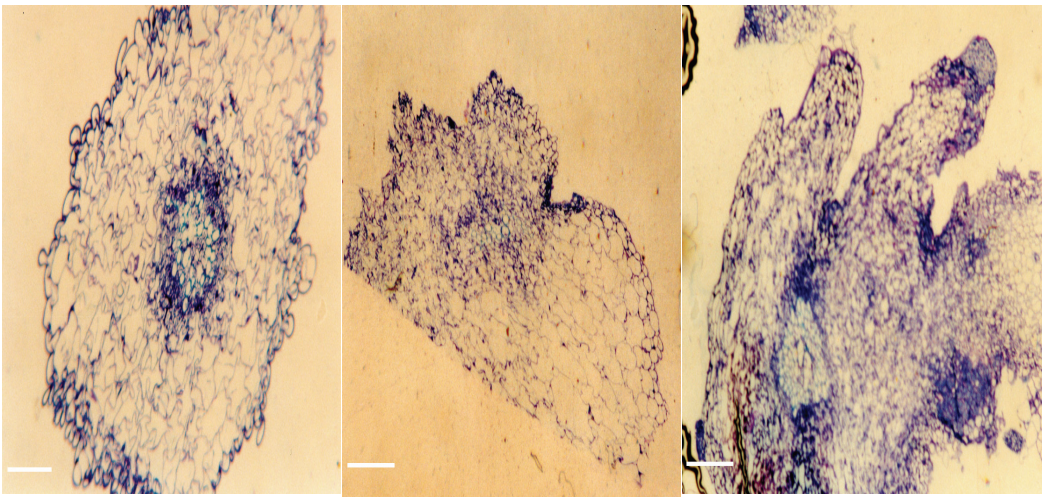

Fig. 2: Light micrographs of sections of NEC, PEC and MEC. Longitudinal and cross sections of 3 different samples for immunolocalisation studies, stained with AzureA/ methylene blue for anatomical analysis and to assist in identification of tissues and cells. NEC, non-embryogenic tissue, Bar= 80μm; PEC, pre-embryogenic tissue, Bar=250μm; MEC, mature embryogenic tissue, Bar=400μm. e, epidermis; C, cortex; P, pith

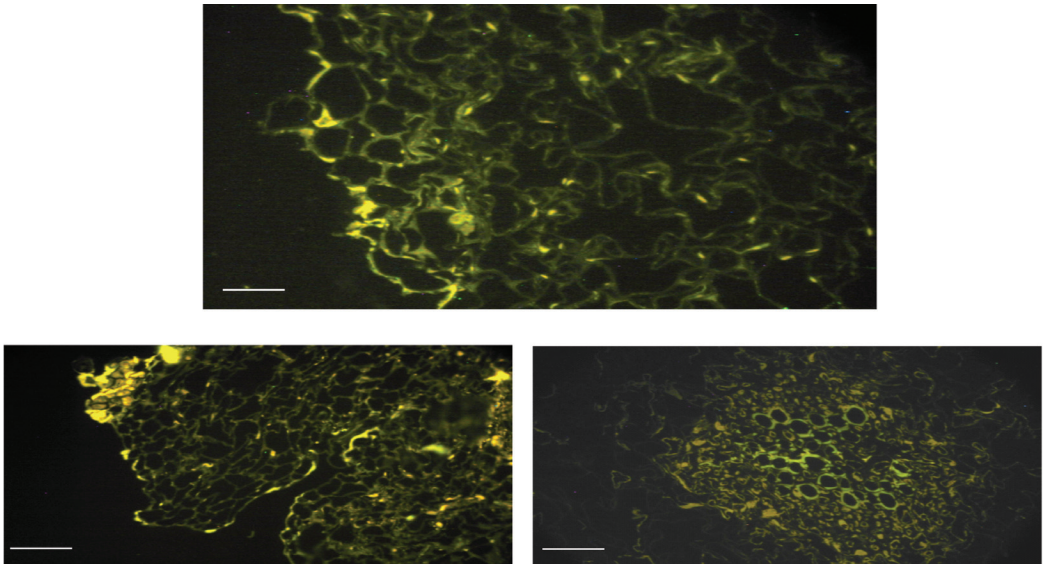

Fig. 3: Immunolocalisation of JIM4 epitope in sections of pre-embryogenic (PEC), mature embryogenic (MEC) and non-embryogenic (NEC) tissue of oilseed rape. A, Immunolabelling of longitudinal sections of PEC tissue showing no signal except for yellow background fluorescence. Bar=40μm. B, Immunolabelling of longitudinal sections of MEC tissue showing no signal except for yellow fluorescence. Bar=60μm. C, Immunolabelling of cross sections of NEC hypocotyl showing greenish yellow autofluorescence in xylem tissues and yellow fluorescence overall. Bar=100μm. C, cortex; ep, epidermis; P, pith; pe, proembryoid

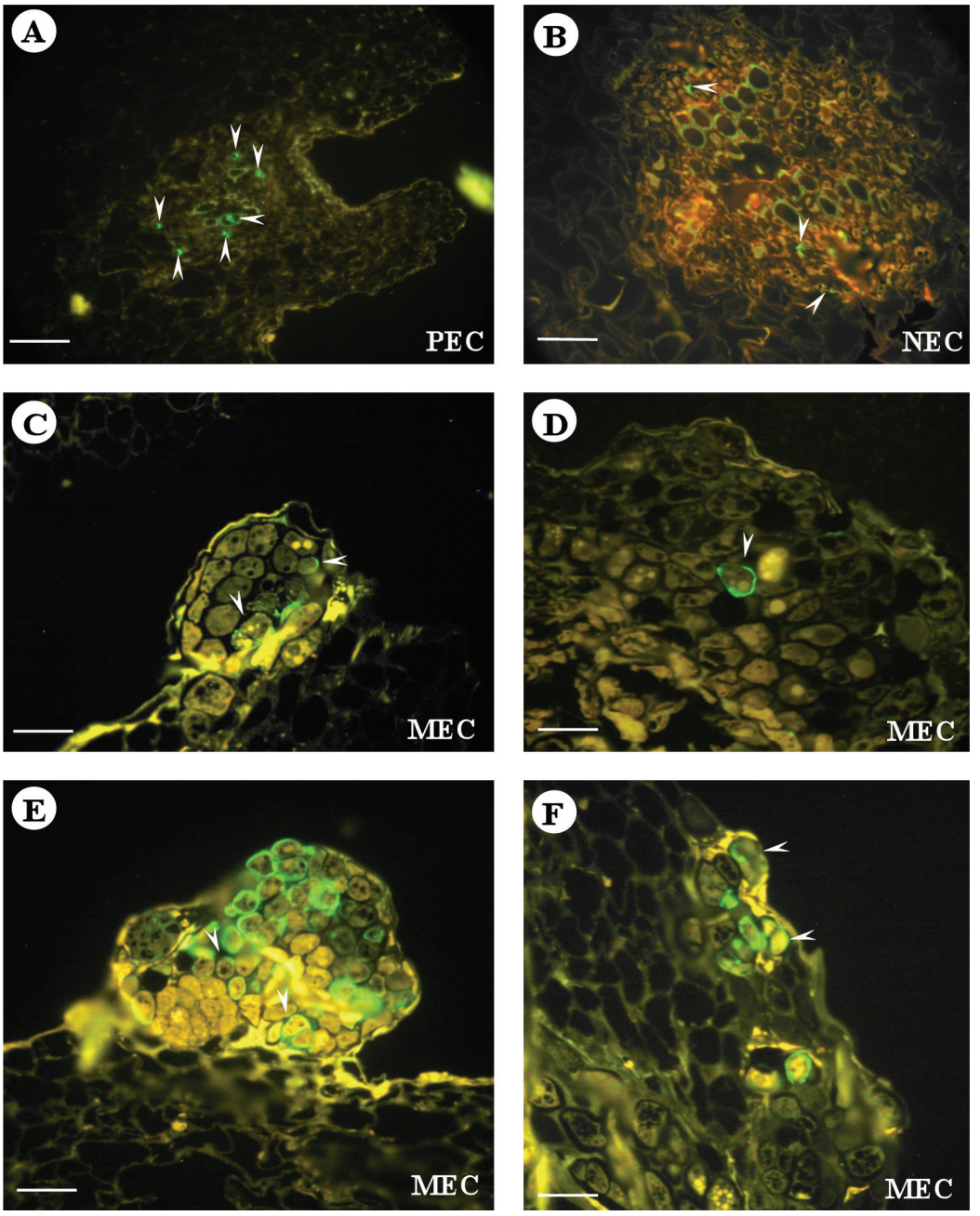

*Fig. 4: Immunolocalisation of JIM8 epitope in sections of pre-embryogenic (PEC), mature embryogenic (MEC) and non-embryogenic (NEC) tissue of oilsedd rape. A, B, Hypocotyls of PEC and NEC showing bright green fluorescence only in the vascular tissues. Bars=200*µm *and 100*µm *respectively. C, E, F, Immunofluorescence over preembryoid on the epidermal cell layer of MEC tissue and arrows denote uneven labelling on the cell wall. Bars= 50*µm, *30*µm *and 50*µm *respectively. D, uneven immunofluorescence signal in the cell wall of one of the subepidermal cells. Bar=50*µm. *C, cortex; ep, epidermis; P, pith; pe, proembryoid*

in the vascular tissues or in the walls of large clusters of cells in the epidermis. Transverse and longitudinal sections of the sample tissues were stained with Azure A/methylene blue for anatomical studies (*Fig. 2*).

Immunolocalisation with JIM4

Immunolocalisation of the JIM4 epitope showed no immunolabelling signal in all tested samples (*Fig. 3A-C*). The mAb JIM4 was originally generated by immunisation with carrot protoplasts. The antibody has been shown to recognise AGP epitopes from the medium of suspension cultured carrot cells, gum arabic, and other AGP-like molecules (Knox *et al.*, 1989). Although fluorescence was observed in the vascular tissues in NEC tissue (*Fig. 3C*) this was attributable to autofluorescence since untreated sections also gave similar results (*Fig. 1A*). An attempt at immunolocalisation of the JIM4 epitope was also negative for the extracellular matrix layer of the PEC (*Fig. 3A*) and MEC (*Fig. 3B*) tissues.

Immunolocalisation with JIM8

JIM8 epitopes were detected in all the tested samples and the pattern of recognition varied between different types of tissues and stages of development. No immunolabelling was ever detected in the ECM layer of all the tissues tested. mAb JIM8 was originally developed from an immunization with sugar beet protoplasts (Pennell *et al*., 1991). JIM8 recognizes a carbohydrate epitope present in the plasma membrane arabinogalactan proteins in sugar beet leaves (Pennell *et al*., 1991) and labels several cell types in carrot embryogenic suspension cultures at the cell wall (Pennell *et al*., 1992; Toonen *et al*., 1996; McCabe *et al*., 1997). In this study, two types of labelling patterns were observed with mAb JIM8. This antibody labelled the walls of certain cells in a few proembryoids of the mature embryogenic tissue (*Fig. 4C, E* and *F*), and also labelled the vascular tissues (*Fig. 4A, B*) (*see* arrowheads) of all the tested samples. In the MEC tissue (*Fig. 4C, E, F*), proembryoids are actually clusters of embryogenic cells comprising small, spherical or oval-shaped cells with dense cytoplasm. Nonetheless, only some of those cells had JIM8-reactive cell walls although all the cells were parts of the same cell cluster. Moreover, no obvious morphological/histological difference was found between the JIM8 reactive and non-reactive cells. In addition, some of the JIM8 reactive cells only contained patches (*Fig. 4 C-F* see arrowheads) of FITC fluorescence in the cell wall. No immunolabelling was observed in any of the randomly distributed and densely cytoplasmic cells in the pre-embryogenic tissue or in epidermal or cortical parenchyma cells of NEC tissues (data not shown). However, bright green fluorescence labelling was detected in vascular tissues of PEC and NEC tissues, as shown in *Fig. 4A* and *B* (*see* arrowheads) and the same labelling pattern of JIM8 was observed in the vascular tissues of MEC (data not shown).

Immunolocalisation with JIM13

Immunofluorescence examination indicated that JIM13 epitope could be detected in all the tested tissues but it showed a pattern of expression restricted to specific tissues and developmental stage. JIM13 mAb was raised against the AGP fraction isolated from conditioned medium of an embryogenic cell suspension of carrot (Knox *et al*., 1991). In particular, JIM13 epitope was frequently found in the MEC tissue, almost throughout the whole region of the pre-embryo stage, labelling majority of the cell walls, cytoplasm and occasionally tiny patches of bright green fluorescence were found in the vacuole (*Fig. 5C*). There was also weak immunofluorescence detected in the ECM layer coating the proembryoid (indicated by the arrowheads in *Fig. 5C*), but not on the ECM layer over the epidermal cells (*see* tiny arrows in *Fig. 5C*). Based on the labelling intensity and binding pattern of JIM13, it appeared that the more vacuolated cells were reactive to JIM13 as compared to densely cytoplasmic cells in proembryoids (compare *Fig. 5C* and *Fig. 5D*). Another notable feature was that

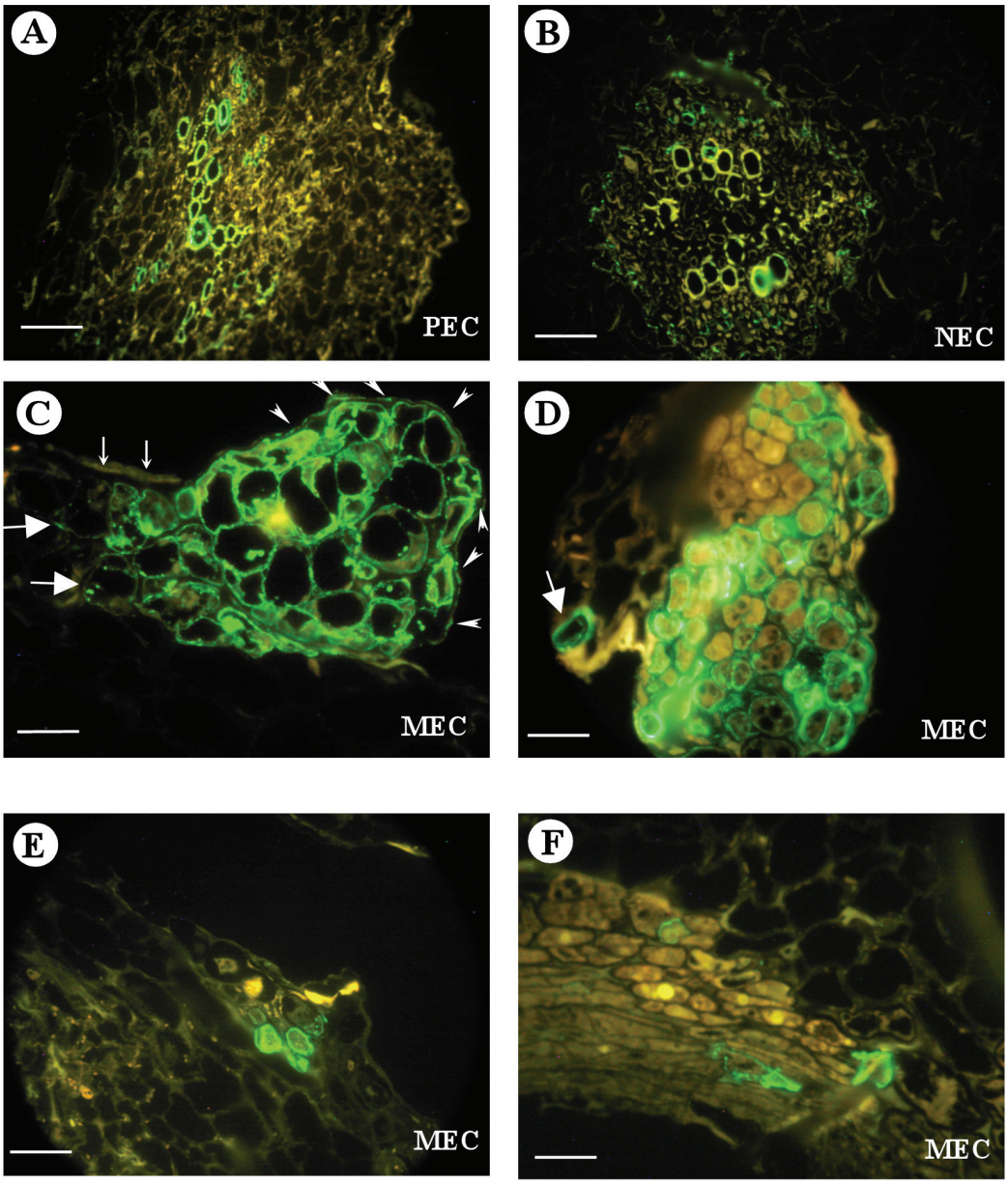

*Fig. 5: Immunolocalisation of JIM13 epitope in sections of pre-embryogenic (PEC), mature embryogenic (MEC) and non-embryogenic (NEC) tissue of oilseed rape. A, B, Immunolabelling of PEC and NEC hypocotyls showing bright green fluorescence particularly in xylem and putative phloem cells in the vascular tissues. Bars = 120um and 50um respectively. C, A proembryoid with bright green fluorescence in all the cells and weak immunofluorescence (shown by arrowheads) in the ECM layer coveing the proembryoid. Bar=25*μ*m. D, A, proembryoid with only some cells that showed positive signal to JIM13 antibodies. Bar=25*μ*m. E, F, Immunolabelling in a few epidermal and subepidermlal cells at the periphery of the MEC tissue. Bars=120*μ*m. C, cortex; e, epidermis; se, sub-epidermis; P, pith; pe, proembryoid*

immunolabelling was not detected throughout the mature or later stages of somatic embryoids, but restricted to some regions of the embryoid (*Fig. 5D*). Furthermore, immunofluorescence was also found in cells adjacent to the developing embryo (*Fig. 5C, D* see big arrows). Bright green fluorescence signal was also observed in a few epidermal and sub-epidermal cells at the periphery of MEC tissue (*Fig. 5E*). In the pre-embryogenic tissue, JIM13 epitope was not detected in the ECM layer or any of the epidermal cells or cells with dense cytoplasm (*Fig. 5A*), indicating that the epitope is not expressed during the early stages of embryogenic tissue development. In addition, for all the tested samples, the labelling experiment also showed the presence of JIM13 epitope in vascular tissues, particularly xylem cells with thick cell walls (*Fig. 5A, B*).

DISCUSSION

In the winter oilseed rape embryogenic culture, immunofluorescent light microscopy investigations using mAbs JIM4, JIM8, and JIM13 revealed that only JIM13 epitope was present within the ECM layer covering the proembryoid. Interestingly, JIM13 epitope was not detectable in the ECM layer at an earlier stage in the pre-embryogenic tissue, suggesting that JIM13 epitope is developmentally regulated. Similar observations were reported for Cichorium where an immunolabelling investigation using mAb JIM13 revealed that AGPs are present within the outer cell layer of young globular somatic embryos and as the embryos developed further, AGP expression was observed in the inner region of the walls of embryogenic cells (Chapman *et al*., 2000). In hybrid fir, specific AGPs containing β-(1→6)galactotetraosyl group was reported to be a component of ECM covering embryogenic cells of gymnosperm (Samaj *et al.*, 2008).

It is surprising that there was no labelling by JIM4 mAb in the ECM layer of the winter oilseed rape embryogenic culture because JIM4 epitope has been shown to be present in the ECM layer of maize embryogenic callus cells (Samaj *et al.*, 1999b). The authors also suggested that JIM4 antibody could be used as an early marker for embryogenic competence in maize callus cells. The absence of JIM4 epitope in the ECM layer of the winter oilseed rape embryogenic culture indicates a possible variability in the composition of the ECM layer among the different plant species. Similarly, JIM4 epitope was not detected in the ECM layer of androgenic callus of wheat (Konieczny *et al*., 2007) and AGPs of sugar beet cultures (Wisniewska and Majewska-Sakwa, 2007). Moreover, there was no positive control for immunolabelling with JIM4. Without it, it is possible that the activity of JIM4 mAb may be too low to detect the presence of JIM4 epitopes in the tissue samples tested.

The monoclonal antibody JIM8 has been shown to react with AGP epitopes in sexual organs, the eight celled embryo and a very limited number of other cell types in oilseed rape (Pennell *et al*., 1991). Recently, Coimbra and Salema (1997) have shown that young zygotic embryos and suspensor cells of *Amaranthus hypochondriachus* are reactive to JIM8. In carrot suspension cultures, JIM8 epitope has been localised on three different cell membrane AGPs (Pennell *et al.*, 1991) and on AGPs secreted into the suspension culture (Knox *et al*., 1991). It has been postulated that JIM8 epitope is a marker for embryogenic capacity of a cell culture as a whole, rather than for a competent cell state (Toonen *et al*., 1996). All these studies support the idea that AGPs play an important role in morphogenesis during zygotic and somatic embryogenesis.

The detection of the green fluorescent labelling with JIM8 and JIM13 in the vascular tissues of both embryogenic and non-embryogenic hypocotyls is consistent with the findings of some previous studies. Among other, JIM13 epitope was found to correlate with the early development of xylem in the roots of Arabidopsis (Dolan *et al*., 1995) and carrot (Knox *et al*., 1991) as well as xylem maturation in maize coleoptiles (Schindler *et al*., 1995), radish roots and carrot roots (Casero *et al*., 1998), and it has also been proposed that AGPs can identify cells committed to programmed cell

death. More recently, JIM8 and JIM13 epitopes were detected in protophloem sieve elements in maize roots (Samaj *et al*., 1998).

JIM8 and JIM13 epitopes were found scattered randomly in some of the densely cytoplasmic cells in the proembryoid and globular stage embryoids in mature embryogenic tissues. Interestingly, those cells expressed these epitopes on the cell wall or plasma membrane only at the later stages of development and not at the earlier pre-embryogenic stage. This implies that the expression of AGP epitopes is being developmentally regulated during the secondary embryogenesis in the winter oilseed rape embryogenic culture, as observed in other plant systems including *Picea abies* (Filonova *et al*., 2000; Egertsdotter and von Arnold, 1995), carrot cultures (Toonen *et al*., 1996; McCabe *et al*., 1997; Stacey *et al*., 1990; Pennel *et al*., 1992) and Cichorium (Chapman *et al*., 2000). However, the significance of AGP epitopes in some of the proembryoid or cytoplasm-rich cells or globular stage embryoids is not clear. There is a possibility that the cells with specific AGP epitopes may be involved in cell-cell recognition and maintenance of close cell contacts during the formation of proembryoids. The fact that anti-AGP mAbs (JIM8 and JIM13) labelled some of the epidermal or sub-epidermal cells in the embryogenic tissue and not the epidermal cells of the non-embryogenic tissue is an interesting observation, implying that AGPs may have a specific role in embryogenic competency or determining the cell fate of embryogenic cells. For future work, it will be interesting to see the three-dimensional immunolocalisation of the AGP antigens on the ECM layer using silver enhanced immunogold scanning electron microscopy (Samaj *et al.*, 1999a).

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Genetic Divergence of Rice on Some Morphological and Physiochemical Responses to Drought Stress

Rajiv, S., Thivendran, P. and Deivanai, S.*
Department of Biotechnology, Faculty of Applied Sciences,
AIMST University, Semeling Campus, 08100 Bedong,
Kedah, Malaysia
**E-mail: deivanai@aimst.edu.my*

ABSTRACT

Twelve Malaysian rice genotypes were evaluated for drought-related morphological and physiochemical responses to determine the degree of genetic divergence. Preliminary investigation showed a considerable reduction in plant growth, total chlorophyll content, chlorophyll stability index (CSI %), and total soluble protein in all the genotypes studied. On the other hand, a sharp increase in the accumulation of proline was also noticed. An analysis of variance revealed significant variations for those traits among the genotypes, which was adequate for the estimation of genetic diversity. Hierarchical cluster analysis of multivariate approach was performed for the genotypes exposed to water deficit stress as well as for the control conditions. Meanwhile, the genotypes were classified into groups based on the deferential responses. However, the analysis was unable to reveal how sensitive or tolerant the genotypes to drought condition, hence a discriminant functional analysis was carried out. The result obtained from canonical discriminant function clearly distinguished the genotypes based on sensitivity to drought stress. Furthermore, the study demonstrated the relevance of morphological and physiochemical responses in screening drought tolerance in rice. Hence, it is suggested that discriminant functional analysis can be used as a potential screening tool to identify drought tolerance genotypes at early stages in rice.

Keywords: Free proline, total soluble protein, genetic divergence, multivariate analysis, drought responses in Malaysian rice

INTRODUCTION

Drought is one of the major factors limiting rice production worldwide. Uneven distribution of rainfall makes rice growers to depend heavily on irrigation. However, increasing the frequency of irrigational input is not possible due to water shortage and inadequate management of infrastructures (Wardlaw, 2000; Llorens *et al*., 2004; Ober *et al*., 2005; Flexas *et al.*, 2006). Therefore, in order to sustaining crop production, it is essential to have improved rice varieties with less sensitivity to water deficit condition.

Improving drought tolerance and productivity is the most difficult task for cereal breeders because of the diverse strategies adopted by plants at various stages of development among the species and cultivars to cope with water stress (Chaves *et al*., 2003). It has been reported (Mansfield and Atkinson, 1990; Nayyar and Gupta, 2006; Yang *et al*., 2006) that the first and foremost response of plants to acute water deficit is the Stomatal closure to prevent transpiration loss, and it has primarily resulted in a reduction in the photosynthesis rate. Fisher

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*Corresponding Author

et al. (1998) found that stomatal conductance and rate of photosynthesis were positively correlated with the increase in yield in wheat cultivars. Meanwhile, Sibournheuang *et al.* (2006) observed genotypic variation among the rice cultivars for leaf water potential (LWP) and suggested that it might be due to the differences in stomatal conductance or differences in the root water uptake.

Several reports (Morgan, 1984; Hoekstra *et al.*, 2001; Ramanjulu and Bartels, 2002; Mahajan and Tuteja, 2005) showed that water deficit condition has resulted in the loss of structural integrity of membrane, disruption in cellular compartmentalization and reduction in enzyme activity. Therefore, to combat the adverse effect, plant synthesis accumulates various compounds such as sugars, amino acids, inorganic ions, and organic acids. These compounds help to maintain their hydrated state in the cell and provide resistance against drought and cellular dehydration.

In general, responses to drought are numerous and interconnected. It is well-established that drought stress impairs numerous metabolic and physiological processes in plant which ultimately resulted in reduce plant growth, loss of chlorophyll pigments, accumulation of osmolytes, etc. (Lima *et al*., 2002; Colom and Vazzana, 2003; Souza *et al*., 2004; Ekmekci *et al.*, 2005; Li *et al*., 2006; Nayyar and Gupta, 2006; Yang *et al*., 2006; Efeoglu *et al*., 2009).

Despite the great deal of research in the physiology of drought, only limited information is available on genetic background. Many scientists (e.g. Rush and Epstein, 1976; Greenway and Munns, 1980; Wyn Jones, 1981; Epistein and Rains, 1987; Cheeseman, 1988; Jacoby, 1999; Shannon and Greve, 1999; Ashraf, 2002; Munns, 2002) have suggested that the physiology of stress would offer valuable information to design efficient and accurate screening techniques for improving drought tolerant traits. Difficulty in breeding complex traits could thus be resolved by identifying reliable morphological and physiological characters that are closely linked to yield in water limiting environment and by integrating the approach of stress physiology with molecular genetics (Tuberosa *et al*., 2002; Ober *et al*., 2005). However, the effectiveness of selection primarily depends on the magnitude of genetic variability present in the breeding material. Pradhan and Ray (1990), as well as Roy and Panwar (1993) emphasized the importance of genetic divergence for the selection of suitable genotypes. Knowledge on genetic diversity maximizes the exploitation of the germplasm resources (Belaj *et al*., 2002; Rasul and Okubo, 2002) and it can be estimated through multivariate approach. Multivariate analysis is a useful tool to quantify the extent of divergence at genetic level. This approach visualizes the interaction between the genotype and traits involved in the study, and it thus provides information about the superior and inferior genotypes (Ober *et al*., 2005). The present study was undertaken to determine the degree of genotypic diversity for drought-related morphological and physiochemical traits such as plant growth, chlorophyll content, chlorophyll stability index, proline and protein using multivariate analysis, and to determine whether these traits can be used to select drought tolerance genotypes at early stage in rice.

MATERIALS AND METHODS

Twelve rice genotypes, namely MR167, MR211, MR219, MR220, MR232, Mahawi, Bahagia, Makmur, Seberang, Ria, Masqia, Gaya, grown in 30 cm x 30 cm plastic pots filled with clay loam soil, were obtained from the Malaysian Agricultural Research and Development Institute (MARDI). The seeds of the rice varieties were planted in pots of 30 cm diameter and 30 cm height, filled with clay loam soil and arranged in randomized block design with three replications. Each replication consisted of five pots. About ten seeds were planted in each pot at 2-3 cm depth. Two weeks after sowing, the seedlings were thinned to five plants per pot. In each pot, about 5 cm of the standing water was maintained and 10 g of slow release commercial fertilizer (15%N, 15%P and 15%K) was then added to maintain a healthy crop stand. Two sets of the experimental materials were maintained; one

was kept as a control and the other for water stress treatment. Drought was initiated 45 days after sowing (45 DAS) by withholding water for a period of 7-10 days. The physiological parameters were measured for both the control and water-stressed plants at the onset of drought initiation. Plants were uprooted carefully after the start of the drought treatment and then separated into root and shoot. Root and shoot lengths were measured (cm), while the number of leaves was counted for each rice genotypes, and the data were also recoded.

DETERMINATION OF THE TOTAL CHLOROPHYLL CONTENT

Chlorophyll content was determined by following the methods of Harbone (1984). Leaf tissue (500 mg) was homogenized in 80% chilled acetone. After appropriate dilution, the chlorophyll levels in the supernatant were determined spectrophotometrically using the following formula:

Total chlorophyll content (mg ml^{-1}) = 17.3 A_{646} + 7.18 A_{663}

Chlorophyll *a* (mg ml^{-1}) = 12.21 A_{663} – 2.81 A.646

ESTIMATION OF CHLOROPHYLL STABILITY INDEX (CSI)

CSI in the leaf was estimated using a spectrometer, following the method of Koleyoreas (1958). Two leaf samples of 250 mg each were put in two test tubes containing 10 ml of distilled water. One of the test tubes was placed in a water bath and heated to 65°C for 30 minutes while the other was kept as a control. Then, the total chlorophyll content was estimated using a spectrophotometer at 652 nm (Koleyoreas, 1958). CSI was calculated using the following formula:

$$\text{CSI (\%)} = \frac{\text{Total chlorophyll content (heated)}}{\text{Total chlorophyll content (control)}} \times 100$$

ESTIMATION OF FREE PROLINE CONTENT

Proline was determined following the procedure by Bates *et al*. (1973). A fresh leaf sample (0.5 g) was homogenized in 5 ml of 3% sulphosalicylic acid and the homogenate was centrifuged at 9000 xg. The reaction mixture consisted of 2 ml of the supernatant, 2 ml of acid ninhydrin (1.25 g ninhydrin dissolved in 30 ml of glacial acetic acid, and 20 ml of 6M orthoposphoric acid) and 2 ml of glacial acetic acid which was boiled at 100°C for 1 h. After termination of the reaction on ice, the reaction mixture was extracted with 4 ml of toluene, and the absorbance was read at 520 nm.

ESTIMATION OF THE TOTAL PROTEIN

About 1.0 g of leaf tissue was ground in cold mortar. The grinding medium (4-6 ml/g fresh mass) consisted of 50 mM Tris-HCL buffer (pH 8.0), 1 mM PMSF, 10% (v/v) glycerol and homogenizing beads. The homogenate was filtered through four layers of cheesecloth and centrifuged at 12000 rpm for 20 minutes at 4°C and the supernatant was taken. An aliquot of the extract was used for protein concentration, following the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

STATISTICAL ANALYSIS

The experiment was performed using a randomized block design with three replications. The multivariate analysis was carried out for control as well as drought stress induced genotypes to assess the differences between the stress induced and control. Meanwhile, the statistical variance analysis was performed using ANOVA and compared with the least significant differences (LSD) at 5% level. Grouping of genotypes was done using the SPSS (Version. 11) statistical programme.

TABLE 1A
Mean (± SE) of the morphological traits of twelve rice genotypes recoded for the control and water stressed conditions

S. no	Rice varieties	Shoot length (cm)			Root length (cm)			Ratio (root/shoot)			No. of leaves/ plant		
		control	stress	C-S	control	stress	C-S	control	stress	C-S	control	stress	C-S
1	MR 167	20.6 ± 2.19	17.8 ± 4.21	2.8	10.4 ±1.14	10.0 ± 1.87	0.4	0.505	0.562	-0.055	5.4 ± 1.14	5.0 ±1.00	0.4
2	MR 211	21.8 ± 2.77	21.2 ± 1.92	0.6	6.6 ± 1.14	7.8 ± 1.92	-1.2	0.303	0.368	-0.065	4.8 ± 0.84	4.6 ± 1.14	0.2
3	MR 232	13.6 ± 1.67	16.6 ± 2.07	-3.0	8.0 ± 1.58	9.4 ± 1.14	-1.4	0.588	0.566	0.022	9.4 ± 2.30	6.6 ± 2.41	2.8
4	MR 219	21.2 ± 1.30	7.6 ± 1.52	13.6	6.2 ± 0.84	8.8 ± 1.30	-2.6	0.292	1.158	-0.865	5.4 ± 1.52	7.8 ± 0.84	-2.4
5	MR 220	25.4 ± 3.85	24.0 ± 1.58	1.4	11.0 ± 0.79	11.9 ± 0.61	-0.86	0.433	0.494	-0.061	6.2 ± 2.49	6.4 ± 2.07	-0.2
6	Mahawi	20.8 ± 1.48	19.0 ± 1.58	1.8	10.9 ± 0.89	9.6 ± 1.52	1.3	0.524	0.505	0.019	3.2 ± 0.84	3.4 ± 0.55	-0.2
7	Bahagia	21.0 ± 3.54	17.2 ± 1.92	3.8	9.6 ± 1.14	9.4 ± 2.07	0.2	0.457	0.546	-0.089	5.2 ± 1.92	4.4 ± 1.67	0.8
8	Makmur	16.2 ± 4.15	16.4 ± 2.07	-0.2	10.0 ± 1.58	10.2 ± 1.92	-0.2	0.617	0.622	-0.005	5.0 ± 1.58	4.8 ± 1.79	0.2
9	Seberang	15.0 ± 3.39	14.0 ± 2.55	1.0	7.2 ± 1.92	7.6 ± 1.50	-0.44	0.480	0.546	-0.06	8.0 ± 2.55	7.6 ± 1.14	0.4
10	Ria	33.6 ± 3.05	9.8 ± 1.48	23.8	7.2 ± 0.84	9.0 ± 1.58	-1.8	0.214	0.918	-0.704	7.2 ± 0.84	6.4 ± 1.14	0.8
11	Masqia	37.2 ± 1.92	9.4 ± 1.52	27.8	6.6 ± 0.55	10.4 ± 1.14	-3.8	0.177	1.106	-0.929	6.8 ± 0.84	6.2 ± 0.84	0.6
12	Gaya	45.2 ± 2.39	41.2 ± 2.17	4.0	7.8 ± 2.28	11.0 ± 2.00	-3.2	0.173	0.267	-0.094	6.8 ± 0.84	5.2 ± 1.64	1.6

TABLE 1B
Mean (± SE) of chlorophyll pigment content of twelve rice genotypes for the control and water stressed conditions

S. no	Rice varieties	Chlorophyll a			Chlorophyll b			Ratio between Chlorophyll a/b		
		control	stress	C-S	control	stress	C-S	control	stress	C-S
1	MR 167	7.86 ± 0.05	6.04 ± 0.11	1.82	5.90 ± 0.37	2.16 ± 0.14	3.74	1.33	2.79	-1.46
2	MR 211	3.88 ± 0.04	2.38 ± 0.08	1.50	1.53 ± 0.06	0.99 ± 0.12	0.54	2.54	2.40	0.14
3	MR 232	8.01 ± 0.04	0.13 ± 0.03	7.87	12.35 ± 0.09	11.88 ± 0.13	0.47	0.65	0.01	0.64
4	MR 219	6.94 ± 0.35	5.39 ± 0.09	1.54	2.48 ± 0.17	1.20 ± 0.05	1.28	2.80	4.49	-1.69
5	MR 220	8.81 ± 0.32	6.80 ± 0.03	2.01	6.38 ± 0.15	3.34 ± 0.08	3.04	1.38	2.03	-0.65
6	Mahawi	9.72 ± 0.14	8.40 ± 0.02	1.32	3.07 ± 0.19	0.60 ± 0.04	2.48	3.17	14.0	-10.83
7	Bahagia	5.09 ± 0.24	3.83 ± 0.04	1.25	1.33 ± 0.22	0.36 ± 0.17	0.97	3.82	10.64	-6.82
8	Makmur	7.58 ± 0.30	5.80 ± 0.08	1.77	3.02 ± 0.14	2.50 ± 0.25	0.51	2.51	2.32	0.19
9	Seberang	6.59 ± 0.80	3.16 ± 0.02	3.43	2.44 ± 0.33	0.25 ± 0.07	2.19	2.70	12.64	-9.94
10	Ria	0.72 ± 0.16	0.60 ± 0.01	0.12	10.11 ± 0.02	9.65 ± 0.15	0.46	0.07	0.06	0.01
11	Masqia	9.55 ± 0.33	5.20 ± 0.02	4.35	4.99 ± 0.02	3.75 ± 0.16	1.24	1.91	1.39	0.52
12	Gaya	4.48 ± 0.11	3.64 ± 0.04	0.84	1.41 ± 0.09	1.41 ± 0.05	0.00	3.18	2.58	0.6

RESULTS AND DISCUSSION

The results obtained in the study revealed that drought had caused considerable morphological and physiochemical changes in plant growth, chlorophyll content, chlorophyll stability index, proline, and protein content (Tables 1a and 1b; *Fig. 1a, b, c* and *d*). Meanwhile, the severity of the drought affects the plant growth and it was measured by reduction in root and shoot length. All the genotypes had registered significant differences ($p<0.05$) for shoot length, root length and root to shoot ratio, but the extent of variation was strongly cultivar dependent (Table 1a). Among the genotypes, Masqia, Ria and MR 291 had shown greater reductions in shoot length under drought stress condition. However, the same genotypes had recorded increases in the root length and root to shoot ratio due to water deficit condition. As for Mukmur, the plant growth was not affected by drought stress.

The study showed that the length of seedling was significantly shorter than the length of the control. In their study, Nayyar and Gupta (2006) reported that leaf growth was inhibited relatively

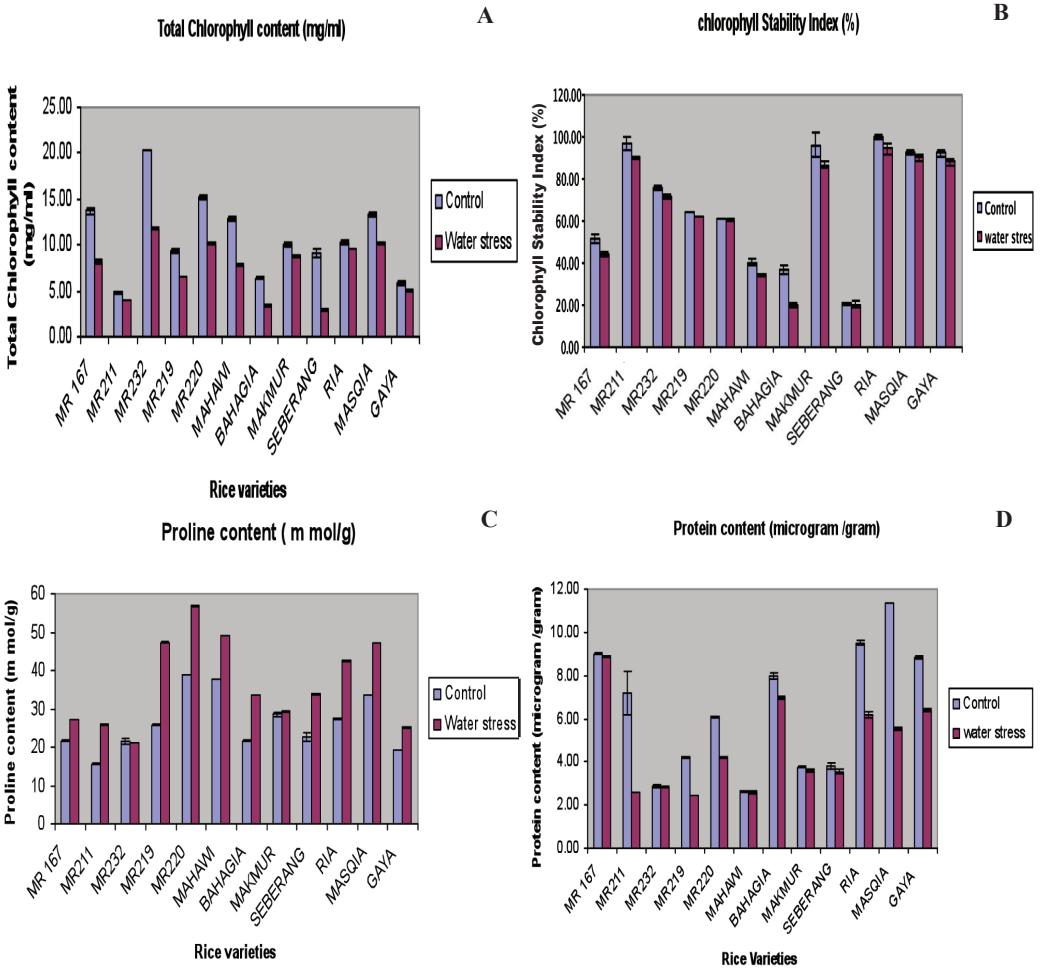

Fig. 1: Effects of drought stress on certain morphological and physiochemical parameters of 12 rice genotypes; A) Total chlorophyll content (mg/g), B) Chlorophyll Stability Index (%), C) Proline concentration (µg/ g) and D) Protein content (µg/ g). All the treatments differed significantly from the control ($p < 0.05$)

more than root growth in a stressed environment. Changes in plant growth were also recorded for Masqia, Ria, and MR219 in response to drought exhibited primary signal for drought adaptation. Plant growth is one of the most drought sensitive physiological processes due to the reduction of turgor pressure. In particular, water stress greatly suppresses cell expansion and growth due to the low turgor pressure (Kartikeyan *et al*., 2007; Jaleel *et al*., 2007; Manivannan *et al.*, 2007).

Therefore, to understand the photosynthetic ability of the genotypes studied under water deficit condition, chlorophyll a, chlorophyll b and total chlorophyll content were determined. A significant reduction in the chlorophyll content (a, b and a + b) was noticed in all the rice genotypes under stressed condition (Table 1b). Meanwhile, the total chlorophyll content showed differences which ranged from 8.60 mg g^{-1} FW in MR 232 to 0.8460 mg g^{-1} FW in Gaya (Table 1b) for both the control and stressed plants. A strong decline in chlorophyll a content was noticed for MR 232, Masqia, and Seberang. Depletion in chlorophyll a indicates that the drought stress impairs photosynthetic reaction centres but the ill effect was compensated by the increase in chlorophyll b for all these genotypes, except for Seberang. Furthermore, the ratio of chlorophyll a/b was also found to be less for these genotypes. On the contrary, a few genotypes, namely Mahawi, Seberang, and Bahagia had very high ratio for this particular trait under water-stressed condition. Some previous studies indicated that drought tolerant genotypes were able to maintain a higher chlorophyll content than the susceptible genotypes. The changes in the chlorophyll a/b ratio were less for these genotypes. Cicek and Cakirlar (2008) reported that the soybean salt stressed cultivars seemed to adapt to the stress by reducing their chl a/b ratio. Accordingly, the genotypes MR 232 and Masqia were found to be tolerant under water stress, whereas Mahawi, Seberang and Bahagia recorded very high chl a/b ratios and they might be sensitive to drought stress.

In addition, the heat stability of chlorophyll pigments has been described as an index for drought tolerance in plants. There was a general decreasing trend observed for CSI (%) in all the genotypes due to drought stress (*Fig. 1b*). A greater reduction was noticed for Bahagia, Makmur, MR 167 and MR 211, whereas slight decreases in CSI (%) were found in Seberang, MR 220, and MR 219.

The high CSI value obtained in the result indicated a better availability of chlorophyll in the plant that helps to withstand stress. On the contrary, Ali *et al*. (2008) reported that low CSI value and high sink strength were found to directly correlate with the productivity of pearl millet cultivars.

Proline is one of the amino acid which appears more commonly in response to stress. There was a steep increase in the proline content in all the genotypes (*Fig.1c*). The differences in accumulation of proline ranged from 0.25 m mol/ FW to 21.49 m mol/ FW. A slight increase in the accumulation of proline was also noticed in MR 232, while the maximum was recoded in MR 219. The synthesis of osmolyte, including proline, is widely used by plants to stabilize membranes and maintain the conformation of proteins at low leaf water potentials. Proline is known to be involved in reducing photo damage in thalakoid membranes by scavenging and/or reducing the production of O_2 (Reddy, 2004). Furthermore, proline plays a role as enzyme stabilizing agent and has the ability to mediate osmotic adjustment and stabilize sub-cellular structure (Hassanein, 2004; Yokota *et al*., 2006). The values of free proline content appear to be related to tolerance, however, the synthesis and accumulation of proline have been found to vary among the cultivars. Zhu (2001) suggested that lower accumulation of osmolyte function in protecting macromolecules either by protecting the tertiary structure of protein or by scavenging ROS (reactive oxygen species) produced in response to drought. The accumulation of proline was invariably observed in all the genotypes under stress and it was

TABLE 2
Analysis of variance for some morphological and physiochemical traits of 12 rice genotypes subjected to water deficit condition and controlled condition

Traits	Df	Mean sum of square		Error sum of square		F ratio		Sig (P<0.05)
		Control	Stress	Control	Stress	Control	Stress	0.00
Root length (cm)	11	16.22	7.45	1.744	2.577	9.30*	2.89*	0.00
Shoot length (cm)	11	459.18	388.99	7.825	4.725	58.68*	82.33*	0.00
No. of leaves/ plant	11	13.54	9.000	2.60	2.117	5.19*	4.25*	0.00
Chlorophyll A (mg/g)	11	34.88	32.19	0.099	1.923	353.00*	16.74*	0.00
Chlorophyll B (mg /g)	11	63.50	70.22	0.035	1.923	1806.38*	4109.20*	0.00
Total chlorophyll (mg / g)	11	98.89	43.30	0.061	0 .009	1593.93*	4561.09*	0.00
CSI (%)	11	3729.66	3894.84	4.593	1.518	812.06*	2565.69*	0.00
Proline (m mol /g)	11	261.1	652.74	0.708	0 .221	368.87**	2949.67*	0.00
Protein (µg/ g)	11	43.17	21.96	0.092	0.005	469.47	4632.08*	0.00

*Significance at p= 0.05 level

found to be higher in stress-sensitive genotype. This result is in accordance with an earlier observation reported for other species such as cassava (Sundaresan and Sudhakaran,1995), Mediterranean scrub (Ain-Lhout *et al*., 2001), European beech (Peuke *et al*., 2002), and wheat (Rampino *et al*., 2006).

There was a general decreasing trend for the total soluble protein content in all the genotypes due to water deficit stress (*Fig. 1d*). A greater reduction was noticed in Masqia followed by MR 211. Sarhan and Perras (1987) suggested that the quantitative changes in polypeptides might be responsible for the adjustments in metabolic pathways under stressed condition. This feature can be used as an indicator for improving stress tolerance (Pareek *et al*., 1997), depending on the nature of cultivar.

The result presented in Table 2 revealed an adequate significant genetic variation ($p<0.05$) for the morphological and physiochemical responses of drought stress. Genetic diversity was then estimated for these responses using the multivariate analysis. Multivariate approach helps to visualize the relationship between the genotypes with traits and presents a picture of superior and inferior genotypes. Meanwhile, clustering of genotypes was established based on the Euclidean distance matrix derived from standardized data and the results obtained are presented in *Figs. 2a* and *2b*. The grouping pattern indicated in *Figs. 2a* and *2b* has revealed the response of the genotypes towards water deficit condition. All the traits tested using the Wilk's criteria have shown pronounced differences among the genotypes. The principal component analysis yielded eight functional eigenvalue for the control and six values for the stress induced genotypes (Tables 3a and 3b). The first two principal axes accounted for 82.1% of the total variation in control, while 68.6% in the stress-induced genotypes. From the data presented in Tables 3a and 3b, it is evident that the characters shoot length, chlorophyll b, total chlorophyll content, CSI (%), proline and protein content recorded greater eigenvalue. In

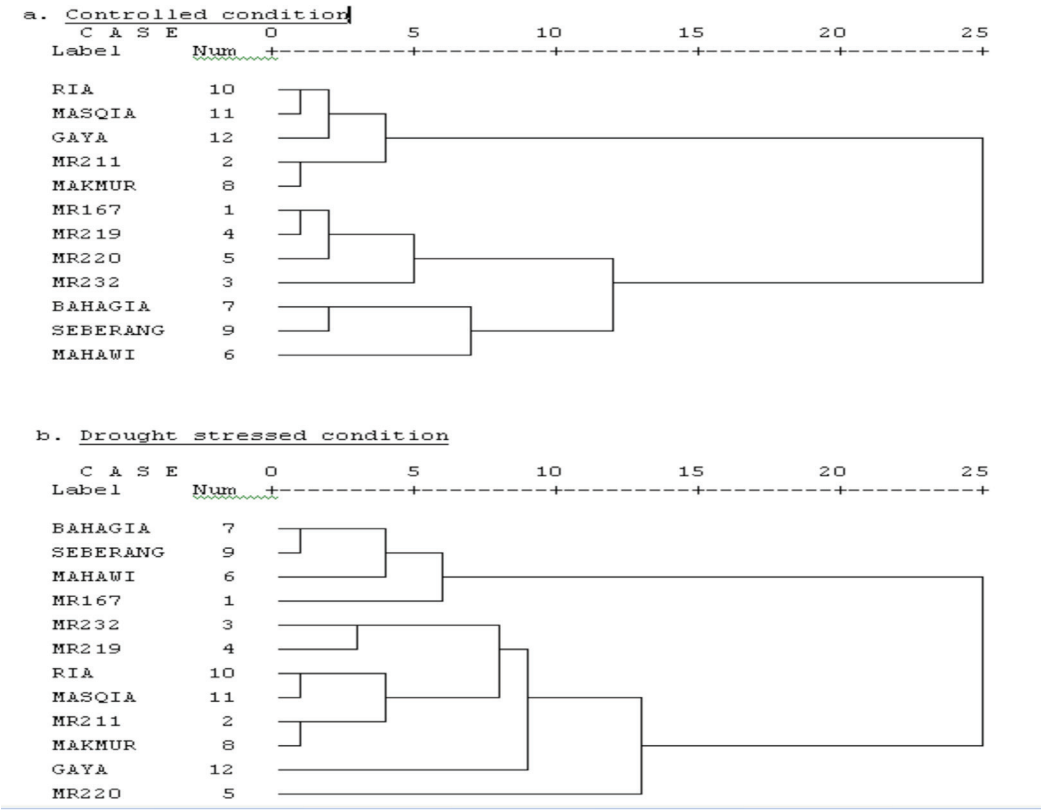

Fig. 2: Dendrogram of hierarchical cluster analysis depicting grouping in rice genotypes based on the morphological and physiochemical responses in the controlled and stress induced conditions. 2a) Grouping of genotypes under controlled condition. 2b) Grouping of genotypes under drought stressed condition

more specific, the large eigenvalue obtained in the study explained that the proportion of variance is associated with a strong function, and it indicates the proportion between the group and within group sum of squares. These values are related to the canonical correlations and they also describe how much discriminating ability a function possesses. Meanwhile, a large eigenvalue is associated with strong function, and thus, the traits involved in the study have contributed more towards genetic divergence. The investigation had further accounted for 53.5% of the first canonical root in controlled condition and 41.9% in stressed condition. Based on the values of the principal component scores, grouping was performed using the hierarchical cluster analysis, as depicted in *Figs. 2a* and *2b.* The position of genotypes in the dendrogram was apparently distributed into three groups in the control and six groups in the drought induced. The result obtained from the cluster analysis clearly revealed the differential responses of the genotypes under stress. However, the hierarchical cluster analysis was unable to state the nature of the genotype responses, i.e. how sensitive or tolerant to drought stresses. The data were further subjected to the discriminant function analysis to determine the magnitude of discriminating abilities of the rice genotypes based on the morphological and physiochemical parameters under drought stress. Hence, a two dimensional scatter diagram was constructed using the values of the first two canonical vectors (functions 1 and function 2) as the coordinates

TABLE 3a
Eigen values and percentage of variation for the morphological and physiochemical characters in 12 rice genotypes in controlled condition

Function	Eigen value	% of Variance	Cumulative %	Canonical correlation
Shoot length	735.225	53.5	53.5	.999
No. of leaves	393.376	28.6	82.1	.999
Chlorophyll A	107.248	7.8	89.9	.995
Chlorophyll B	89.610	6.5	96.4	.994
Total Chlorophyll	40.473	2.9	99.4	.988
CSI (%)	5.896	0.4	99.8	.925
Proline	2.436	0.2	100.0	.842
Protein	.467	0.0	100.0	.564

TABLE 3b
Eigen values and percentage of variation for the morphological and physiochemical characters in 12 rice genotypes in water stressed condition

Function	Eigen value	% of Variance	Cumulative %	Canonical correlation
Shoot length	1913.800	41.9	41.9	1.000
Chlorophyll B	1223.488	26.8	68.6	1.000
Total chlorophyll	817.711	17.9	86.5	.999
CSI (%)	427.251	9.3	95.9	.999
Proline	173.596	3.8	99.7	.997
Protein	15.297	.3	100.0	.969

for the graphical presentation (*Figs. 3a* and *3b*), where F_1 served as x axis and F_2 as y axis. The grouping which was obtained by hierarchical clusters was compared with two dimensional representation of the canonical discriminant function analysis. It is interesting to note that the results obtained matched with the magnitude of divergence measured by dendrogram. Moreover, it is evident from the scatter plot diagram that the response of the genotypes varies widely as indicated by the change in the localization of genotypes in both the control and drought stressed conditions. The positions of Seberang and Mahawi in the graph have shifted far below the central axis, indicating a drastic reduction in their function and revealing that they are considered as sensitive to stress. Meanwhile, MR 232 and Masqia were found close to the axis, and this indicated stabile in their performance. Meanwhile, the positions of Ria, Gaya, and MR 167 were shown to be above the central axis and they might be tolerant to water deficit stress. Furthermore, the scatter plot of canonical discriminant function reflected the relative importance of character contributing towards divergence.

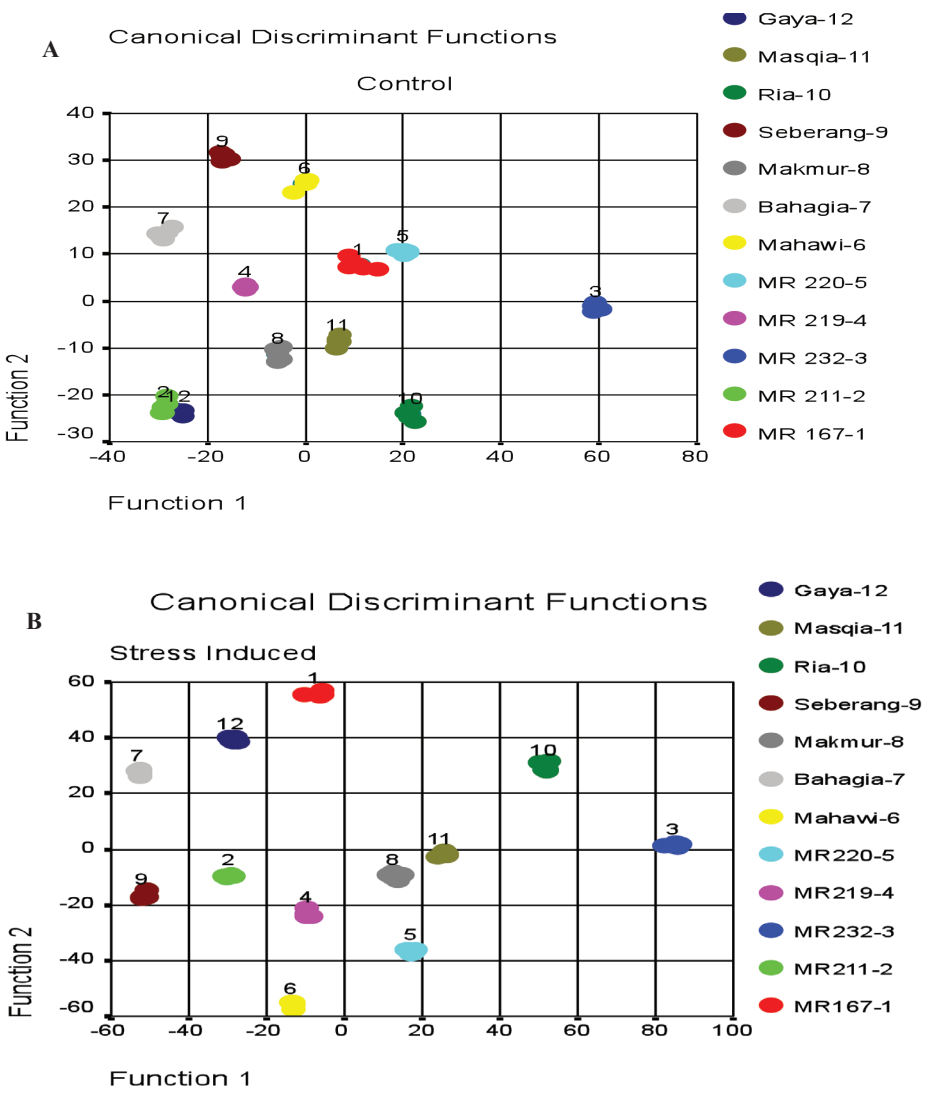

Fig. 3: Two dimensional representation of drought stress response of 12 rice genotypes obtained from the canonical discriminant functions; a) Genotypes response obtained under controlled environment, b) Genotype response obtained under water stressed condition

CONCLUSIONS

The study has demonstrated that the morphological and physiochemical traits investigated have greater relevance to future breeding programme, particularly for screening drought tolerance at early stage. The traits total chlorophyll content, chlorophyll stability index, proline and protein have contributed the maximum towards genetic divergence under stressed condition and classified the genotypes as tolerance and sensitive to drought stress. Furthermore, the correlation coefficient presented in Tables 3a and 3b shows a strong association with those physiochemical traits. Therefore, it is suggested that these traits can be employed as potential indicators for screening

drought tolerance at early stage. Therefore, the present investigation has demonstrated that laboratory-based measurements on growth rate, total chlorophyll content, CSI %, proline and protein enable large number of genotypes to be screened in a shorter period time. The traits examined are promising and can be used as potential selection criteria for improving drought tolerance in rice. More importantly, the techniques used to screen the genotype at the laboratory level are economical and effective alternatives to select drought and stress tolerant genotypes at early stage.

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Gender Identification of Domesticated Chicken Using a PCR-based Method

Richard Teh Swee Aun* **and Jayaraj Vijaya Kumaran**
Department of Biotechnology, Faculty of Applied Sciences,
UCSI University, No.1, Jalan Menara Gading, UCSI Heights,
56000 Kuala Lumpur, Malaysia
**E-mail: richard_12501@hotmail.com*

ABSTRACT

The monomorphism of newly hatched chicks poses a problem for farmers who need to separate them. Currently, the most widely used technique is the cloacal sexing method which has a low accuracy rate. Thus, a PCR based gender determination protocol was established to obtain a higher accuracy rate than conventional sexing methods. Feathers of a day up to a week old chicks were collected for the molecular analysis using the PCR targeting the *CHD-W* and *CHD-Z* genes in the chicken's chromosome Z and chromosome W, respectively. The results of this study showed that the PCR based gender determination protocol was a sensitive and accurate method for determining the gender of monomorphic chicks as compared to the conventional sexing method.

Keywords: Monomorphism, PCR based gender determination protocol, *CHD-W* and *CHD-Z*

INTRODUCTION

Chicken sexing has been an integral part in the breeder, broiler, and layer industries since 1935. An early determination of chick's gender will enable farmers to reap more profits based on the difference in growth rates exhibited by the gender of chickens and the female's role in producing eggs in the layer industry. This, in turn, will reduce unnecessary cost in raising unwanted chicken such as food, water, and vaccination of chicken. The first sexing technique was vent sexing which was introduced by Professor Masui and Mashimoto (Cerit and Avanus, 2006) in 1935. Vent sexing is a sexing method which is based on the appearance of the chick's sexual organs. Unfortunately, this widely practiced method is not very accurate due to certain factors such as the morphological variation of the sex organs in different breeds of chicken and the personnel's experience in handling the sexing exercise. The accuracy of vent sexing has been determined at 98% (Phelps, 2001). Unfortunately, this 2% margin of error may cost big monetary loss considering the fact of the current scale of the poultry industry.

Chromosomally, the gender of chicken is determined by the sex chromosome ZW with males having ZZ (Saitoh *et al*., 1991; Saitoh *et al*., 1993). On the other hand, females are heterogametic WZ (Griffith and Tiwari, 1996; Griffith and Korn, 1997; Griffith *et al*., 1998; Ellegren, 2001). In 1996, Griffith developed universal primers for PCR based application of sex determination on non-ratite birds. These primers targeted the Chromodomain Helicase DNA binding protein (*CHD*) gene. The protein itself is an ATP-dependent chromation remodelling factor that controls how DNA is being packed. Cerit and Avanus (2006) state that the *CHD* gene chromosomes Z and W are avian specific sex linked gene. Subsequently, Griffith *et al*. (1996) designed a pair of primers (P8 and

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*Corresponding Author

P2) that are able to amplify both *CHD-W* and *CHD-Z* simultaneously with different product sizes for both sex chromosome linked genes in non-ratite avian. This results in females having double bands and males with a single band of PCR products after agarose gel electrophoresis due to the amplification of both targeted genes. Thus, an attempt was done in this study to test the application of the existing universal primers, described by Griffith *et al*. (1998), in determining the gender of a day up to a week old chicks, as well as to compare the accuracy rate of this PCR-based sexing method with the conventional sexing method. Subsequently, two sets of primers were also designed to confirm the results obtained from the primers described by Griffith *et al*. (1998) and Richard *et al*. (2009).

MATERIALS AND METHODS

Collection of DNA Sources and DNA Extraction

Primary feathers were collected from 30 chicks and each chick was tagged for verification exercise after four weeks beginning from the date of samples collection. DNA was extracted from these 30 samples using the Wizard® Genomic DNA Purification Kit (Cat. No. A1125, Promega, Madison, USA) and the resultant DNA extract was qualitatively checked and quantified at 260nm/280nm using a spectrophotometer. This was followed by standardisation of all DNA extractions into concentration of 0.1µg/µl DNA each. All the samples were also electrophoresed in a 1% agarose gel to determine the intensity of the bands for the DNA extracts.

PCR

The PCR was done following the protocol by Mulis (1987) and Sambrook *et al*. (1989). Meanwhile, the final reaction mixture of 25µ1 was prepared as follows: 1x PCR buffer; 0.25mM dNTP; 1U/25µl *Taq* DNA polymerase; 1pmol/µl of primers; 0.2 µg/25µl DNA template and topped up with ddH_20. The PCR primers used in this study were P8 (5'- CTY CCR AGR ATG AGA AAC TG -3') and P2 (5'- TCT GCA TCA CTA AAT CCT TT -3'), adapted and modified from Griffith *et al*. (1998); 2 sets of primers were designed from the National Centre for Biotechnology Information (NCBI) Genbank sequences *viz*. JVK1 (5'- CTC AGG AGA TGG ATA TAG-3') and JVK2 (5'-GTT AGC TAC CTT GAA CTG -3') from the accession number AF00659; ZR (5'- CAG AGG TCT CCT TAT GGT TC -3') and ZF (5'- GTC CTT GAG AGT TCT CTA CC -3') were from the accession number AF006660. The product sizes for the primer pair P2 and P8 are 345 bps for the *CHD-W* gene and 362 bps for the *CHD-Z* gene, respectively. The primer pair JVK1 and JVK2 yielded a PCR product with a size of 92 bps from the *CHD-W* gene, whereas the primer pair of ZF and ZR yielded a PCR product with the size of 527 bps from the *CHD-Z* gene, respectively. *Figs. 1* and *2* show the amplicon sites of the primers used in this study.

The optimisation of the PCR conditions was done using both the adult male and female chicken archived DNA extracts which were available at the Molecular Biology Laboratory, Biotechnology Department in UCSI University. The PCR was performed in a Mastercycler Gradient thermocycler (Eppendorf, Germany), with the following temperature profile: initial denaturation at 95°C for 5 mins, followed by 30 repeating cycles of denaturation at 95°C for 1 min 30 sec, annealing at 41.5°C for 1 min 30 sec, extension at 72°C for 1 min 30 sec, and a final extension of 72°C for 3 mins. The PCR products were then separated in 3% agarose gel electrophoresis stained with ethidium bromide at 90 V for an hour, and they were then visualised under UV illumination and photographed.

RESULTS AND DISCUSSION

Apart from extracting DNA from feathers, other non-invasive approaches such as DNA extraction from egg shell membranes (Yun and Xiao, 2008) and DNA from faeces (Wasser *et al*., 1997) can be good sources of DNA (Taberlet *et al*., 2006) for the molecular analysis of chicken sex determination. The spectrophotometric ratios of 260nm/280nm in this study were found to range

***Gallus gallus* chromosome W chromodomain helicase DNA binding protein 1 (*CHD-W*) mRNA.**

1741 gctagagctc ataggattgg accaaagaaa caggttaata tttatcggct agtcaccaaa

1801 ggatcagtag aagaagatat tcttgaaaga gccaagaaaa agatggtgtt agatcattta

1861 gtgattcaga gaatggacac cacagggaaa actgtactac atacaggctc tactccttca

1921 agctcaacac cttttaataa ggaagagtta tcagcaattt tgaagtttgg tgctgaggaa

1981 ctttttaaag aacctgaagg ggaggaagag gagcctcagg agatggatat agatgaaatc

2041 ctgaagaggg ctgaaactcg agaaaatgag tcaggcctat taactgtagg agatgagtta

2101 ctttcacagt tcaaggtagc taacttttcc aatatggatg aagatgacat tgaattggaa

2161 ccagaacaaa atctaagaaa ctgggaagaa atcattccag aagttcagtg gcgacgaata

2221 gaagaggagg aaagacaaaa agaacttgaa gaaatatata tgcttccaag aatgagaaac

2281 tgtgcaaaac agatcagctt taatggaaat gaagggagat gcagtaggag cagaagatat

2341 tctggatctg atagtgattc catctcagaa agaaaacgac caaaaaaacg tggacgacca

2401 cgaactattc cccgtgaaaa cattaaagga tttagtgatg cagagattag acgatttatc

2461 aagagttaca agaaatttgg tggcccactt gaaaggttag atgctatagc tagagatgct

2521 gagctagttg ataaatctga aacagacctt agacgtctgg gagaacttgt acataatgga

2581 tgcattaagg ctttaaatga taatgacttt ggtcaaggaa gaacaggtgg tagatttggg

2641 aaagttaaag gcccaacatt ccgaatagca ggagtgcagg tgaatgcaaa gctagtcatt

2701 tctcacgaag aagagttggc accattgcat aaatcgattc cttcagatcc agaagaaagg

2761 aaaagatatg tcatcccata ccacaccaaa gcagctcatt ttgatataga ttggggtaaa

2821 gaagatgatt ccaatctgtt aataggcatc tatgaatatg gttatggcag ctgggaaatg

Fig. 1: Nucleotides in grey show primers JVK1 and JVK2 aligned to the CHD-W in G. gallus genome (Accession number AF181826), while nucleotide (in yellow) indicates the locations of primers P8 and P2 align in CHD-W. The estimated size for the product of primers JVK1 and JVK2 was 92 bps

***Gallus gallus* chromo-helicase-DNA-binding on the Z chromosome protein (*CHD-Z*) mRNA.**

3241 cattgactgt aggggatgag ttgctttcac agttcaaggt ggcgaacttt tccaatatgg

3301 atgaagatga tattgagttg gaaccagaaa gaaattcaag aaattgggaa gaaatcatcc

3361 cagaatccca acggagaagg atagaggagg aggaaagaca aaaagaactt gaagaaatat

3421 acatgctccc gaggatgaga aactgtgcaa aacagatcag ctttaatggg agtgaaggaa

3481 gacgcagtag gagcagaaga tattctggat ctgatagtga ctccatcaca gaaagaaaac

3541 ggccaaaaaa gcgtggaaga cctcgaacca ttcctcgaga aaatattaaa ggatttagtg

3601 atgcagagat caggcggttt atcaagagtt acaagaaatt tggtggccct ctggaaaggt

3661 tagatgctgt agctagagat gctgaactgg ttgataaatc tgagacagac cttagacgtt

3721 tgggtgaact tgtacataat ggatgcatta aggctttaaa ggacaattca tctggacaag

3781 aaagagcagg aggtagactt gggaaagtta aaggcccaac gtttcgaatc tcaggagtgc

5461 aatcttcgag agattataga taccactcag actggcaaat ggaccacaga gcttctggta

5521 gtggcccgag gtcaccacta gatcagaggt ctccttatgg ttcaagatct cccctaggac

5581 acagatctcc atttgaacac tcatcagatc acaaaagtac acctgaacat acatggagta

5641 gccggaagac ataacaaaga ctgacatttt ctggaccttc tttttagcca tatacagtaa

5701 actaacacag taattgcctt acatgacttg aaagatatgg actggatatt ctatcagtag

5761 cagtattgtt acttctttcc aggatgcaag gtctattatc ccaacagaag aaaaatattt

5821 ttgtatttaa agtttatgct gcactgtgct gcaaatgttg tggcactttt tttttaagaa

5881 atggaagatg tttacttttа cagggacctc aacactgccc ctttcagact ggatcttact

5941 ataaaactct tcatgtcaaa gtggttctag gctgaacaca gattaaatta tgtttgtaaa

6001 tgaacactta aacactgacc tgtgcttatg tttcaggaaa gaatggggga tttattttgt

6061 tttatttctt ggtagagaac tctcaaggac tttgttcact ttccaaagct acttgtttac

Fig. 2: Nucleotides in grey show primers ZF and ZR align to the CHD-Z in G. gallus genome (Accession number AF004397). On the other hand, nucleotides (in yellow) show the location of the primer pair P8 and P2 aligned to the CHD-Z gene. The estimated size for the product of primer pair ZF and ZR was 527 bps

from 1.7 to 2.3, with the values falling below 1.8 due to protein contamination in the extracted DNA sample (Joseph, 2007). Meanwhile, the values more than 2.0 might indicate the presence of RNA contamination or the presence of high GC content substances in the extracted DNA (Sambrook and Russell, 2001). The authors were able to extract high yield and high purity DNA using primary feathers in this study. Subsequently, if the bands of the DNA extract were too intense, further dilution was then carried out to ensure that all the samples had the DNA concentration of 0.1µg/µl and that their intensities were comparable to one another.

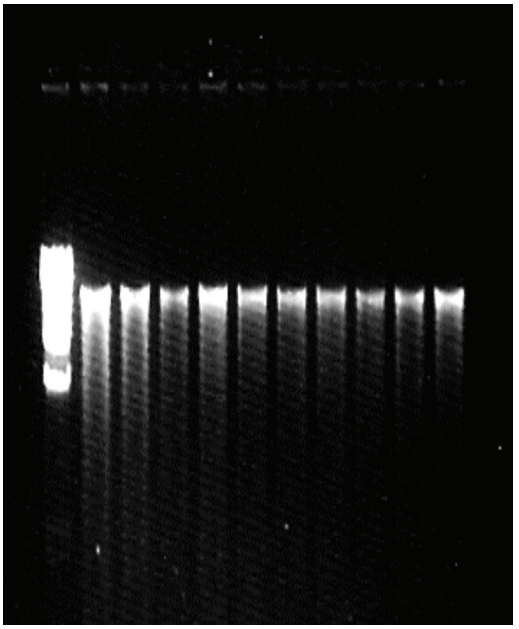

Fig. 3: The results of DNA extraction performed on 10 primary feather samples

Then, the positive controls underwent PCR optimisation (Vernon *et al*., 2001) and the results are shown in *Fig. 4*. The female sample produced two bands at the estimated size of 345 bps and 362 bps, respectively, whereas the adult male chicken produced a single band of 345 bps size, which is congruent with the finding by Griffith *et al*. (1998).

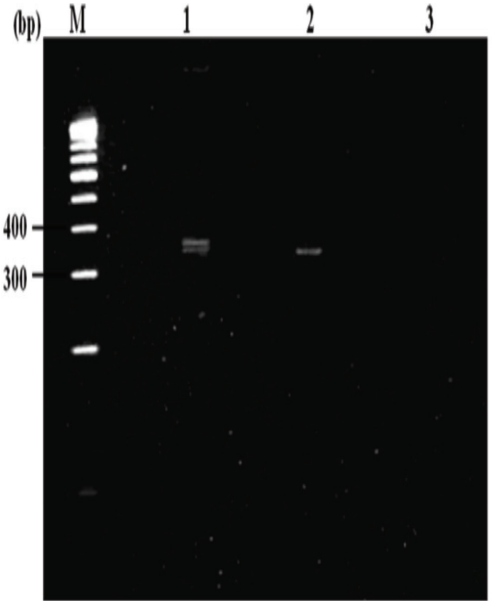

Fig. 4: A female sample in lane 1 was with double bands due to the CHD-Z (345 bp) and CHD-W (362 bp), while a single banded male sample CHD-Z (345 bps) in lane 2; M is the 100 bps molecular marker and lane 3 is the negative control

The results gathered for the PCR amplification of both the male and female samples, using the JVK1/JVK2 primer set which targeted the CHD-W gene, are shown in *Fig. 5*. The female samples produced a single band at 92 bps, and this is congruent with the predicted amplicon size designed using the NCBI Genbank accession no. AF181826. On the other hand, the male samples did not produce any band as they do not have chromosome W in their karyotype. *Fig. 6* shows the PCR amplification of both the male and female samples using the ZF/ZR primer set. Both the samples produced bands as the primers targeted the CHD-Z genes in both the male and the female chicken genome. The size of the products, on the contrary, was about 527 bps, which is the same as the predicted amplicon size designed using the NCBI Genbank accession no. AF004397.

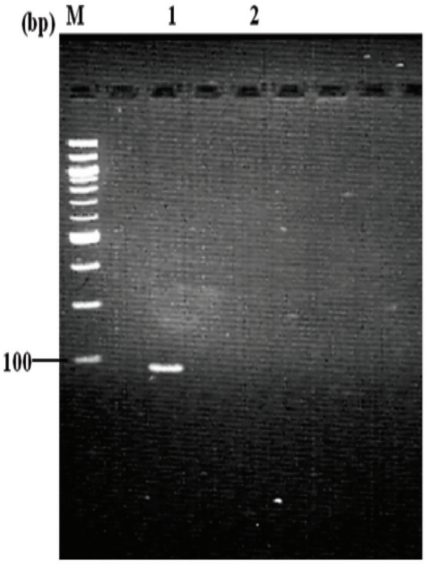

Fig. 5: A positive sample was detected in lane 1 where CHD-W (92 bps), while lane 2 shows the absence of CHD-W, and M is the 100 bps molecular marker

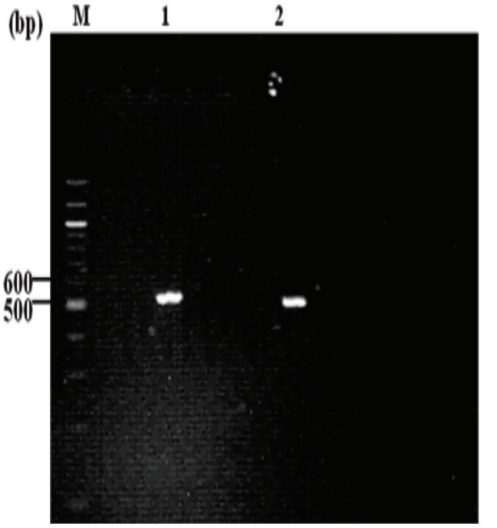

Fig. 6: Positive results of CHD-Z for both the samples using the ZF/ZR primer set due to presence of CHD-Z in both the male and female chicken genomes

Both the positive control samples showed positive results as CHD-Z (527bp) was present in both the male and female genomes (*Fig. 6*). *Fig. 7* reveals the PCR results of 10 out of the 30 samples of chicks using the P8/P2 primer set. In this study, the molecular analysis results were verified by observing the chickens that were sampled earlier. As a result, out of 30 the chicks, there were 16 male chicks and 14 female chicks. The PCR-based results are congruent with the results collected from the farm site after four weeks of captivity. Therefore, this method has been proven to be reliable and is 100% accurate as the gender for all the 30 samples has been correctly identified using the established PCR-based protocol. Thus, it is a better sexing method compared to vent sexing which only has 98% accuracy rate. Apart from that, using feathers as a source of DNA is a good approach as it is a non-invasive method compared to other sources, such as blood, biopsy, and liver samples.

As the PCR-based sexing method is an accurate method, it should be proposed to be used by farmers from various industries to replace the conventional vent sexing method which has an accuracy rate of 98% only. This will increase productivity and reduce cost associated with loss due to raising chicks not desired in the poultry industry. Despite all the advantages and benefits of using this method, the cost incurred is higher than the vent sexing method due to the equipment, reagents and laboratory setup, which will become more profitable in a long run, especially for the large-scale poultry farms. In addition, the PCR screening is also capable to sex 10,000 chicks within 6 days or less, without any error. Thus, this may also serve as a potential new job opportunity for biotechnology graduates as trained personnel in the field of molecular biology are required to use the proposed method in conducting tests.

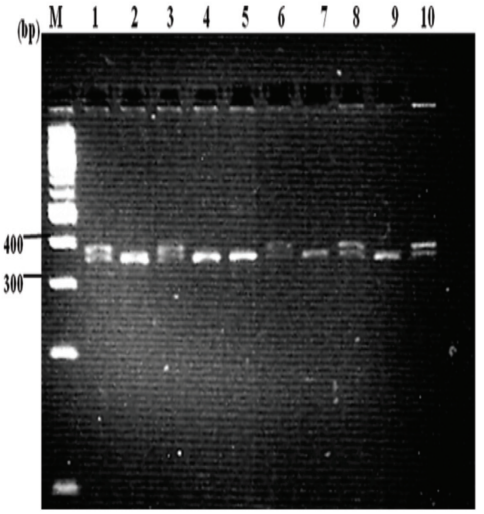

Fig. 7: 10 samples of chicks and M is the 100bp molecular marker. Samples 2, 4, 5, 7, and 9 were males while the rest were females

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TSPY as a Genetic Marker for Sex Determination of Cattle Spermatozoa

Ying-Ju Tan[1*], Mahanem Mat Noor[2], Ainu Husna M.S Suhaimi[1], Wan Somarny Wan Md Zain[1], Sharif Haron[1] and Johari Jiken Abdullah[1]

[1]Strategic Livestock Research Centre,
Malaysian Agricultural Research and Development Institute
[2]Faculty of Science and Technology, Universiti Kebangsaan Malaysia,
43600 UKM, Bangi, Selangor, Malaysia
**E-mail: yjtan@mardi.gov.my*

ABSTRACT

The beef industry prefers male calves as they tend to have higher growth rates and feed efficiency for meat production. The dairy industry, however, prefers female calves which produce offspring and milk for dairy products. Thus, methods are required to determine the sex of spermatozoa in order to manipulate the sex of the offspring of livestock. The ability to sex spermatozoa has great potential for commercialization in the beef and diary cattle, thus a lot of the research has been conducted to develop and refine spermatozoa sexing technology. In this study, fluorescence *in-situ* hybridization (FISH) was performed on decondensed bovine spermatozoa using testis-specific protein, Y-encoded (*TSPY*) marker, as a screening method for the detection of Y-chromosome bearing bovine spermatozoa. The PCR-produced fragments of *TSPY* were cloned, transformed, cultured, and extracted according to a standard protocol. *TSPY* fragment-bearing plasmids were labelled with SpectrumRed-dUTP by nick translation labelling. The labelled probes were hybridized onto the pre-washed slides containing decondensed bovine spermatozoa for 72 hours. After post hybridization, the washed slides were counterstained using 4,6-diamino-2-phenylindole (DAPI II) and the FISH images were captured and analysed. The results showed only certain spermatozoa cells were hybridised with red signals, indicating the presence of Y-chromosome bearing spermatozoa. It is important to note that this chromosome-specific marker can be used to verify the sex of the flow cytometrically sorted spermatozoa and open the way of elucidation for *TSPY* to be used as a marker in sex determination.

Keywords: FISH, sex determination, *TSPY*, spermatozoa

INTRODUCTION

Altering the sex ratio for a particular population may be necessary for maintaining the profitability of the dairy and beef cattle industries. Thus, a predetermination of sex prior to conception is the most cost-effective means to achieve a desired result. This particular approach is useful in cattle breeding programmes, where a specific sex of calves is required. The X- and Y-chromosome bearing spermatozoa can be used in artificial insemination (AI), in-vitro fertilization (IVF) or embryo transfer (ET) programme. However, the only proven method for sexing is cell sorting which is based on DNA differences by flow cytometry (Parati *et al*., 2006). The predetermination the sex of calves after spermatozoa sexing using flow cytometry obtained between 85% and 95% accuracy (Seidel, 1999).

The validation of the proportions of the X- and Y-chromosome bearing spermatozoa

*Corresponding Author

sorted by flow cytometry is essential. A practical approach is to use the fluorescence *in-situ* hybridization (FISH) technique. This technique is effective for validating the sorted X- and Y-chromosome bearing spermatozoa since it can be used to examine a large number of sperm cells by directly viewing sperm chromosomes without relying on flow cytometry (Kawarasaki *et al.*, 1998).

The objective of this study was to develop the FISH method using the chromosome Y-specific DNA probe which could determine the efficiency of spermatozoa sexing techniques.

MATERIALS AND METHOD

Semens were collected from using artificial vagina. Fresh semens were cryopreserved and stored in liquid nitrogen (N_2). The frozen semen were thawed and the spermatozoa was washed once with an equal volume of normal saline containing 0.9% NaCl by centrifugation for 10 minutes at 900 rpm. This was followed by smearing and fixing the spermatozoa on glass slides. The slides were then incubated with decondensation solution containing dithiotreitol (DTT) for 1.5 minutes to make the sperm nuclei accessible to the probes.

The PCR amplification was performed using primers 5'-CCC GCA CCT TCC AAG TTG TG-3' and 5'-AAC CTC CAC CTC CTC CAC GAT G-3' that is specific to testis-specific protein (*TSPY*) gene in Y-chromosome. The template DNA was prepared by PCR with the final volume of 25µl reaction mix, containing template sperm DNA, dNTP mix, 10× PCR buffer, as well as $MgCl_2$ and Taq DNA Polymerase. The amplification was performed using the following thermocycling conditions: an initial denaturation at 94°C for 4 min, followed by 35 step cycles of denaturation (94°C for 30 sec), annealing (60°C for 30 sec) and extension (72°C for 1 min), with the final extension at 72°C for 10 min. The DNA band of interest was cut and purified from agarose gel. The product was cloned into qPCR® 2.1-TOPO® plasmid using Invitrogen Cloning Kit. The extracted pasmids was labelled with SpectrumRed-dUTP by nick translation labelling.

The labelled product was mixed with blocking DNA and precipitated overnight. The DNA pellet was mixed with 10µl hybridization

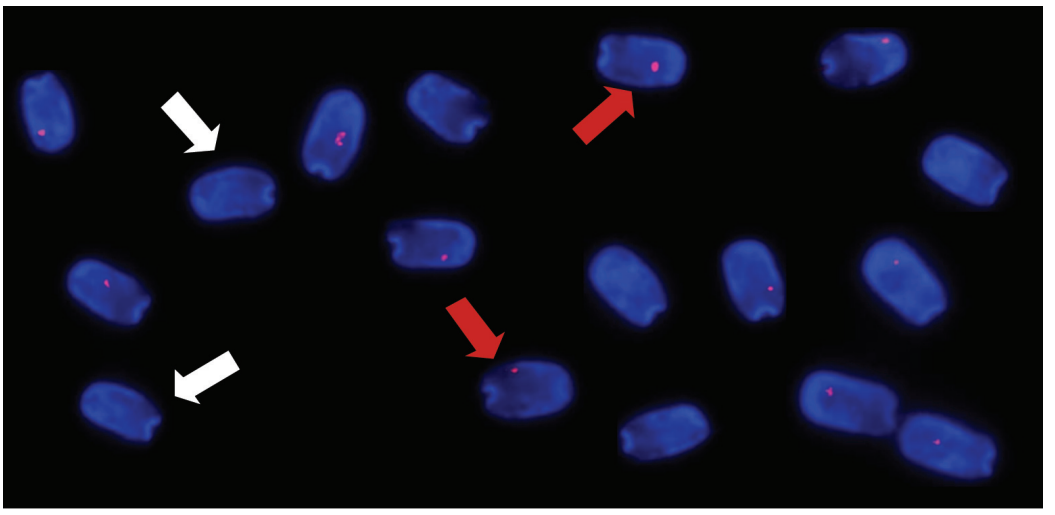

Fig. 1: Spermatozoa with red signal indicated Y-chromosome bearing spermatozoa (red arrows). Spermatozoa without red signal indicated X-chromosome bearing spermatozoa (white arrows)

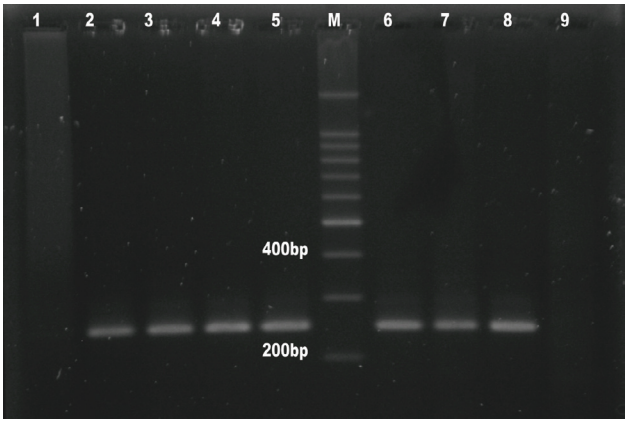

Fig. 2: DNA band from male and female bovine blood. The 260bp Y-specific bands were present in males bovine DNA (lane 2-8). M=100bp marker; lane 1=female DNA; lane 2-4=male DNA; lane 5-7=bovine's sperm DNA; lane 8=positive control, DNA from Y-chromosome bearing spermatozoa; Lane 9=negative control, without DNA template

buffer and denatured at 80ºC for two minutes. Then, the probe mixture was hybridized onto the decondensed spermatozoa slides in a moist chamber at 37ºC overnight.

The slides were washed using the washing buffer containing Nonipet-40. The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI II). After that, these slides were sealed with nail polish and the spermatozoa were observed using a phase-contrast epifluorescence microscope. The optical sections of each field of view were obtained individually and analysed.

RESULTS AND DISCUSSION

In this study, a total of 300 spermatozoa were examined. The spermatozoa with chromosome Y was indicated by the presence of red signal. The rate of Y-bearing spermatozoa was calculated by the number of sperm cells with the red signal divided by the number of the total sperm cells. The percentage of the Y-bearing spermatozoa was 50.8% and the percentage of X-bearing spermatozoa was 49.2% (*Fig. 1*). The ratio of the X- and Y-bearing spermatozoa for the whole sperm populations was found to be as expected.

The Y-encoded, testis-specific protein (*TSPY*), is a Y-specific gene (Lemos *et al.*, 2005). Meanwhile, the number of the *TSPY* copies was ranged from 20 to 60 in men and up to 200 in bull (Manz *et al*., 1998). The higher repetitive probe enhanced the signals from Y chromosome. In cattle, *TSPY* expression is apparently restricted to male germ cells and their precursors begin during foetal development. The cellular site of expression suggested a function in spermatogonial proliferation (Vogel *et al*., 1997). In this study, the specificity of *TSPY* marker was confirmed by the PCR on DNA from the blood samples of the male and female cattle. These DNA were screened for the presence of *TSPY* using the specific PCR primers amplifying a 260-bp segment of *TSPY*. All the male samples (blood and spermatozoa) are *TSPY*-positive, while all the female samples are *TSPY*-negative (*Fig. 2*).

The de-condensing of the spermatozoa head using DTT allows DNA probes to hybridize in the chromosome. One problem that normally occurs when FISH is performed on spermatozoa is the degree of condensation of the DNA. Obtaining an optimal decondensed of sperm head size for an optimum hybridization is difficult as the

time required for sperm decondensation varies considerably among species (Perreault *et al.,* 1988). In particular, a short decondensation time will cause the failure of DNA probe to access the chromatin. In contrast, if the nuclei swells more than twice of the original size, the signal from one chromosome will split and appear as two or more signals causing false score (Parrilla *et al.,* 2003).

The present study used direct DNA probe labelling by nick translation. If the reaction is optimally controlled, nick translation can give the highest sensitivity. This method does not involve extensive manipulation of the sample, and therefore the risk of contamination is low when compared to other method such as the PCR labelling (Parrilla *et al.,* 2003). Therefore, in order to increase the specificity of sex determination method using FISH, it is better to use dual colour labelling with two fluorescence colours on autosomal and Y chromosome. The time of hybridization could also be shorten to save time and improved efficiency.

CONCLUSIONS

In conclusion, FISH using *TSPY* probe can be used to determine the X- and Y-chromosome bearing spermatozoa. This technique can be used to validate the purity of the sexed spermatozoa sorted via flow cytometry.

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Morphometric Variation among the Three Species of Genus *Acetes* (Decapoda: Sergestidae) in the Coastal Waters of Malacca, Peninsular Malaysia

S.M. Nurul Amin[1*], Aziz Arshad[1, 3], Noor Hashida Ismail[2], Mohd Hanafi Idris[2], Japar Sidik Bujang[2] and Siti Shapor Siraj[3]

[1]Laboratory of Marine Science and Aquaculture, Institute of Bioscience,
[2]Department of Biology, Faculty of Science,
[3]Department of Aquaculture, Faculty of Agriculture,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: smnabd02@yahoo.com

ABSTRACT

Small sergestid shrimps of the genus *Acetes* are locally known as 'udang garagau' which can be found along the coastal region of Malacca. A total of three species of the genus *Acetes* (namely, *A. indicus*, *A. japonicus* and *A. intermedius*) are found along the coastal waters of Malacca. The morphometric data of the three species were analyzed using one-way ANOVA and PRIMER software to examine the degree of similarity among the three species. All the morphometric characters, which include total length, carapace length, standard length, abdominal length, telson length, first antenular peduncle, second antenular peduncle, third antenular peduncle, eye length, eye wide, and eye diameter of the three species were significantly found to be different ($P < 0.05$). The dendrogram of both the male and female populations showed three major clusters indicating the three species of genus *Acetes.*

Keywords: *Acetes* shrimps, morphometric variation, Peninsular Malaysia

INTRODUCTION

Although the shrimps of the genus *Acetes*, family sergestidae, are a minor planktonic crustacean group represented by a small number of species, they are one of the economically important organisms in Asia and East African waters (Omori, 1975). It is mainly used in subsistence fisheries and is, therefore, commercially important in Peninsular Malaysia. Six species of *Acetes* from the Malay Peninsula and Singapore, namely *A. erythraeus* Nobili, *A. indicus* Milne-Edwards, *A. japonicus* Kishinouye, *A. sibogae* Hansen, *A. serrulatus* Hansen, and *A. vulgaris* Hansen are briefly reported (Pathansali, 1966). The two latter species, *A. serrulatus* and *A. vulgaris,* were recorded from Singapore waters, while the other four species of the genus *Acetes* were recorded only from the Malay Peninsula.

Morphometric characters are powerful tools for measuring discreteness and relationships among stocks (Ihssen *et al*., 1981; Melvin *et al*., 1992). In the present study, the morphometric data were used to clarify the intra-population variation in the genus *Acetes* from the coastal water of Malacca. Nonetheless, the information on the morphometric variation among the three species of *Acetes* (*A. indicus*, *A. japonicus,* and *A. intermedius*) has not been reported from the region. The population biology of *Acetes* have been reported in many studies by different

[*]Corresponding Author

authors (Deshmukh, 2002; Oh and Jeong, 2003; Arshad *et al*., 2007; Amin *et al*., 2008a,b). Therefore, the present study is a pioneering attempt in this direction.

MATERIALS AND METHODS

Collection of Data

The fresh samples of *Acetes* were collected monthly between February 2005 and January 2006 from the commercial push net catches landed at Klebang Besar (N $02^0$13.009/ and E 102^0 11.921/) in Malacca (*Fig. 1*). *Acetes* shrimps were caught using a push net (triangular shape) known locally as 'Sungkor' (Omori, 1975) in the coastal waters of Klebang Besar, Malacca. The dimensions of the net are 5-6 m in length, 4.0 - 4.5 m in width, and 3.0 - 3.5 m in height. The mean mesh size was 3.2 (± 0.27) cm at the anterior section, 0.75 (±0.05) cm at the middle, and 0.5 (±0.08) cm at the cod end (stretched). After collection, the samples were fixed in 10% formalin solution in the field and they were analyzed after 2-3 days of preservation. In the laboratory, these specimens were identified using a 'Nikon' dissecting microscope. Their sexes were determined by the presence or absence of petasma on the first pleopod and clasping spine on the lower antennular flagellum (Omori, 1975). The identification of the different species of *Acetes* was according to the keys developed by Omori (1975).

Data Analysis

A total of 180 specimens in the size ranging from 10 to 30 mm were used for the morphometric measurements; 60 (30 males and 30 females) specimens each from *A. japonicus*, *A. intermedius,* and *A. indicus*. Eleven selected morphometric characteristics, as shown in *Fig. 2*, were measured using the KEYENCE Digital microscope (VHX-500) for each sample. The following morphometric characters, which include the total length (TL), carapace length (CL), standard length (SL), abdominal length (AL), telson length (TLL), first antenular peduncle (P1), second antenular peduncle (P2), third antenular peduncle (P3), eye length (EL), eye wide (EW), and eye diameter (ED) were measured (*Fig. 2*). The morphometric data were analyzed using the one-way analysis of variance (ANOVA) while the PRIMER software was used for the cluster analysis of the species.

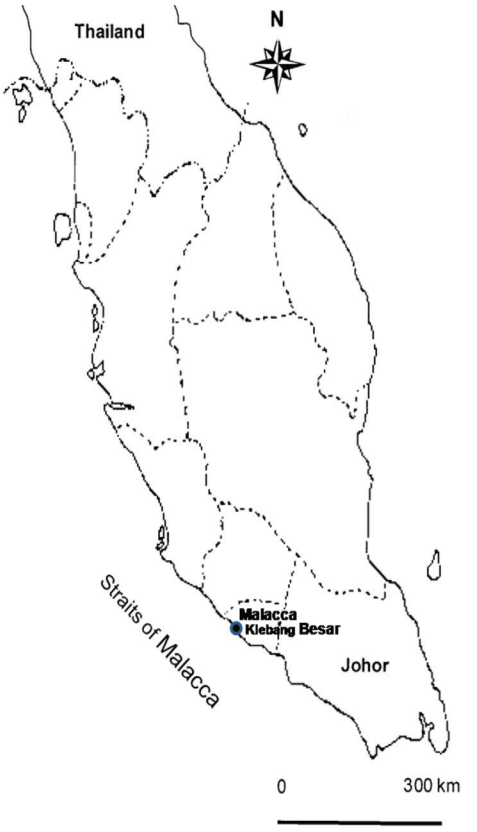

Fig.1: Sampling station (dot) and location of Klebang Besar in Malacca, Peninsular Malaysia

RESULTS AND DISCUSSION

The range and mean ± standard error values of the morphometric characters for the three species of *Acetes* (*A. japonicus, A. intermedius,* and *A. indicus*) are presented in Tables 1 and 2. The ANOVA showed that the mean differences in the total length (TL), standard length (SL), carapace length (CL), abdominal length (AL),

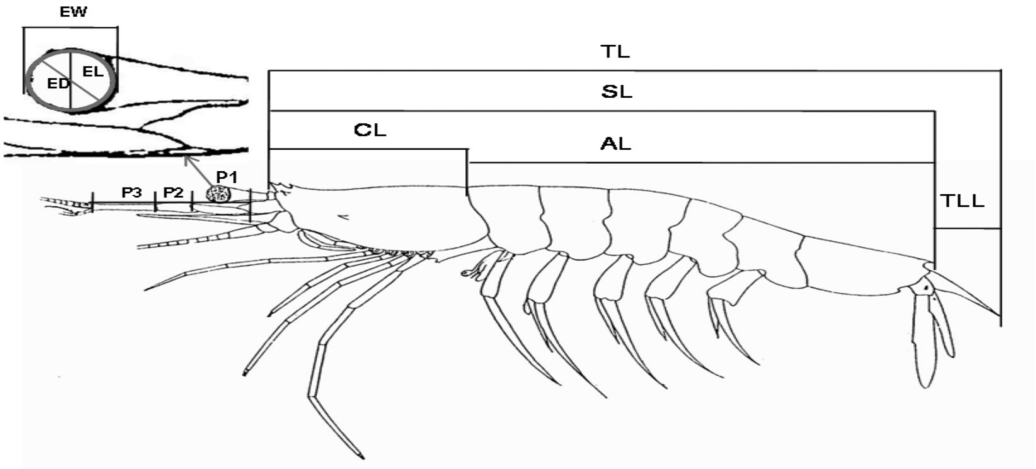

Fig. 2: Morphometric characters used for Acetes *shrimp*

telson length (TLL), first antennular peduncle (P1), second antennular peduncle (P2), third antennular peduncle (P3), eye length (EL), and eye wide (EW) among the three species were highly significant ($P < 0.05$). Nonetheless, no significant difference was observed for the male eye diameter of the different species. Meanwhile, the dendrograms (*Figs. 3* and *4*) showed three major clusters of the *Acetes* species in the coastal waters of Malacca. The similarity of matrix indicated that there were about 90 – 93% similarity between *A. japonicus* and two other species (namely, *A. indicus* and *A. intermedius*). There was about 97- 98% similarity between *A. indicus* and *A. intermedius,* based on the male and female morphometric characteristics which covered *A. japonicus* in one group and the other group that comprised two species of *A. intermedius* and *A. indicus*.

Moreover, there is no previous record on morphometric variation between the different species of *Acetes.* However, the morphometric analysis of the Malaysian Oxudercine Goby, *Boleophthalmus boddarti* was studied by Daud *et al*., 2005. The analyses of various morphometric characters showed significant differences among the three species of *Acetes.* The results shown by ANOVA had high significant differences ($P < 0.05$) of all the morphometric characters except for the eye diameter ($P > 0.05$) of the males in the three species. Meanwhile, the female population also showed highly significant differences ($P < 0.05$) for all the morphometric characters. In more specific, two major groups were observed for both the male and female of the three *Acetes* species from the study area (*Figs. 3* and *4*). The first group consists of *A. japonicus*, while the second group has *A. intermedius* and *A. indicus*.

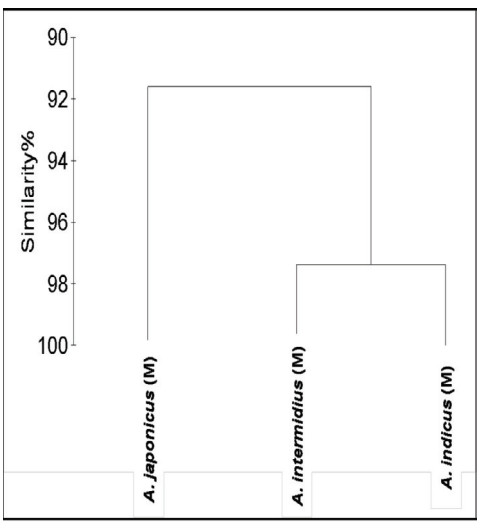

Fig. 3: Dendrogram of the three Acetes *species on the basis of morphometric characters of the male collected from Malacca coastal waters*

TABLE 1
Mean ± standard error, ranges (in parentheses) and F-values (derived from the analysis of variance) of each morphometric character (mm) in three species of genus *Acetes* (male)

MC	*A. japonicus* (M)	*A. intermedius* (M)	*A. indicus* (M)	N	F-values	P
TL	12.93^{a} ±0.16 (11.5-15)	18.90^{b} ±0.30 (17-22)	18.15^{b} ±0.59 (12.5-24)	30	69.27	0.000*
CL	3.24^{a} ±0.04 (2.98-3.61)	4.85^{b} ±0.06 (4-5.80)	4.69^{b} ±0.16 (3.41-6.45)	30	71.65	0.000*
SL	11.44^{a} ±0.13 (10-13.25)	16.75^{b} ±0.27 (15-19.50)	16.04^{b} ±0.52 (11-21)	30	78.33	0.000*
AL	8.41^{a} ±0.10 (7.5-9.75)	12.12^{b} ±0.20 (10.5-14.25)	11.61^{b} ±0.38 (8-15)	30	40.23	0.000*
TLL	1.56^{a} ±0.0.05 (1-2.25)	2.36^{b} ±0.05 (2-3.12)	2.38^{b} ±0.10 (1.66-3.54)	30	63.33	0.000*
P1	0.85^{a} ±0.02 (0.6-1.17)	1.07^{b} ±0.03 (0.75-1.50)	1.10^{b} ±0.04 (0.6-1.53)	30	12.61	0.000*
P2	0.77^{a} ±0.02 (0.58-1.17)	0.60^{b} ±0.01 (0.44-0.75)	0.97^{c} ±0.05 (0.60-1.67)	30	27.30	0.000*
P3	2.10^{a} ±0.04 (1.66-2.70)	1.05^{b} ±0.02 (0.65-1.35)	2.38^{c} ±0.10 (1.39-3.38)	30	101.72	0.000*
EL	0.60^{a} ±0.01 (0.44-0.75)	0.78^{b} ±0.01 (0.64-0.93)	0.74^{b} ±0.02 (0.5-1.11)	30	23.87	0.000*
EW	0.62^{a} ±0.01 (0.50-0.78)	0.80^{b} ±0.02 (0.58-1.07)	0.74^{b} ±0.03 (0.51-1.03)	30	22.12	0.000*
ED	0.63^{a} ±0.01 (0.50-0.73)	0.81^{a} ±0.01 (0.64-0.97)	0.75^{a} ±0.02 (0.56-1.06)	30	2.24	0.113^{NS}

For each morphometric variable, means with the same letter superscript are not significantly different.
* The mean difference is significant at 5% level; NS = not significant at 5% level

TABLE 2
Mean ± standard error, ranges (in parentheses) and F-values (derived from the analysis of variance) of each morphometric character (mm) in three species of genus *Acetes* (female)

MC	*A. japonicus* (F)	*A. intermedius* (F)	*A. indicus* (F)	N	F-values	P
TL	17.31^{a}±0.30 (14-19.75)	22.82^{b}±0.39 (19-27)	24.42^{b}±0.52 (18-30)	30	68.63	0.000*
CL	4.59^{a}±0.09 (3.5-5.38)	5.94^{b}±0.12 (4.9-7.09)	6.42^{b}±0.17 (5-8.29)	30	68.11	0.000*
SL	15.44^{a}±0.27 (12.5-17.50)	20.33^{b}±0.34 (17-24)	21.75^{b}±0.46(16-27)	30	50.34	0.000*
AL	11.06^{a}±0.19 (9-12.50)	14.50^{b}±0.25 (12-17)	15.73^{c}±0.34 (12-20)	30	23.90	0.000*
TLL	2.00^{a}±0.07 (1-2.52)	2.65^{b}±0.08 (1.97-3.25)	2.90^{b}±0.13 (1-4.1)	30	70.24	0.000*
P1	0.89^{a}±0.03 (0.55-1.29)	1.20^{b}±0.03 (0.9-1.60)	1.29^{b}±0.05 (0.80-1.81)	30	27.40	0.000*
P2	0.49^{a}±0.02 (0.33-0.72)	0.59^{b}±0.02 (0.39-0.81)	0.77^{c}±0.03 (0.44-1.13)	30	27.78	0.000*
P3	1.06^{a}±0.0 (0.61-1.92)	1.06^{a}±0.03 (0.80-1.38)	1.41^{b}±0.06 (0.94-2.39)	30	11.84	0.000*
EL	0.67^{a}±0.02 (0.47-0.87)	0.88^{b}±0.02 (0.72-1.07)	0.87^{b}±0.02 (0.66-1.17)	30	30.10	0.000*
EW	0.69^{a}±0.02 (0.50-0.93)	0.90^{b}±0.02 (0.72-1.04)	0.90^{b}±0.02 (0.67-1.18)	30	30.21	0.000*
ED	0.70^{a}±0.02(0.47-0.87)	0.91^{b}±0.02 (0.72-1.07)	0.90^{b}±0.02 (0.7-1.14)	30	30.9	0.000*

For each morphometric variable, means with the same letter superscript are not significantly different.
* The mean difference is significant at 5% level

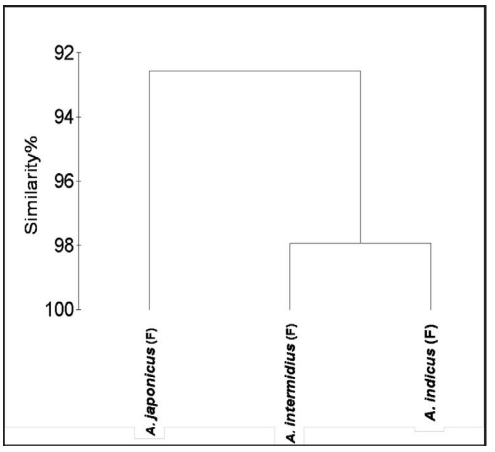

Fig. 4: Dendrogram of the three Acetes *species on the basis of morphometric characters of the female collected from Malacca coastal waters*

CONCLUSIONS

Both the similarity matrix and cluster analysis revealed that there are three different species of *Acetes* (*A. japonicus, A. intermedius,* and *A. indicus*) recorded from the coastal waters of Malacca, Malaysia. A more detailed study on systematics of the *Acetes* spp. is therefore needed from more geographical locations in Malaysia to obtain updated information on the systematic accounts and resources of *Acetes* from this country.

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