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Use of Plant Residues for Improving Pod Chemical Composition, Biochemical Quality and Pod Yield of Okra *(Abelmoschus esculentum* L.*)*

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Keywords: Plant residues, *Abelmoschus esculentum* L., *fertilizer treatment*

ABSTRAK

*Kesan abu kayu, habuk gergaji, sekam koko, bijian terpakai dan dedak digunakan ke atas lenggai okra, komposisi kimia, kualiti biokimia dan hasil lenggai okra (*Abelmoschus esculentum *Moench) kepelbagaian NHAe-47 dikaji. Lima jenis rawatan baja organik dibandingkan dengan baja kimia (1,600kg/ha NPK 15-15-15) dan kawalan yang tidak berbaja dalam empat lapangan eksperimen. Rawatan-rawatan tersebut direplikatkan empat kali dan disusun dalam reka bentuk blok lengkap secara rawak. Komposisi kimia bahan organik, nutrien lenggai, kualiti biokimia dan hasil lenggai okra telah ditentukan untuk rawatan yang berbeza. Keputusan menunjukkan penggunaan 6t/ha sisa tumbuhan secara signifikan ($P<0.05$) meningkatkan hasil lenggai, N, P, K, Ca, Mg, Na, abu, protein mentah dan hasil lenggai okra berbanding rawatan kawalan. Abu kayu, sekam koko, bijian terpakai merupakan bahan yang paling efektif dalam pembaikan hasil lenggai, status nutrien lenggai dan kualiti biokimia manakala dedak dan habuk gergaji adalah bahan yang paling tidak efektif. Sisa tumbuhan mengeluarkan Ca, Mg dan Na kandungan lenggai dengan lebih baik berbanding rawatan baja NPK 15-15-15. Bijian terpakai meningkatkan Ca, Mg dan Na lenggai okra dengan lebih 98, 94 dan 69% berbanding baja NPK. Hanya bijian terpakai yang meningkatkan lenggai abu berbanding baja NPK. Dalam kalangan sisa tumbuhan, abu kayu mempunyai nilai N, P, K, Ca dan Mg tertinggi, dan diikuti dengan rawatan sekam koko, bijirin terpakai, dedak dan habuk gergaji. Abu kayu meningkatkan N, K, Ca dan Mg lenggai dengan 50, 70, 72 dan 52% berbanding habuk gergaji. Bijian terpakai mempunyai nilai hasil lenggai okra tertinggi, diikuti dengan sekam koko dan abu kayu, manakala kedua-dua habuk gergaji dan dedak mempunyai nilai yang terendah. Bijian terpakai meningkatkan hasil lenggai dengan 50, 49, 65 dan 52% melebihi rawatan habuk kayu, sekam koko, dedak dan habuk gergaji. Baja NPK meningkatkan hasil lenggai dengan 25, 24, 48 dan 48.2% lebih tinggi daripada abu kayu; sekam koko, dedak dan habuk gergaji. Bijian terpakai juga meningkatkan hasil lenggai dengan 33% berbanding baja NPK. Pekali kolerasi (r) di antara hasil lenggai dan lenggai N, hasil lenggai dan abu lenggai, hasil lenggai dan protein mentah adalah 0.81, 0.73 dan 0.64 pada aras 1% ($P\geq0.01$) manakala pekali regresi (R^2) bagi hubungan antara hasil lenggai okra, komposisi kimia dan kualiti biokimia adalah 0.83. Implikasinya adalah lenggai N, P, K, Ca, Mg, Na, protein mentah dan abu dihitung dengan 83% hasil variasi dalam okra.*

ABSTRACT

*The effect of wood ash, saw dust, ground cocoa husk, spent grain and rice bran used ordinarily on the okra pod, chemical composition, biochemical quality and pod yield of okra (*Abelmoschus esculentum *Moench) variety NHAe-47 was studied. Five organic fertilizer treatments were compared to a chemical fertilizer (1,600 kg/ha NPK 15-15-15) and unfertilized controls in four field experiments. The treatments were replicated four times and arranged in a randomized complete block design for each experiment. The chemical composition of the organic materials, pod nutrients, biochemical quality, and pod yield of okra were determined for the different treatments. The results showed that the application of 6 t/ha of plant residues significantly ($P<0.05$) increased the pod yield, N, P, K, Ca, Mg , Na, ash, crude protein and pod yield of okra compared to the control treatment. Wood ash, cocoa husk and spent grain were the most effective in improving pod yield, pod nutrient status and biochemical quality while the rice bran and saw dust were least effective.The plant residues produced better pod Ca, Mg and Na contents than the NPK 15-15-15 fertilizer treatment.*

Spent grain increased okra pod Ca, Mg and Na by 98, 94 and 69% more respectively compared to NPK fertilizer. Only spent grain significantly increased the pod ash compared to NPK fertilizer. Among the plant residues, wood ash had the highest values of pod N, K, Ca and Mg followed by cocoa husk, spent grain, rice bran and saw dust treatments respectively. Wood ash increased pod N, K, Ca and Mg by 50, 70, 72 and 52% more respectively compared to the saw dust. Spent grain had the highest value of okra pod yield followed by cocoa husk and wood ash while both saw dust and rice bran had the least values. The spent grain increased pod yield by 50; 49, 65 and 66% more compared to wood ash, cocoa husk, rice bran and saw dust treatments respectively. NPK fertilizer increased the pod yield by 25, 24, 48 and 48.2% more compared to wood ash; cocoa husk, rice bran and saw dust respectively. Spent grain also increased the pod yield by 33% compared to NPK fertilizer. The correlation coefficients (r) between pod yield and pod N, pod yield and pod ash, pod yield and crude protein were 0.81, 0.73 and 0.64 respectively at 1% level ($P \geq 0.01$) while the regression coefficient (R^2) for the relationship between okra pod yield, chemical composition and biochemical quality was 0.83. The implication is that pod N, P, K, Ca, Mg, Na , crude protein and ash accounted for 83% of yield variation in okra.

INTRODUCTION

Okra *(Abelmoschus esculentum* L.) is an annual herb and fruit vegetable crop which belongs to the family Malvaceae. Okra is grown throughout the tropical and subtropical parts of the world either as a sole crop or intercropped with major staple crops such as yam and maize.

Okra plays an important role in the human diet by supplying additional carbohydrate, protein, fats, minerals and vitamins which are usually deficient in the staple food (Oyenuga, 1968). The nutritional importance of okra has led to renewed interest in bringing the crop into commercial production.

In spite of the above nutritional importance of okra, its optimum yield and the quality have not been attained partly because of continued decline in soil fertility. However, efforts to increase the yields of okra through the use of the inorganic fertilizers have been hampered by acute scarcity, high cost and continued deterioration of soil properties (Adeoye, 1986).

Sanchez *et al.* (1989) reported that an important method to improve nutrient recycling and conserve soil fertility is through the use of applied organic inputs.

This work investigated the effect of plant residues such as wood ash, spent grain, ground cocoa husk, rice bran and saw dust on pod chemical composition, biochemical quality and yield of okra in Akure, South West Nigeria.

MATERIALS AND METHODS

Source and Preparation of the Organic Materials

Cocoa pod husk and wood ash were collected from the cocoa farm plots and cassava processing units of Federal College of Agriculture, Akure. Rice bran was collected from the OS-6 variety processed at the college rice mill while the sorghum based spent grain was collected from the International Breweries Nig. Plc., Ilesa, Osun State, Nigeria.

The saw dust was obtained from the nearby saw mill industry at Akure township, specializing in cutting obeche *(Triplochiton scleroxylon)* log trees into pieces.

The organic materials were processed to allow for decomposition. The dried cocoa pod husk were ground with a hammer mill while the rice bran was chopped into pieces, wetted and allowed to decompose.

The college has 300 ha of cocoa plantation from which quantities of cocoa pod husk were obtained. There is also a 200 ha rice field from which sizeable quantities of rice bran were obtained. The processing of harvested tubers from 250 ha of cassava generated high quantities of woodash derived from fuel wood and planks purchased from the nearly saw mill.

Generally, all the organic residues used were easily available, sustainable and cheap for growing okra commercially.

Field Experiments

The experiments were carried out at Akure, Nigeria in the tropical rainforest zone. The

soil is a sandy loan texture and belongs to Akure soil series, Iwo Association and is classified as a skeletal, Kaolinitic Ishohyperthermic Paleustalf (Alfisol) USDA or Ferric Luvisol (FAO) Harpstead (1972).

The soil had pH (H_2O) of 5.1, 0.53% organic matter, 0.02% N, 4.6 mg kg^{-1} Bray P1 extractable P, 0.05 mmol kg^{-1} exchangeable K, 0.1 mmol kg^{-1} exchangeable Ca and 1.12 mmol kg^{-1} exchangeable Mg.

The soil was under arable crops for 10 years. The field experiments were conducted four times between April 6 1998 and August 23 1999 at the same site. Each experiment spanned for four months.

Five organic fertilizer treatments were applied to each crop of okra at the start of the experiment, in-addition, to 400 kg/ha kg^{-1} (1,600 kg ha^{-1} for four experiments) NPK 15-15-15 fertilizer and the control (no manure or fertilizer) treatments.

The five plant residues were wood ash, saw dust, ground cocoa pod husk, rice bran, spent grain (sorghum based brewery waste) and saw dust.

The seven treatments were replicated four times on each of the four consecutive okra crops and arranged in a randomized complete block design. The size of each plot was 16 m^2 (4 m x 4 m) and soils were ploughed and harrowed to maintain good tilth for the okra crop. The residues and NPK fertilizer were incorporated into the soil two weeks before planting using a garden fork to allow better decomposition.

Four seeds of early maturing okra variety (NHAe 47-4) were planted per hole of 2 cm depth and at a spacing of 60 cm x 30 cm (20 kg/ha). There were five vertical rows of okra seeds planted in each plot and germination took place five days after planting. Thinning to one plant per stand was done.

The plots were manually weeded thrice starting from second, fourth and sixth weeks after planting. The insect pests were controlled by spraying Vetox 85 at the rate of 28 g a.i in 9 L of water starting from the second week after planting (WAP).

Twenty six plants were selected and tagged out of 88 plants per plot for sample pod chemical composition, biochemical quality and pod yield determination.

Harvest of mature pods started at 35 days after planting and it continued at every four days interval until senescence. The yields of the remaining 62 plants for okra in each treatment plot were weighed, put into labeled envelopes and oven dried at 70°C for 48 hours. At the end of each experiment, all okra plants were uprooted.

Analysis of the Okra Pods for Nutrients Chemical Composition and Biochemical Quality

The dried okra pods were ashed for 6 hours in a muffle furnace. The chemical composition (P, K, Ca, Mg and Na) in the ashed pods were extracted with water. The % P was determined by using vanado-molybdate solution and it was read on spectronic 20 at 442 um (Udo and and Ogunwale, 1979). The % K, Ca and Na were read on the flame photometer using appropriate element filters (Jackson, 1958). The Mg was determined on atomic absorption spectrophotometer (Novaspec II visible spectrophotometer, manufactured by Pharmacia Biotech (Biochrom Ltd) Cambridge, England).

The crude protein content was determined by multiplying % N N x 6.25 and the total ash was determined by using the formula.

$$\% \text{ total ash} = \frac{\text{ash weight}}{\text{Sample weight (oven dry)}} \times 100$$

The % N was determined by Microkjedahl method (Jackson, 1964).

Chemical Analysis of Organic Materials

The processed forms of the organic materials were analysed. The determination of nutrients in organic materials were done using wet digestion method based on 25-5-5 ml of NHO_3-

H_2SO_4-$HC10_4$ acids respectively. The filtrates collected were used for the determination of %, P, K, Ca, Mg and Na. The % P was determined using vanado molybdate colorimetry and read on specronic 20 while % K, Ca and Na were read on flame photometer. Mg was read on atomic absorption spectrophotometer. The % N was determined by microkjedahl method (Jackson, 1964).

Statistical Analysis

The mean data for pod nutrients N, P, K, Ca, Mg, Na crude protein, ash content and pod yield of okra crops were subjected to ANOVA F-test and their levels of significance were determined for the residue treatments using Duncan's Multiple range test (DMRT) at 5%. Multiple regression coefficient showing the nutrient use relationship between the okra pod yield, pod nutrients and biochemical quality was calculated.

RESULTS

Table 1 presents the chemical composition of the organic materials used for the cultivation of okra. Among the plant residues, wood ash had the best nutrient status with regards to C, N, K, Ca, Mg and Na while cocoa husk had the highest total P.

Wood ash supplied the highest P while cocoa husk had the highest K. NPK supplied moderate amounts of N, P and K nutrients and very low Ca and Mg to the soil (Table 2).

Effect of Plant Residues on Okra Pod Chemical Composition and Biochemical Quality

The mean percent values of okra pod N, P, K, Ca, Mg, Na, total ash and crude protein for the four crops of okra under the different manure treatments are presented in Table 3. The plant residues increased the okra pod status of N, P, K, Ca, Mg, Na, total ash and crude protein significantly ($P<0.05$) relative to the control. Wood ash increased the okra pod N, P, K, Ca, Mg, Na, total ash and crude protein more by 98, 88, 98, 79, 92, 77, 84 and 98% respectively compared to the control treatment.

The plant residues produces better pod Ca, Mg and Na contents than the NPK 15-15-15 fertilizer treatment. Spent grain increased okra pod Ca, Mg and Na by 98, 94 and 69% more respectively compared to NPK 15-15-15 fertilizer. Only spent grain significantly increased the pod ash compared to NPK fertilizer.

NPK fertilizer had the highest values of pod N, K and crude protein than all residues while pod P values were the highest in cocoa husk and NPK fertilizer treatments.

Among the plant residues, wood ash had the highest values of okra pod N, K Ca, and Mg followed by cocoa husk, spent grain, rice bran and saw dust treatments respectively. Cocoa husk and spent grain had the highest values of pod P and ash respectively. For instance, wood ash increased percent pod N, K, Ca and Mg by 50, 70, 72 and 52% respectively compared to the saw dust.

Rice bran, cocoa husk and wood ash had the highest values of pod Na compared to others while only wood ash and cocoa husk had the highest values crude protein in okra.

Effect of Plant Residues on Pod Yield of Okra

The plant residues and NPK fertilizer increased pod yield (gross) of okra significantly ($P<0.05$) relative to the control treatment (Table 4). Wood ash, cocoa husk, rice bran, spent grain, saw dust and NPK fertilizer increased the okra pod yield by 97, 98, 96, 99, 96 and 98% respectively compared to the control treatment.

Among the plant residues, spent grain had the highest value of pod yield of okra followed by cocoa husk and wood ash while both saw dust and rice bran had the least values. Spent grain increased the pod yield by 50, 49, 65 and 66% compared to wood ash, cocoa husk, rice bran and saw dust treatments respectively. Generally, the residues had cumulative effect on pod yield and chemical composition.

NPK 15-15-15 fertilizer increased okra pod yield compared to plant residues except in

TABLE 1
The chemical composition of the organic materials used in the treatments of okra

Organic Materials	C %	N %	C/N ratio	Total P(mg/kg)	Na	Ca	K	Mg
					Mg/L			
Cocoa husk	16.0	1.44	11.1	100.0	4.41	9.34	20.59	7.10
Wood ash	18.0	1.53	11.76	86.0	8.26	9.40	23.02	8.52
Spent grain	10.0	0.78	12.82	76.0	4.57	0.13	7.86	3.10
Rice bran	14.0	0.60	23.23	56.0	4.43	0.12	6.93	1.80
Saw dust	8.0	0.42	18.96	10.0	4.39	0.10	5.12	1.30

spent grain. NPK fertilizer increased the pod yield by 25, 24, 48 and 48.2% compared to wood ash, cocoa husk, rice bran and saw dust respectively. Spent grain alone increased the pod yield by 33% compared to the NPK fertilizer.

The correlation coefficients (r) between okra pod yield, pod N, P, K, Ca Mg, ash and crude protein were significantly and positively correlated at 1% and 5% levels (Table 5). The r values between pod yield and pod N, pod yield and pod ash, pod yield and crude protein were 0.81, 0.73 and 0.64 respectively at 1% level ($P \geq 0.01$).

The multiple regression analysis showing the relationship between okra fresh pod yield, pod Ca, Mg, Na, K, N, P, ash and crude protein is presented in Table 6. The R^2 value (coefficient of determination) was 0.83 and the implication is that pod N, P, K, Ca, Mg, Na crude protein and ash accounted for 83% yield variation in okra.

DISCUSSION

In the control treatment (no fertilizer and residues), the pod yield of okra was the least compared to those of the five plant residues (cocoa husk, spent grain, rice bran, wood ash and saw dust). This could be due to the initial low soil nutrient status of the field before application of residues. Agboola (1982b) reported poor growth and yield responses in unfertilized soils.

The nutrient contents in the okra pod under the control treatment were very far below the critical level of 0.25% P, 1.19% K, 0.8% Ca and 0.7% Mg as reported by Jones Eck (1973). Thus, the okra plants showed deficiency symptoms of P (purple colouration), K (burnt leaf margin), Ca (stunted root growth) and N (yellow leaf colouration).

Spent grain produced the best pod yield of okra, although, it had lower nutrient status compared to wood ash and cocoa husk. Spent grain also gave relatively low pod nutrient composition in okra. Therefore, the best crop performance associated with the use of spent grain could be attributed to possible improvement in the soil physical properties (bulk density and low porosity). Folorunso (1999) reported that spent grain better reduced the soil bulk density compared to the other plant residues. The reduction in the soil bulk density enhanced root growth and subsequently enhanced uptake of nutrients from the soil for sustainable yield.

Cocoa husk followed spent grain with regards to the enhancement of okra pod yield. It had the least C/N ratio, which implies that it decomposed and released its nutrients faster when compared with the wood ash, rice bran and saw dust. The better effect of cocoa husk on okra pod yield compared with rice bran and saw dust is consistent with the fact that it had better nutrient composition (Table 1).

Cocoa husk had the highest total P among the plant residues and better Ca, K and Mg than rice bran and saw dust. Cocoa husk improved okra pod N, K, Ca, Mg and crude

TABLE 2
The total quantity of residues and amount of nutrients in plant residues sources

Fertilizers	N	P	K	Ca	Mg Kg	Total quantity of residue applied per for each planting	Total quantity of residues applied for the four plantings
NPK fertilizer	240	240	240	16	8	400kg	1600kg
Cocoa husk	346	250	285	638	16	6 Tonnes	24 Tonnes
Rice bran	144	104	119	59	35	6 Tonnes	24 Tonnes
Saw dust	101	93	103	43	30	6 Tonnes	24 Tonnes
Spent grain	187	260	163	158	68	6 Tonnes	24 Tonnes
Wood ash	367	272	262	642	212	6 Tonnes	24 Tonnes

TABLE 3
Effect of plant residues on okra pod chemical composition and biochemical quality

Treatments	Pod N	Pod P	Pod K	Pod Ca	Pod Mg %	Pod Na	Pod ash	Pod crude protein
Control (no fertilizer)	0.031a	0.052a	0.024a	0.22b	0.019b	0.016a	1.142a	0.193a
NPK 15-15-15	2.804g	0.615e	1.50f	0.011a	0.0087a	0.02a	7.262e	17.67f
Wood ash	1.568f	0.418d	1.395e	1.028f	0.233g	0.07d	7.237e	9.80e
Cocoa husk	1.488e	0.603e	1.053d	0.975e	0.152f	0.071d	7.156d	9.304e
Rice bran	0.506b	0.354c	0.433b	0.298c	0.123d	0.072d	6.625c	3.167b
Spent grain	1.357d	0.221b	0.601c	0.609d	0.145e	0.064c	7.345f	8.484d
Saw dust	0.791c	0.212b	0.423b	0.292b	0.113c	0.051b	5.355b	4.945c

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

TABLE 4
Effect of different plant residues on pod yield of okra kg/ha (gross plot)

Treatments	Pod yield (kg/ha)
Control (no fertilizer)	18.72a
NPK 15-15-15	988.58e
Wood ash	746.19c
Cocoa husk	754.56d
Rice bran	512.80b
Spent grain	1483.20f
Saw dust	511.56b

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

TABLE 5
The linear correlation coefficient (r) between the okra pod yield, %, N, P, K, Ca, Mg, Ash and crude protein

Parameters	"r" values
Pod yield vs % pod K	0.50*
Pod yield vs % pod P	0.53**
Pod yield vs % pod Ash	0.73**
Pod yield vs % pod N	0.81**
Pod yield vs % pod crude protein	0.64**
Pod yield vs % pod Ca	0.48*
Pod yield vs % pod Mg	0.43*

* - significant at 5% level
** - significant at 1% level

TABLE 6
Multiple regression coefficient (R^2) between okra fresh pod yield and % Ca, Mg, Ash, Na, K, N, P and crude protein

Amount of residues applied	Regression equation $Y = a+bx_1 + x_2 + x_3 \ldots x_8$	Regression coefficient (R^2)
$6tha^{-1}$	Y= 13.25 + 18.35x1 + 63.50x2 +6.15x3 – 20.73x4 + 40.28x5 +43.28x6 + 5.94x7 + 10.51x8	0.83

x_1 = %Ca, x_2 = %Mg, x_3 = %Ash, x_4 = %Na,
x_5 = %K, x_6 = %N, x_7 = %P and x_8 = % crude protein.

protein better than the other plant residues except wood ash. Cocoa husk has been found to be good source of K for maize (Adu-Daap *et al*, 1994).

Although, wood ash had the highest macro nutrient contents and a relatively low C/N, it had lower effect on okra yield compared to spent grain and cocoa husk. This might be due to leaching of the easily available nutrients since ash is highly water soluble compared with other plant residues. Ojeniyi (1998) reported that wood ash increased maize yield.

Saw dust and rice bran respectively were least efficient in the supply of nutrients to the okra crops. Accordingly, they had the least values of pod N, P, K, Ca, Mg, crude protein and ash. The low nutrient contents of rice bran and saw dust are consistent with the least values of okra pod yield in the field. The poor performance attributed to saw dust and rice bran could be due to the fact that these residues had the least nutrient contents compared with wood ash, cocoa husk and spent grain.

Saw dust had the least values of C, N, P, K, Ca, Mg and rice bran had relatively low N, Ca and Mg. This might be due to their high C/N ratio which slowed degradation rate and subsequent slow rate of nutrient release to soil.

The increase in pod N, P, K status under NPK fertilizer compared to the plant residues might be due to the fact that N, P and K in the fertilizer were easily available than those supplied by organic sources. The R^2 values of 0.83 showed that pod N, P, K, Ca, Mg, Na, crude protein and ash were responsible for 83% yield variation in okra. This implied that

the plant residues were efficient in improving the chemical composition and biochemical quality of okra for human consumption. This observation is supported by Oyenuga (1968) who reported that okra played an important role in the human diet by supplying additional carbohydrate, proteins, fats, minerals (Ca, P and Fe) and Vitamins (Vitamins A, C, thiamine, niacin and folic acid) which are usually deficient in the staple food of Nigeria (Oyenuga, 1968).These are required for body growth, reproduction and maintenance of health.

CONCLUSIONS AND RECOMMENDATIONS

Plant residues such as wood ash, cocoa husk and spent grain are effective sources of nutrients for okra because their addition to the soil enhanced the okra pod nutrients composition, biochemical quality and pod yield. Saw dust and rice bran were less effective.

Therefore, plant residues such as wood ash, spent grain and cocoa husk applied at rates of 6 t/ha are very useful as fertilizer materials for improving the nutrient availability and ensuring sustainable cultivation of okra on low fertility soils in humid tropics as well as improving the nutritional quality of okra.

This is particularly important considering the fact that inorganic fertilizers are scarce and expensive for resource poor farmers who are the producers of vegetable crops in most developing countries. In addition the increasing deep interest in organic farming in developed countries further justifies the recommendation for the use of plant residues for sustainable crop production.

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Wooden Household Furniture: Does Brand Matter?

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Keywords: Wooden, furniture, household, brand, consumer

ABSTRAK

Pengguna menilai sesuatu keluaran berdasarkan kepada pelbagai atribut apabila membuat satu keputusan pembelian. Atribut yang diambil kira berbeza di antara individu serta keluaran. Makalah ini melaporkan penemuan daripada satu kajian penerokaan terhadap kepentingan jenama dalam keputusan pembelian perabot kayu isi rumah dalam kalangan pengguna di Malaysia. Satu soal selidik berstruktur digunakan untuk mengumpul data daripada responden. Keputusan menunjukkan pengguna tidak menitikberatkan jenama malahan lebih mementingkan harga serta atribut ketara perabot kayu isi rumah berkenaan. Kebiasaan jenama dalam kalangan responden adalah rendah. Makalah ini membincangkan bagaimana pembuat perabot kayu isi rumah boleh menjenamakan keluaran mereka dengan berkesan.

ABSTRACT

Consumers evaluate a product based on various attributes when making a purchase decision. The attributes considered and their importance varies among individuals and differs between products. This paper reports the findings from an exploratory study on the importance of brands in wooden household furniture purchase decision among Malaysian consumers. A structured, self-administered questionnaire was used to collect data from the respondents. Results indicated that the respondents are not concerned about brands, but are instead price-conscious and placed more importance on the tangible attributes of the wooden household furniture items. Brand familiarity among the respondents was low. This paper discusses how the wooden household furniture manufacturers can brand their product in an effective way.

INTRODUCTION

The wooden furniture-manufacturing sector in Malaysia has experienced an impressive growth in recent years. The impetus and priority given to the sector in the national industrial development programs during the past decade saw more than 3,500 wooden furniture manufacturing mills established in the country (Jegatheswaran, 2002). These manufacturing mills, which range from small cottage operations to large automated plants with sophisticated machinery, sell their products both to the local and export markets.

In addition to the large number of manufacturers, there are also some salient characteristics of the sector that contribute to the intense competition among the manufacturers serving the local market. There is a common practice in Malaysia whereby some popular designs are continually being made by many manufacturers, plus their tendency to copy designs from one another. Furthermore, a large number of furniture workshops/dealers obtain their supplies of unfinished furniture from the same manufacturing mills, thus resulting in products sold in the market being of similar designs.

As competition intensifies, the wooden furniture manufacturers have to find ways of differentiating their products from each other. There is an emerging trend among these manufacturers to give and promote the brands

of their products, using either the company's name or specific name for a particular furniture design. An evaluation of a brand's influence on consumer decisions to purchase wooden household furniture is therefore helpful to the furniture manufacturers in marketing their products in the local market. This paper reports the results of an analysis on the importance of brands in consumers' wooden household furniture purchase decision. The following section discusses the importance of brands to consumers. The methodology adopted in the study is explained in the third section. The fourth section presents and discusses the results from the study. The paper concludes with a discussion on implications of the findings for marketers of wooden household furniture in the Malaysian market.

IMPORTANCE OF BRANDS TO CONSUMERS

Many products found in the market are not only offered with problem-solving functions sought by the consumers, but also with other forms of tangible attributes such as color, size, style and quality. These products would also carry other less tangible features like product warranty and after-sales services. Thus, seemingly similar products with a similar core benefit may become differentiated to the consumers with these augmented features.

Consumers employ criteria such as price, tangible and intangible product attributes, and place of purchase when evaluating product alternatives (Buell, 1985). Consumers vary with regards to which attributes they consider relevant, and they will pay most attention to those attributes connected with their needs (Kotler and Armstrong, 1997). Effective marketing, therefore, begins with an understanding of the needs and wants of the consumers. In essence, to provide a product that meets the needs of the consumers, marketers must assess the importance of various attributes of the product from the perspective of the consumers.

However, the consumers may not be fully aware of all the attributes of the various alternatives to help them make an evaluation during a purchase. They would normally use search features as indicators of benefits, of which brands have been commonly used as the primary indicator. Marketers believe brands are important because they shape customer decisions, and have been reported to be a key factor in purchase decision in both consumer and business-to-business markets of the US, Europe and Asia (Court and Freeling, 1996).

Brands are used to communicate a single or a range of positive attributes about a product or service (Betts, 1994). Brands tell the consumer something about the quality of a product, as a brand's reputation is normally used as a proxy when consumers are not adequately informed about the quality of a product (Sullivan, 1998). In addition, consumers buying the same brand know that they will get the same quality each time they buy (Kotler and Armstrong, 1997). Consumers can also reduce the risk they would face when buying something they know little about by buying branded products (Montgomery and Wernerfelt, 1992). Furthermore, consumers buying branded products often think that they are getting a special guarantee that the product meets their needs better than other similar products (Seperich *et al.*, 1994). And it would be quite difficult to change consumers' brand preference once they are convinced of the quality and value of a particular brand (Crispel and Brandenburg, 1993).

METHOD

Survey Instrument

A structured questionnaire was used to collect the responses from the respondents on the importance of a product's brand, and several other attributes, in their wooden household furniture purchase decision. The questionnaire used a five-point numerical scale in which the respondents were asked to indicate the importance of each attribute. The

scale ranged from a score of 1 (not at all important) to a score of 5 (very important) responses.

Sampling Frame

A convenience sampling approach was adopted with a goal to include both gender, and a broad range of age and income groups. Seventy-seven personnel of a public university who indicated their willingness to participate in the survey were given the questionnaire at their workplace. The questionnaires were self-administered and the interviewer was present to clarify any doubts or queries. All responses were collected on the spot. Demographic information was also collected. Respondents were asked to indicate their gender, age and income in the questionnaire. A summary of the demographic profile of the respondents is shown in Table 1.

TABLE 1
Demographic characteristics of the respondents

Characteristics	Frequency (%) (n = 77)
Gender:	
Male	66.2
Female	33.8
Age:	
Young (< 30 years)	5.2
Middle (30 - 40 years)	49.5
Matured (> 40 years)	45.5
Monthly gross household income:	
Low (< RM2,000)	13.0
Middle (RM2,000 – 4,000)	49.4
High (> RM4,000)	27.6

RESULTS AND DISCUSSION

Relative Importance of Brands

The following analysis concerns the evaluation of the relative importance of several wooden household furniture attributes, based on their mean scores. The list of attributes was by no means exhaustive, as the main objective is to determine whether brand is an important factor influencing consumer purchase decision. The mean score was calculated as $\sum Si/n$, where Si is the observed raw score for the ith individual in a sample of n respondents. As the scale used in the study ranged from 1 to 5, a score above 3 (the midpoint) indicates that the attribute is important, while a score below 3 indicates that the attribute is not important. Table 2 shows the relative importance of the different wooden furniture attributes, as perceived by the respondents. While Table 3 shows the relative importance of brands for sub-samples of the respondents.

Evidently, brand was not considered an important attribute in the respondents' wooden household furniture purchase decision. The unimportance of brands is also prevalent for the various sub-samples of the respondents as shown in Table 3. Even though brand is not an important attribute to purchase decision of the respondents, the low household income and mid-aged groups of respondents in the study placed a relatively higher importance on brand compared to other groups in their respective sub-samples. The results show that the respondents are more concerned about price and the tangible attributes of the furniture. Owners of household furniture in Malaysia are reported to consider factors like quality, design and colour; and price in their purchase decision (Anon, 1998). There is, therefore, a need to study the consumer's perception on the association between these attributes and the existing wooden household furniture brands in the market. Further studies can consider the consumer psychographic variables (Lin, 2002) in explaining their brand preference as brand, and price, are normally used as expressions of self and/or to indicate prominence and status (Wickliffe and Pysarchik, 2001).

Brand Familiarity

The respondents were also asked to identify the brand or names of wooden household furniture companies they knew to gauge their familiarity of the various brands existing in the

TABLE 2
Distribution of respondents' responses (in percentages of total respondents) and mean importance scores

Attributes	Level of importance					Mean importance score[a]
	5	4	3	2	1	
Price	64.9	11.7	19.5	5.2		- 4.34
Design	41.6	37.7	19.5	1.3		-4.19
Wood material	23.4	42.9	16.9	13.0		-3.69
Finishing	33.8	26.0	16.9	10.4	13.0	3.56
Brand	2.6	7.8	24.7	33.8	31.2	2.17

[a]Scale of 1 (not at all important) to 5 (very important)

TABLE 3
Relative importance of brand in wooden household furniture purchase decision for sub-samples of respondents

Sub-sample	Mean importance score[a]
Gender[b]	
Male	2.22
Female	2.08
Age[b]	
Young	1.50
Middle	2.32
Matured	2.08
Monthly gross household income[b]	
Low	2.44
Medium	2.22
High	1.93

[a]Scale of 1 (not at all important) to 5 (very important)
[b]Not significantly different (X^2 test at $p = 0.05$)

local market. The results of the study indicated that brand familiarity among the respondents is low, as only 35 percent of them were able to identify several common brands or company names of wooden household furniture existing in the local market. Most of them (89.5%) even indicated that the furniture items owned do not carry any brand or names of the manufacturer. This is reflective of the fact that brand is not considered an important attribute in the purchase decision of the respondents.

CONCLUSION

The results indicate that Malaysian consumers did not consider brands when deciding on a purchase of wooden household furniture items. Instead, they placed higher importance on price and, to a relatively lesser importance, on the other tangible attributes of the product. A casual observation of the many advertisements on wooden household furniture posted in local newspapers attest to the greater emphasis on price, and sometimes with minimal details on the other attributes of the furniture items. However, this does not

preclude the importance of branding in marketing of wooden household furniture to Malaysian consumers.

Although choosing an appropriate name for a product is important (Rooney, 1995; Kohli, 1997), branding goes beyond deciding on the name. It must be noted that the ultimate aim of branding is to build a level of awareness and knowledge in consumers, so as to create confidence in their purchase decision (Betts, 1994). Except the name is only for identification purposes, it should then move through several phases before a bond is finally created between the name or brand and the consumer.

Repeated advertising and promotion will introduce the name to the consumers (Kotler and Armstrong, 1997; Alreck and Settle, 1999). Consumers, up to this point, would be familiar with the name, and should be able to associate the name with its content —*name recognition*– (Court *et al.*, 1997). Name recognition, however, will not necessarily attract consumers especially when the product is not readily differentiated from those they are currently using or have used before.

The next step is to build preference for the name, or to be precise, for the product which the name is attached to. Alreck and Settle (1999) suggested several strategies for building consumer tastes and preferences, which range from linking the name to a particular consumer need to providing attractive models for consumers to emulate. For durable, large-ticket consumer products like furniture, where consumers are highly involved in the purchase decision process, the use of cognitive processing preference-building strategy is suggested. Whichever strategy is adopted, a link between the product's attributes with the benefits looked for by the consumers has to be established.

The name would then turn into a brand once the consumers associate a set of tangible or intangible benefits they obtain from the product (Court *et al.*, 1997). A brand is, thus, everything that a customer gains when purchasing a product or service; both the tangible aspects of the brand including product features and physical attributes as well as the intangible dimensions of the brand, which include the associations with the product (Zajas and Crowley, 1995). Marketers should then make a conscious effort to ensure that what they tell about their brands is what they actually deliver, if a bond is to be created between the brand and the consumer.

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Measurements of Leaf Area Index Using Optical Method (LAI-2000) in Oil Palm Plantation: Accuracy and Limitation Assessment

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Keywords: Leaf area index, Plant Canopy Analyzer, accuracy and limitation

ABSTRAK

Indeks kawasan daun (LAI) merupakan parameter penting untuk pengkarakteran struktur kanopi tanaman. LAI kerap digunakan sebagai pemboleh ubah kritikal untuk menyerupai model ekosistem yang berbeza, sukar untuk diukur secara terus dalam minyak sawit. Dalam kajian ini, kaedah optik untuk menyatakan variasi kuantiti dalam LAI di bawah keadaan berbeza dinilai. Didapati ketepatan bacaan bergantung pada faktor-faktor yang berbeza seperti teknik pengukuran, penglihatan sudut litupan, pembolehubahan ruang dan ketinggian titik pengukuran. Teknik pengukuran memberikan kesan kepada pengukuran LAI. Keputusan menunjukkan kaedah Zigzag mencatatkan kurang anggaran LAI berbanding kaedah lain. LAI menggunakan kaedah Zigzag mencatatkan 11.6% kurang berbanding teknik "satu di atas dan empat di bawah" dan 5.7% kurang berbanding teknik "satu di atas dan lapan di bawah". LAI memperoleh 6.2% kurang dengan menggunakan teknik "satu atas dan lapan di bawah" berbanding teknik "satu atas dan empat di bawah". Keputusan daripada siasatan yang dibuat terhadap kesan daripada penglihatan litupan ukuran LAI menunjukkan penglihatan litupan mempengaruhi pengiraan LAI dan LAI menurun dengan peningkatan pada sudut penglihatan litupan. Nilai LAI PCA juga turut dipengaruhi oleh pembolehubahan ruang dan ketinggian sensor. Nilai LAI PCA meningkat dengan peningkatan ketinggian sensor di permukaan tanah dengan nilai LAI maksimum (2.77) pada ketinggian 2.5 meter di permukaan tanah dan nilai minimum LAI (0.932) pada ketinggian 0 meter di permukaan tanah. Nilai LAI maksimum diperoleh pada semua arah pada jarak 0.5 meter daripada batang dan nilai minimum LAI diperoleh berdekatan dengan hujung pelepah. Nilai PCA LAI meningkat 5% dengan peningkatan jarak dari hujung pelepah ke tangkai.

ABSTRACT

Leaf area index (LAI) is an important parameter for characterizing the canopy structure of a crop. The LAI, which is often used as a critical variable to simulate different ecosystem models, is difficult to measure directly in oil palm. In this study, optical methods for quantifying variation in LAI under different conditions were evaluated. It was found that the accuracy of the readings depended on different factors, such as measuring technique, view cap angle, spatial variability, and height of the measuring point. The measuring technique had an effect on the LAI measurement. Results showed that the Zigzag method underestimated the LAI compared to other methods. The LAI by the Zigzag method was 11.6% less than the LAI by the "one above and four below" technique and 5.7% less than the LAI by the""one above and eight below" technique. The LAI obtained by the "one above eight below" technique was 6.2% less

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than the LAI obtained with the "one above four below" technique. Results from the investigation of the effect of view cap on LAI measurement showed that the view cap strongly influenced the LAI calculation and LAI decreased with increase in the view cap angle. PCA LAI values were also affected by spatial variability and height of the sensor. PCA LAI values increased with increase in sensor height above ground with a maximum LAI value (2.77) at 2.5-meter height above ground and minimum LAI value (0.932) at 0-meter height from ground. Maximum values of LAI were obtained for all directions at 0.5-meter distance from the trunk and minimum LAI values were obtained near the tip of the frond. The PCA LAI values increased by about 5% - 50% with increase in distance from the frond tip to frond base.

INTRODUCTION

Leaf area index (LAI) is a dimensionless index, which can be defined as the assimilative leaf area relative to the projected ground area for a plant community (one-side area for broad-leaved trees) (Lang *et al.*, 1991). Accurate and fast non-destructive measurements of leaf area index (LAI) of plant canopies are essential to environmental applications such as water and carbon cycle modeling. A commonly used technique to acquire LAI in situ is based on measurements of radiation transmittance through the canopy with optical instruments. The LAI-2000, which obtains measurements of effective LAI based on gap fraction at five view angles, is designed to work under diffuse light conditions (Leblan and Chen, 2001). Direct methods, such as destructive sampling, may provide the best estimates of LAI, but they are time consuming, difficult, with higher labor cost. Several non-destructive methods have been developed that utilize light attenuation through the plant canopy to estimate the amount and, in some cases, the orientation of foliage (Feldkirchner and Gowe, 2001). In the past few years, a number of systems for making indirect canopy structural estimates have become commercially available. These include linear sensors that require specific illumination conditions, such as the DEMON (CSIRO, Center for Environmental Mechanics, GPO Box 821, Canberra, ACT, Australia), Line quantum sensors (Decagon Devices, Box 835, Pullman, WA 99163, USA), and hemispherical sensors, such as the LAI-2000 ((Plant Canopy Analyzer, LI-COR, Lincoln, Nebraska, USA), the Leaf Laser, and CI-100 (4018 NE 112th Ave. Suite D-8, Vancouver, WA 98682, USA). The performance of these instruments as reported in the literature is reviewed for forest, row crops and individual trees (Welles and Cohen, 1996).

One of the instruments is the LAI-2000 (Plant Canopy Analyzer, LI-COR, Lincoln, Nebraska, USA), which makes use of diffuse light and should, in principle, avoid direct sunlight. Therefore, the measurements should be taken on uniform sky conditions found on overcast days, or near sunset or sunrise to avoid the interference of direct sunlight. The LAI-2000 has been used in a wide range of plant canopies: coniferous and deciduous species (Gower and Norman, 1991), different pines (Law *et al.*, 2001) and boreal forests in Canada (Chen *et al.*, 1997). Many of these studies showed that the LAI-2000 generally underestimates the LAI from direct measurements. Indirect methods of determining LAI which relate total leaf area to the radiation environment below the canopy are generally less time consuming as well as non-destructive. However, indirect LAI measurements are sensitive to a range of external and internal factors, often inducing difficult-to-define errors in the final LAI estimate at the scale of interest, such as the oil palm plantation. The objectives of this study were to determine the effects of different factors such as PCA measuring technique, sky condition, height and direct light on the measured LAI.

Study Site

Measurements were made at the Malaysian Palm Oil Board (MPOB) ENOVECY research plot in Bangi situated about 30 km South from Kuala-Lumpur, (Latitude 2° 58′ 0.36" N, Longitude 101° 44′ 26" E) at an average altitude of 66.5m above sea level. The

commercial D x P palms with 148 planting density were planted in 1998 and managed according to the standard estate practices. The 6 years old uniform palms were used for this study.

Instrument Description

The LAI-2000 Plant Canopy Analyser (LI-COR, USA) is an instrument designed to measure LAI of green canopies. The instrument's sensor is incorporated with fisheye optics to project a hemispheric image onto five silicon detectors, arranged in concentric rings. The sensor also contains an optical filter to restrict sensed radiation of wavelengths below 490 nm, in order to minimize the contribution of radiation that has been scattered by foliage. The control box records the sensor's data and executes the calculation necessary for determining LAI. The basic technique combines a measurement of sky brightness from a levelled sensor above the canopy with a second measurement taken beneath the canopy. The ratio of each ring's signals (below to above) is then assumed to be equivalent to the canopy's gap fraction at that ring's viewing angle. Although the LAI-2000 potentially "views" a full 360° of azimuth, it can be restricted by a view cap of 270°, 180°, 90°, and 45°, attached onto the sensor head to limit sensor view for special purposes (Welles and Norman, 1991). The LAI-2000 consists of five sensors, which simultaneously measures the PAR light intensities in five concentric Field of Views (FOVs) centred at zenith angles of 7, 23, 38, 53 and 68 degrees, and respectively referred to as PCA Sensors 1, 2, 3, 4 and 5. Usually, below and above-canopy readings are simultaneously acquired to calculate the canopy gap fraction, which represents the probability of light penetration. Gap fraction values are then converted to contact frequency values that are used for further analysis (LI-COR 1992).

Basic Theory Related to Light Interception and Leaf Area Index

In an ideal diffusing medium, receiving a radiant flux I at the surface, the flux I at an optical depth F is an inverse exponential function of the extinction coefficient k,

$$I = I \exp(-k * F). \quad (1)$$

Monsi and Saeki (1953) equated LAI with F, explored extensively both the theoretical geometric and practical reasons for variation of k in different plant covers; k being a function of leaf angle and direction of the incident beam. When leaves are grouped in clumps, or regularly rather than randomly distributed, k changes. The combination of theory and practice of measurement of the various components, the foliage area F, flux I, extinction coefficient k, and foliage angle has since been extensively explored (Warren, 1963).

Light traveling through a vegetation canopy is attenuated by leaves interception. The fraction of photosynthetically active radiation (PAR) transmitted through the canopy is related to the distribution and amount of leaves in the canopy. If the leaves are assumed to be randomly distributed in the canopy and opaque in the PAR wavelengths of the irradiance at the bottom of the canopy, then Equation (1) can be written as:

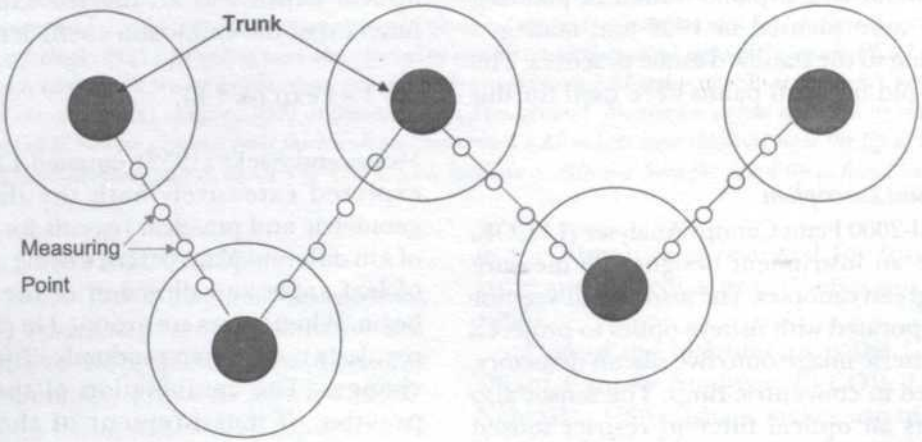
$$I = I_o * \exp(-k * LAI) \quad (2)$$

Where I_o is the incoming irradiance, LAI is the leaf area index, and k is an extinction coefficient. The exponent, (k * LAI), is the area of the shadow of the leaves projected onto a horizontal plane. Assuming a spherical leaf angle distribution, then the distribution of leaf inclination and orientation angles is similar to those found on the surface of a sphere and k, can be calculated from the solar zenith angle, Θ:

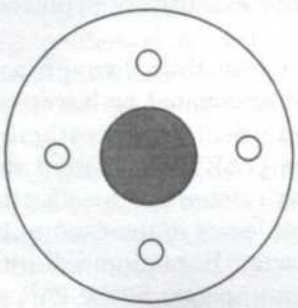
K is a function of zenith angle (Θ) and the leaf inclination angle distribution. Theoretically, k simplifies to

$$K = 1/ 2 \cos \Theta \quad (3)$$

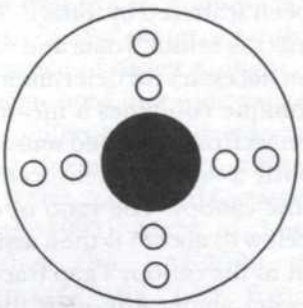
If a spherical leaf angle distribution is assumed (Campbell, 1986), these equations



i) Zigzag methods



ii) One above four below methods



iii) one above eight below methods

Fig. 1: Experimental design for below reading measurements

can be combined to solve for LAI from line sensor measurements of PAR transmittance from measurements above and below the canopy.

MATERIALS AND METHODS

The experiments were conducted in May to June 2003 at the Malaysia Palm Oil Board (MPOB) research plot. Six-year old palms were chosen for this investigation. Five uniform palm trees were chosen at random for measurement. Four different types of tests were conducted in this study. For each test, an above-canopy sensor (A) logged readings in a nearby large opening (no vegetation at greater than 15 degrees above the horizon) while a below-canopy sensor (B) was used to log readings within the experimental plot. Various factors affecting the LAI measurement were also evaluated by the experiments.

Different Measuring Technique

Three techniques were used for accuracy assessment of the PCA LAI-2000, i.e. the Zig-zag method, "one above reading - four below readings" and "one above - eight below readings". Lamade and Setiyo (1996) used zigzag methods for indirect measurement of LAI by PCA (LAI-2000). They selected eight measuring points between two adjacent palms. Roslan *et al.* (2002) used one above canopy

reading followed by four readings under canopy at $^1/_2$ frond length distance from the palm base. In this study, four equal measuring points between two adjacent palms were selected for the zigzag method (*Fig. 1*). For the "one above four below" technique, one above reading followed by four below readings was chosen. Below readings were chosen accordingly in the North-South and East-West direction. Sometime, it is difficult to choose the justified sensor position for below reading measurements along the frond. A homogeneous position was chosen for the below reading measurements. The readings represented the whole palm tree. For the "one above eight below" technique, data were taken in same compass directions as in the previous method. For this technique, two below readings were taken at 1/3 and 2/3 of frond length from frond base (*Fig. 1(iii)*). All PCA LAI-2000 readings were taken with the sensor-facing north at 1.4 meters above the ground and with a 180-degree view cap attached.

Sensor Height

The effect of vertical height (sensor distance from ground to lowest frond) on PCA LAI was evaluated at six different heights under the palm canopy. The first measurement was taken at ground surface and the last measurement taken at least 0.5-meter distance below the lowest frond (*Fig. 2*). Other measurements were then taken at every 0.5-meter interval from the first measurement.

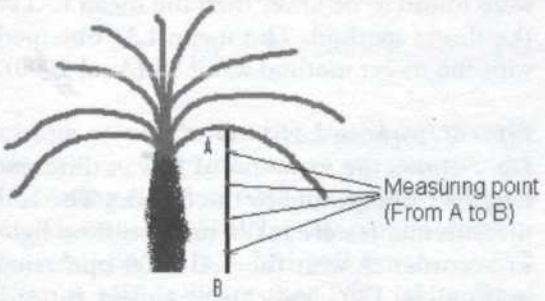


Fig. 2: Vertical distance between ground to the lowest frond

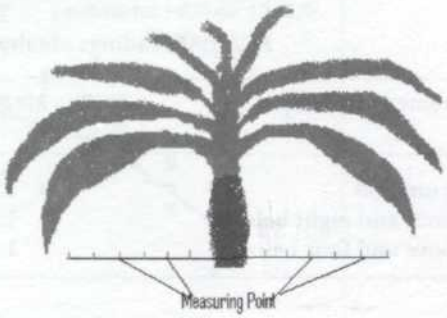


Fig. 3: Spatial variation of PCA reading under canopy

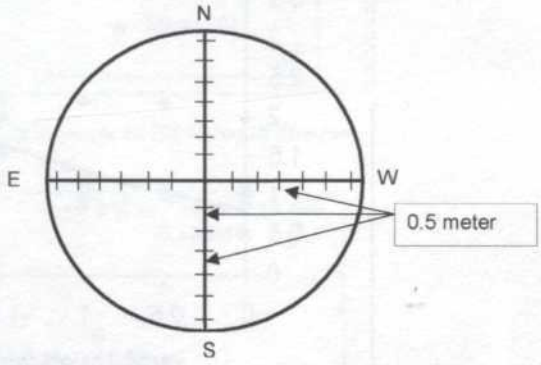


Fig. 4: Experimental design for spatial variation of PCA reading

Distance from Trunk to Frond Tip

To evaluate the effects of spatial variation on LAI at different positions under the palms, measurements were taken at 0.5-meter intervals distance from trunk (*Fig. 3*). Measurements were taken in the North, South, East and West side of the trunk (*Fig. 4*).

View Cap

As Welles (1990) pointed out, the use of a view cap is required to prevent direct sunlight from hitting the sensor and causing increased variability and underestimation in LAI measurements with the PCA instrument. Lastly, for testing the effect of view cap, different view cap sizes (7°, 45°, 90°, 180° and 270°) were used under the same conditions (i.e. same height, same palm, same direction

TABLE 1
PCA LAI readings obtained from the different techniques

Measurement technique	Mean PCA LAI	Reference LAI (LAI obtained with direct method)
Zig-zag method	1.14±0.09	
One above and eight below	1.21±0.09	2.38
One above and four below	1.29±0.15	

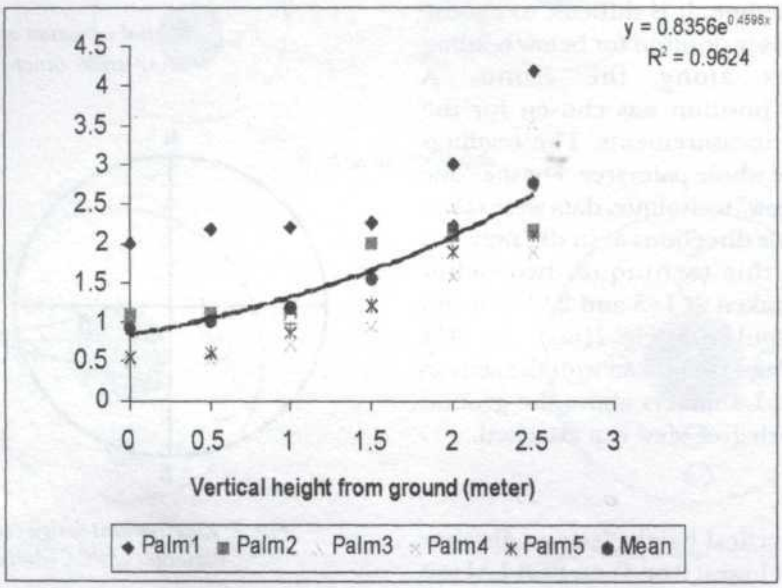


Fig. 5: Variation of PCA reading (LAI) with different height from ground to the lowest frond

and same sky condition). The canopy diameter was 8-meter (the distance between trunks to frond tip was 4 meters) and the lowest average canopy height was 3.5-meter from the ground.

RESULTS AND DISCUSSION

Effect of Different Measuring Technique

Three techniques were compared to investigate the effects on PCA LAI. The results in Table 1 show that the LAI measured with the Zig-zag method was lower as compared to two other methods. The LAI obtained with zig-zag method was 11.6% less than the LAI obtained with the "one above and four below" technique and 5.7% less than the LAI obtained with the "one above and eight below" technique. The LAI obtained with the "one above eight below" technique was 6.2% less than the LAI obtained with the "one above four below" technique. The mean PCA LAI values for the three techniques investigated were found to be lower than the mean LAI of the direct method. The mean LAI obtained with the direct method was 2.38 (Awal 2006).

Effect of Height on LAI

Fig. 5 shows the variation of LAI at different heights from ground to the frond. The LAI measurements were taken under diffuse light in accordance with the LAI-2000 operating manual. A 180-view cap was used for the measurement and the "above-canopy" reference measurements made outside the

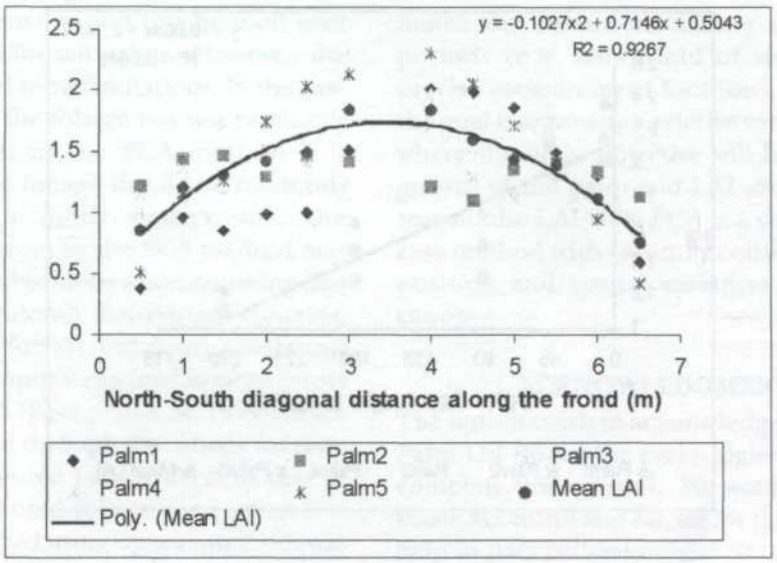


Fig. 6: PCA LAI with spatial variation of PCA sensor in North-South direction

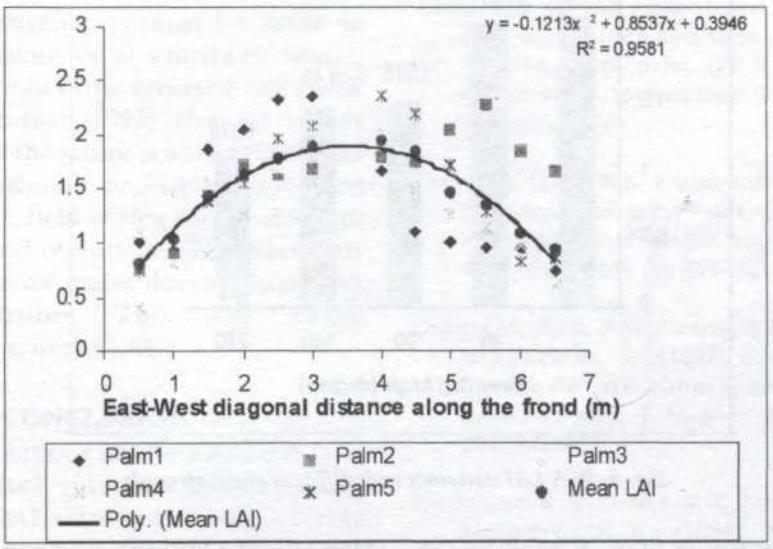
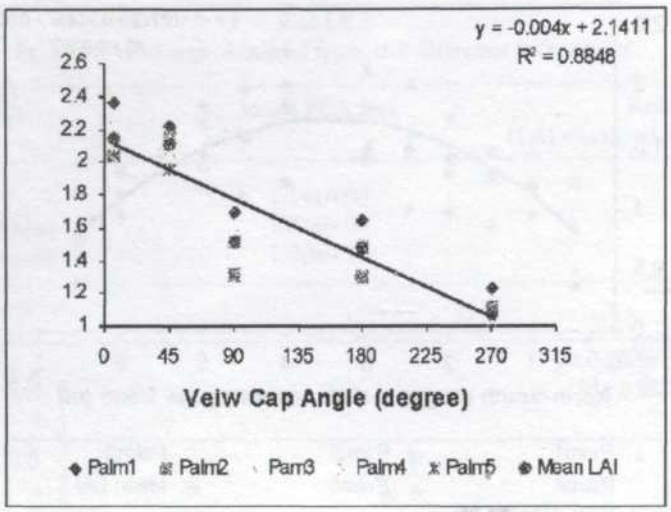


Fig. 7: PCA LAI with spatial variation of PCA sensor in East-West direction

canopy were taken at the same time. Results show that the LAI values increased with increasing distances from ground to the lowest fronds and a strong (R^2 =0.98) relationship was found between PCA LAI and Sensor height. Results also indicate that PCA LAI related exponentially to the sensor height from ground.

Effect of Distance from Trunk to Frond Tip

The assessment of the variation of LAI at different positions beneath the canopy along the North -South and East-West axis are illustrated in *Figs. 6* and *7*. Results show that the LAI decreased from frond base to frond tip in both North-South and East-West directions. Data showed that LAI increased on



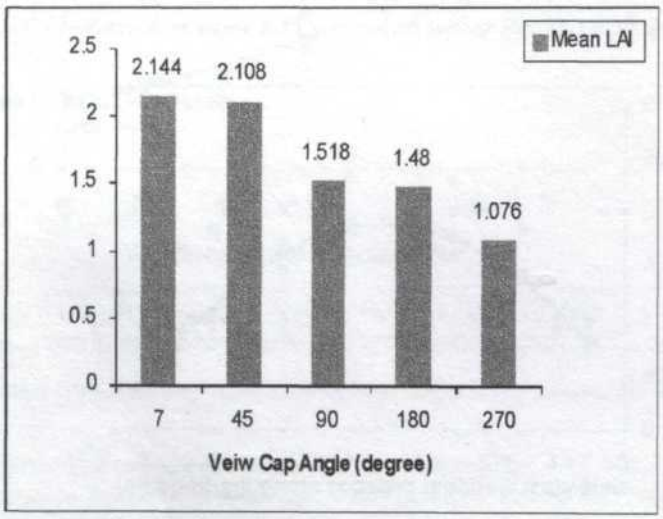


Fig. 8: PCA LAI variation with different view cap angle

average by about 5% to 50%. A good linear relationship (R^2 = 0.84 for N-S and R^2 = 0.84 for E-W) was found between PCA LAI and position of the sensor along the frond.

View Cap Effect on Measuring LAI

Different angles of view cap (7°, 45°, 90°, 180° and 270°) were used to investigate their effects on LAI measurement. *Fig. 8* shows the relationships between view cap angle and PCA LAI. Results showed that the view cap angle selection had great effect on PCA LAI value. Maximum LAI value was obtained for 7° view cap and minimum LAI value for 270° view cap. A linear relationship (R^2 = 0.94) was found between PCA LAI and view cap. Results showed that the view cap angle strongly influenced o PCA LAI. PCA LAI decreased with increasing view cap angle.

The performance of the LAI-2000 Plant Canopy Analyser (PCA) was evaluated under different conditions for indirect LAI measurements of oil palm. Compared to the direct methods, the PCA method was more

rapid, non-destructive and can be used over larger areas and for tall palms. However, the PCA method had some limitations. In the case of the oil palm, the foliage was not randomly distributed, but in the PCA method it is assumed that the foliage should be randomly distributed. In many ecosystems, the measurement errors by the PCA method may be within acceptable limits when assuming that the foliage is randomly distributed. However, in open-canopy forests and many coniferous forests, this assumption can lead to large errors in excess of 100% (Fassnacht *et al.*, 1994). Since light transmission through the canopy for non-randomly distributed foliage exceeds that of randomly distributed foliage for a given leaf area, LAI calculated using optical methods was often underestimated for non-random or clumped, foliage distributions. Other limitations to the use of the LAI-2000 PCA were that the measurements must be made in overcast conditions or at sunrise or sunset; direct sunlight can cause errors of up to 50% (Welles and Norman, 1991). User errors may be substantial if the sensor is not levelled, held at the correct azimuth, or if view caps that are used to limit the field of view do not match in terms of size and orientation. Measurements will not be accurate under direct light or very sunny condition. This may cause underestimation of the LAI.

CONCLUSION

The main limitations of the LAI-2000 PCA method was that it gave underestimated and inconsistent LAI values, and was severely affected by sunlight conditions, spatial variability, and sensor position. However, the ability to easily obtain data without having to correct for limitations of the LAI-2000 PCA method or other optical methods will likely depend on the ultimate use of the data. For example, the LAI-2000 PCA data can be used as an effective means of comparing relative differences among treatments within a system or for examining changes throughout or among seasons, provided that care is taken to match the conditions among measurement periods (e.g. same field of view, azimuth, precise measurement location). Moreover, if the goal is to measure relative variation in LAI, where the main objective will be to monitor growth of the palm and LAI variation within season, the LAI-2000 PCA is a very quick and easy method with carefully considered sensor position and spatial variation under palm canopy.

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The Use of LP-RAPD for Assessing Genetic Relatedness among Selected Banana Cultivars

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ABSTRAK

Analisis LP-RAPD (Primer panjang DNA Polimorfik Gandaan Rawak) telah dijalankan ke atas lapan jenis pisang iaitu Mas (AA), Berangan (AAA), Raja (AAB), Rastali (AAB), Awak (ABB), Nipah (BBB), Kapas (AAB) dan Nangka (AAB) untuk menilai hubungan genetik antara jenis-jenis tersebut. Dua puluh lima individu daripada setiap jenis telah disampel dari negeri Perak, Selangor, Melaka dan Negeri Sembilan. Lima primer panjang terpilih iaitu PEH A3, ERICIR, PUCMI3F, BOXAIR dan PEH A6 telah digunakan untuk menggandakan genom DNA. Corak jalur DNA diperhatikan dan dianalisis. Keputusan menunjukkan fragmen terbesar ialah 2500 bp manakala fragmen yang terkecil ialah 100 bp. Primer ERICIR adalah paling polimorfik (26.5%) manakala PEH A6 merupakan primer yang paling kurang polimorfik (20.8%). Dendrogram menunjukkan terdapat tiga kumpulan utama. Kumpulan I terdiri daripada Berangan, Rastali, Mas, Nangka dan Raja. Kumpulan II merangkumi Kapas dan Awak yang mempunyai nilai jarak genetik terendah (0.3633), di mana kedua-duanya adalah jenis pisang untuk dimasak. Kumpulan I dan II mempunyai perhubungan genetik yang rapat dengan nilai jarak genetik 0.396. Pisang Nipah (BBB) jelas terasing daripada kedua-dua kumpulan tersebut dengan nilai jarak genetik 0.795. Walaupun semua jenis pisang ini berbeza antara satu sama lain secara morfologi, hasil kajian ini menunjukkan darjah keserupaan genetik antara jenis-jenis pisang tersebut adalah selari dengan kumpulan genotip, sama ada A atau B, yang dikongsi.

ABSTRACT

LP-RAPD (Long primer Random Amplified Polymorphic DNA) analysis was carried out on eight banana cultivars namely Mas (AA), Berangan (AAA), Raja (AAB), Rastali (AAB), Awak (ABB), Nipah (BBB), Kapas (AAB) and Nangka (AAB) to assess their genetic relationships. Twenty five individuals of each cultivar were collected from the Malaysian states of Perak, Selangor, Melaka and Negeri Sembilan. Five long-primers, namely PEH A3, ERICIR, PUCMI3F, BOXAIR and PEH A6 were selected to amplify the genomic DNA. The DNA banding patterns were observed and analyzed. The results showed that the largest fragment was 2500 bp and the smallest 100 bp. The ERICIR was found to be the most polymorphic primer (26.5%) whilst PEH A6 was the least polymorphic (20.8%). The dendrogram revealed three major groups. Group I consisted of Berangan, Rastali, Mas, Nangka and Raja cultivars. Group II included Kapas and Awak, which had the lowest genetic distance (0.3633) and are known as plantains by sharing the B genotype. Groups I and II were clustered closely together with a genetic distance of 0.3961 indicating a close relationship between the two groups. The Nipah (BBB) cultivar alone was distinctly separated from both Groups I and II with a genetic distance of 0.795. Although all cultivars differ morphologically, the findings agree with the degree of shared genotypes, A or B, among the cultivars.

INTRODUCTION

Cultivated bananas (including plantains) belong to the *Eumusa* section of the family Musaceae and are natural hybrid polyploids (diploid, triploid or tetraploid) of two species of Musa: *Musa acuminata* (genome A) and *Musa balbisiana* (genome B). These tallest monocotyledons (Stover and Simmonds, 1987) have become the premier fruiting plants of Southeast Asia and are considered to be of great socioeconomic importance in the countries of the region. Bananas rank second or third in importance among the industrial fruits of India, Malaysia and Taiwan, and are important export commodities for countries such as Malaysia and those in Central America (Valmayor, 1987). All cultivars are classified into various genomic groups such as AA, AAA, AB, AAB and ABB based on the morphological scoring method (Simmonds, 1987). Cultivars containing the B genotype have starchy and acidic fruits and they are usually eaten boiled, fried or roasted. Cultivars containing the A genome have sweet and fine textured fruits, and they are mainly eaten raw or as a dessert. Among the popular dessert bananas in Malaysia are Pisang Mas (AA), Berangan (AAA) and Rastali (AAB) while the popular cooking types are Pisang Raja (AAB), Nangka (AAB), Awak (ABB), Nipah (BBB) and Kapas (AAB) (Jamaluddin, 1990).

Mas has a small fruit, 8.0-12.0 cm in length and 3.0-4.0 cm in diameter. The peel is thin and golden yellow in colour when ripe. The pulp is firmly attached, yellow in colour, aromatic and very sweet. The fruit of Berangan is medium to large in size and the peel is thick, golden yellow in colour and covered with slight to heavy blemishes. The flesh of Berangan is very aromatic and sweet. Rastali has a thin peel, yellow orange in colour when ripe and covered with moderate to heavy black blemishes and slightly sour in taste. The fruit of Raja is angular and the skin is thick and coarse and develops an orange-yellow colour when ripe. The flesh of Raja is coarse in texture. The Nangka plant is short to medium and the fruit is long, pointed and angular. The peel is thick and remains green when ripe. The pulp of Nangka is creamy, starchy and slightly sour in taste. The Awak has a small to medium fruit and becomes yellow when ripe. The skin of Awak is thick and the pulp is whitish, firm and consistently sticky. The Nipah has a short, stout and angular fruit with a thick skin that turns yellow when ripe. The fruit of Kapas is shorter than the Awak and the skin turns yellow in colour when ripe. The taste is sweet with slight subacid flavour (Jamaluddin, 1990).

The classical approaches for the identification of banana cultivars are based on morphological characters. However, morphological changes caused by environmental factors are major obstacles to accurately identify the varieties (Kaemmer *et al.*, 1992). DNA markers have proven to be useful, efficient and reliable methods for genetic characterization, studying genetic diversity and relationships among populations and varieties because they are not affected by environmental conditions (William *et al.*, 1990).

Random Amplified Polymorphic DNA (RAPD) is widely and successfully used for determining genetic diversity in a number of plant species, such as plum (Ortiz *et al.*, 1997), lemon (Deng *et al.*, 1996) and grapes (Qu *et al.*, 1996). The RAPD technique is relatively quick, inexpensive and requires no prior sequence information of the target genome. Small amounts of DNA are sufficient. It requires the use of no radioactive isotopes and yet can detect a good number of polymorphisms (William *et al.*, 1990; Welsh and McClelland, 1990). However, this technique requires careful optimization that can affect the reproducibility of the results (Yang and Quiros, 1993). To overcome the reproducibility problem, the long primer Random Amplified Polymorphism DNA (LP-RAPD) technique (Gillings and Holley, 1997) is used. This technique provides a more sensitive and reproducible PCR method because longer primers can make the PCR amplification more selective and thus be able to easily distinguish closely related organisms.

TABLE 1
Primers used in the LP-RAPD method (Gillings and Holley, 1997)

Primers	Sequence (5''Æ3')
PEH A3	CAGCAGAACCCGCGCCTGATCCAG
PUCM13F	CGCCAGGGTTTTCCCAGTAGTCAC
BOXAIR	CTACGGCAAGGCGACGCTGACG
PEH A6	ATCGCACTTGATGATGCGCAGGCCGTT
ERICIR	ATGTAAGCTCCTGGGGATTCAC

The objective of this study was to assess the genetic relatedness among eight local banana cultivars by using the LP-RAPD technique.

MATERIALS AND METHODS

Plant Material

Eight local banana cultivars, namely Pisang Mas (AA), Berangan (AAA), Rastali (AAB), Raja (AAB), Nangka (AAB), Awak (ABB), Kapas (AAB) and Nipah (BBB), were used in this study. Twenty five samples of each cultivar were collected from the Malaysian states of Perak, Selangor, Melaka and Negeri Sembilan.

DNA Isolation

Young leaves from each individual sample were stored at -20°C prior to DNA extraction. Total DNA was extracted following the CTAB method of Doyle & Doyle (1990) with some modifications.

One tenth gram (0.1 g) of leaves was ground by using cold mortar and pestle in 1 ml of 65°C preheated CTAB buffer [2% (w/v) CTAB, 1.4M NaCl, 0.2% mM EDTA, 100mM Tris-HCl (pH 8.0) and 1% (v/v) PVP-40] and incubated at 67°C for 60 minutes. The lysate was extracted with 0.79 ml of chloroform/ isoamylalcohol (24:1) and centrifuged for 15 min at 1000 rpm at 4°C. In order to precipitate the DNA, the aqueous portion was mixed with an equal volume of cold isopropanol.

The DNA precipitate was washed in 1 ml of 70% ethanol with 3M ammonium acetate and air dried before being resuspended in 200ml of TE buffer [5 mM Tris, 0.1 mM EDTA, pH 7.5].

The DNA purity was determined by using a spectrophotometer. The absorbance was read at wavelengths of 260 nm and 280 nm. DNA purity is indicated by the ratio of absorbance at 260 nm and 280 nm of 1.8 to 2.0.

DNA Amplification

The amplification was performed as described by William *et al.* (1990) with some modifications. The amplification reactions consisted of 50 ng of template DNA; 1X PCR reaction buffer [10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1% Triton®X-100], 3.0 mM $MgCl_2$, 0.2 mM each of dATP, dTTP, dCTP and dGTP; 1.5 units of Taq DNA Polymerase and 1 μM primer. The primers used in this study are shown in Table 1 (Gillings and Holley, 1997). Sterilized deionized water was added to make up 10 μl of total reaction volume. Samples were amplified in a MJ Research PTC –100 thermal cycler. The thermal cycler was programmed for predenaturation at 94°C for 5 min. Samples were processed through 39 cycles consisting of denaturation at 94°C for 30 sec, annealing at 52°C for 1 min and extension at 72°C for 2 min. For the last cycle, the extension step at 72°C was extended to 10 min. The amplified fragments were separated on 2.0 % agarose gel using 1x TBE buffer. After staining with ethidium bromide the bands were visualized and photographed using Alpha Imager Digital Imaging System (Siber Hegner Sdn. Bhd.).

TABLE 2
Size range of fragments (bp), number of amplified bands, number of monomorphic bands and percentage of polymorphic bands produced by five primers in eight banana cultivars

Primer	Fragments size (bp)	No. of amplified band	% of monomorphic band	% of polymorphic band
Peh A3	100-2500	44	74.6	25.4
ERICIR	100-2500	36	73.5	26.5
PUC M13F	150-1600	38	79.2	20.8
BOXAIR	230-2000	33	73.9	26.1
PEH A6	140-1500	41	76.9	23.1

Analysis of DNA Amplification

Clear and sharp bands of the amplified DNA using different primers were compared among banana cultivars. Each band was scored as present (1) or absent (0). Genetic distances (D) were calculated using the formula of Nei & Li (1979). A dendrogram was constructed based on the genetic distance matrix data by using the Unweighted Pair Group with Arithmatic Mean (UPGMA) method (Sneath and Sokal, 1973).

RESULTS

From the 10 long primers tested, five primers gave clear and high resolution banding patterns. A combination of five selected primers produced a total of 192 scorable bands ranging in size from 100 bp to 2500 bp. Among these, 145 were monomorphic and 47 were polymorphic in the eight banana cultivars. Each primer generated between 33 and 44 scorable bands (Table 2). The highest number of scorable bands was generated by PEH A3 while the lowest was by BOXAIR. The most informative primer was ERICIR which produced the highest percentage of polymorphic bands (26.5%) whilst the lowest percentage (20.8%) was produced by PUCM13F (Table 2).

The average number of polymorphic bands detected per primer was 29. Among the cultivars, Kapas had the highest percentage of polymorphic bands whilst Raja had the lowest. Table 3 shows that the PEH A3 primer produced 300 bp, 400 bp and 1000 bp bands as common bands in all cultivars. The Raja, Rastali, Nipah, Mas, Awak and Nangka cultivars are distinguishable among themselves by the presence of unique 1500 bp, 366 bp, 320 bp, 1250 bp, 800 bp and 550 bp bands, respectively. Rastali also can be marked at 325 bp.

The ERICIR primer produced a 750 bp band which was present in all cultivars as a common band. The Mas was clearly distinguished from the other cultivars by the presence of a 2500 bp band. The 1375 bp band only existed in the Nangka cultivar. All cultivars had 1125 bp except for Mas. The 416 bp band was only absent in the Nipah cultivar while the 375 bp band was observed in the Raja cultivar. The 400 bp, 450 bp and 500 bp bands can also be used to distinguish Nipah from the other cultivars.

The BOXAIR primer showed that all cultivars had a 250 bp band. The Nipah cultivar can be distinguished from the other cultivars by the absence of a 325 bp band which existed in all other cultivars. The 600 bp band was present in all AAB cultivars except for Kapas while Berangan, Raja and Mas cultivars shared a 1000 bp band. The Rastali cultivar can be discriminated from the other cultivars by the presence of the 1125 bp and 1375 bp bands. The PEH A6 primer revealed that all cultivars had a 217 bp band (*Plate 1*). The Nipah was clearly distinguished from the other cultivars by the absence of 225 bp band. Awak and

TABLE 3
Distribution of LP-RAPD markers within eight banana cultivars.
'+' indicates presence and '-' indicates absence of band

Primer/Locus	bp	Cultivar							
		Berangan	Nangka	Raja	Rastali	Nipah	Kapas	Awak	Mas
PEH A3	1500	-	-	+	-	-	-	-	-
	1250	-	-	-	-	-	-	-	-
	1125	+	-	+	-	-	+	-	-
	1000	+	+	+	+	+	+	+	+
	900	+	+	-	-	+	+	-	+
	800	-	-	-	-	-	-	+	-
	750	+	+	-	-	-	+	-	-
	700	+	+	+	+	+	+	+	+
	650	+	+	-	-	-	-	-	+
	550	-	+	-	-	-	-	-	-
	475	-	+	+	+	-	+	-	-
	400	+	+	+	+	+	+	+	+
	366	-	-	-	+	-	-	-	-
	320	-	-	-	-	+	-	-	-
	300	+	+	+	+	+	+	+	+
	325	-	-	-	+	-	-	-	-
ERICIR	2500	-	-	-	-	-	-	-	+
	1500	-	-	-	+	-	+	+	+
	1375	-	+	-	-	-	-	-	-
	1125	+	+	+	+	+	+	+	-
	900	-	-	-	+	-	+	+	-
	850	-	-	-	-	+	+	+	-
	800	-	-	+	-	-	-	-	-
	750	+	+	+	+	+	+	+	+
	700	-	-	-	-	+	-	-	-
	650	+	-	+	+	+	-	+	-
	600	-	-	+	-	-	-	-	-
	500	-	-	-	-	+	-	-	+
	450	-	-	-	-	+	-	-	-
	433	+	+	+	+	-	-	+	+
	416	+	+	+	+	-	+	+	+
	400	-	-	-	-	+	-	-	-
	375	-	-	+	-	-	-	-	-
	312	-	-	+	+	+	+	-	+
	300	+	-	+	-	-	-	-	-
PUCM13F	1250	-	+	-	+	-	-	-	+
	1000	-	-	-	+	+	-	+	-
	925	-	-	-	-	-	-	-	+
	900	+	+	+	-	-	-	-	-
	750	-	-	-	+	-	-	-	-
	700	+	+	+	-	-	-	+	+
	575	-	-	-	-	-	-	+	-
	550	+	+	+	+	+	+	-	-

Table 3: Continued

Primer/Locus	bp	Cultivar							
		Berangan	Nangka	Raja	Rastali	Nipah	Kapas	Awak	Mas
	525	-	-	-	-	-	-	-	+
	500	-	-	+	-	+	+	+	-
	450	-	+	-	-	-	-	-	-
	425	+	+	+	+	+	+	+	+
	375	+	+	+	-	-	-	-	-
	350	+	+	-	-	+	-	-	+
	266	+	+	+	-	+	+	-	+
	250	+	+	+	-	+	-	-	-
	216	+	+	+	-	+	+	-	-
	171	+	-	-	+	-	-	-	-
	128	-	-	-	+	-	-	-	-
BOXAIR	2000	-	-	+	+	-	-	-	+
	1500	+	+	+	-	-	-	-	-
	1375	-	-	-	+	-	-	-	-
	1250	+	-	+	-	-	+	+	-
	1125	-	-	-	+	-	-	-	-
	1000	+	-	+	-	-	-	-	+
	900	-	-	-	-	+	-	-	+
	800	+	+	-	-	-	-	+	+
	750	-	-	+	-	-	-	+	-
	700	-	-	-	-	-	+	-	-
	650	+	-	+	+	-	+	+	+
	600	-	-	-	+	-	-	-	+
	550	-	-	-	+	-	-	-	+
	500	-	+	-	-	+	-	+	+
	475	-	+	-	-	-	-	-	-
	450	+	-	-	-	+	+	+	+
	400	-	+	+	+	-	+	+	+
	350	-	-	-	-	+	-	-	+
	325	+	+	+	+	-	+	+	+
	250	+	+	+	+	+	+	+	+
PEH A6	1500	+	-	-	-	-	-	-	-
	1250	+	-	-	-	-	+	-	-
	1000	+	-	-	-	-	-	+	-
	700	+	-	-	-	-	+	+	-
	650	+	-	-	-	-	-	+	-
	600	-	-	-	-	-	-	+	-
	500	+	+	-	-	-	-	-	-
	350	-	-	-	-	-	-	+	-
	267	-	-	-	+	+	-	-	-
	225	+	+	+	+	+	+	+	-
	217	+	+	+	+	+	+	+	+
	175	+	+	+	-	+	+	-	-
	150	+	+	-	-	-	+	+	+
	125	-	+	+	+	+	+	-	+

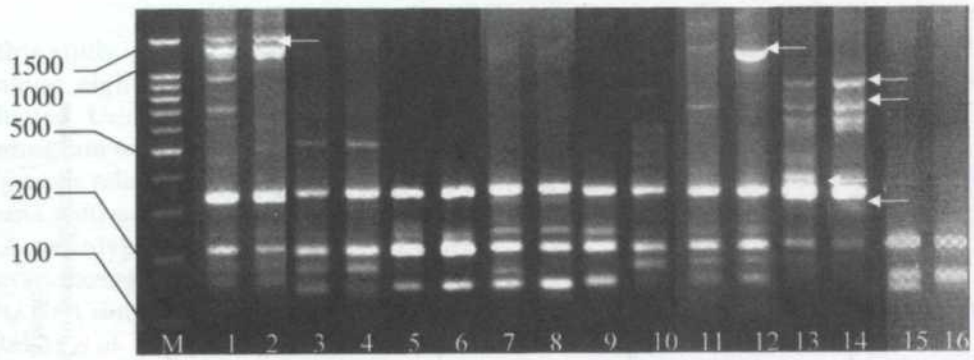

Plate 1: DNA banding patterns among eight banana cultivars obtained by using the long-primer PEH A6 (M = 100 bp ladder; lane 1 and 2 = Berangan; lane 3 and 4 = Nangka; lane 5 and 6 = Raja; lane 7 and 8 = Rastali; lane 9 and 10 = Mas; lane 11 and 12 = Kapas; lane 13 and 14 = Awak; lane 15 and 16 = Nipah

TABLE 4
Genetic distance values among eight banana cultivars

	BERANGAN	NANGKA	RAJA	RASTALI	NIPAH	KAPAS	AWAK	MAS
BERANGAN	0							
NANGKA	0.74823	0						
RAJA	0.78977	0.79455	0					
RASTALI	0.74226	0.78955	0.74786	0				
NIPAH	0.82475	0.79233	0.79806	0.78011	0			
KAPAS	0.80261	0.81485	0.82394	0.80412	0.79628	0		
AWAK	0.76815	0.76134	0.77332	0.74654	0.79111	0.72661	0	
MAS	0.7507	0.77489	0.78525	0.76092	0.79213	0.80595	0.76868	0

Berangan both shared the 1000 bp and 650 bp bands. The Awak cultivar was characterized by the presence of 350 bp and 600 bp bands while Berangan had a 1500 bp band. Kapas was distinguished from the other cultivars by the presence of a 1250 bp band.

The PUCM13F primer produced a 425 bp band in all cultivars. The 375 bp band can be used to distinguish the Raja, Berangan and Nangka cultivars from the other cultivars. The 750 bp and 128 bp bands were observed only in the Rastali cultivar while the 575 bp band was only present in the Awak cultivar. The 925 bp and 525 bp bands were only exhibited in the Mas cultivar and can be considered as marker bands.

The values of the genetic distance between pairs of banana cultivars are presented in Table 4. The dendrogram constructed by using the UPGMA method demonstrated that three major groups existed in the collection. Group I consists of Berangan (AAA), Rastali (AAB), Mas (AA), Nangka (AAB) and Raja (AAB) cultivars. Group II included Kapas (AAB) and Awak (ABB), which had the lowest genetic distance (0.3633) and are known as plantains by sharing the B genotype. Group I and II are clustered closely together with a genetic distance of 0.3961, indicating a close relationship between the two groups. The Nipah (BBB) cultivar alone was distinctly separated from both Groups 1 and II with a genetic distance of 0.795.

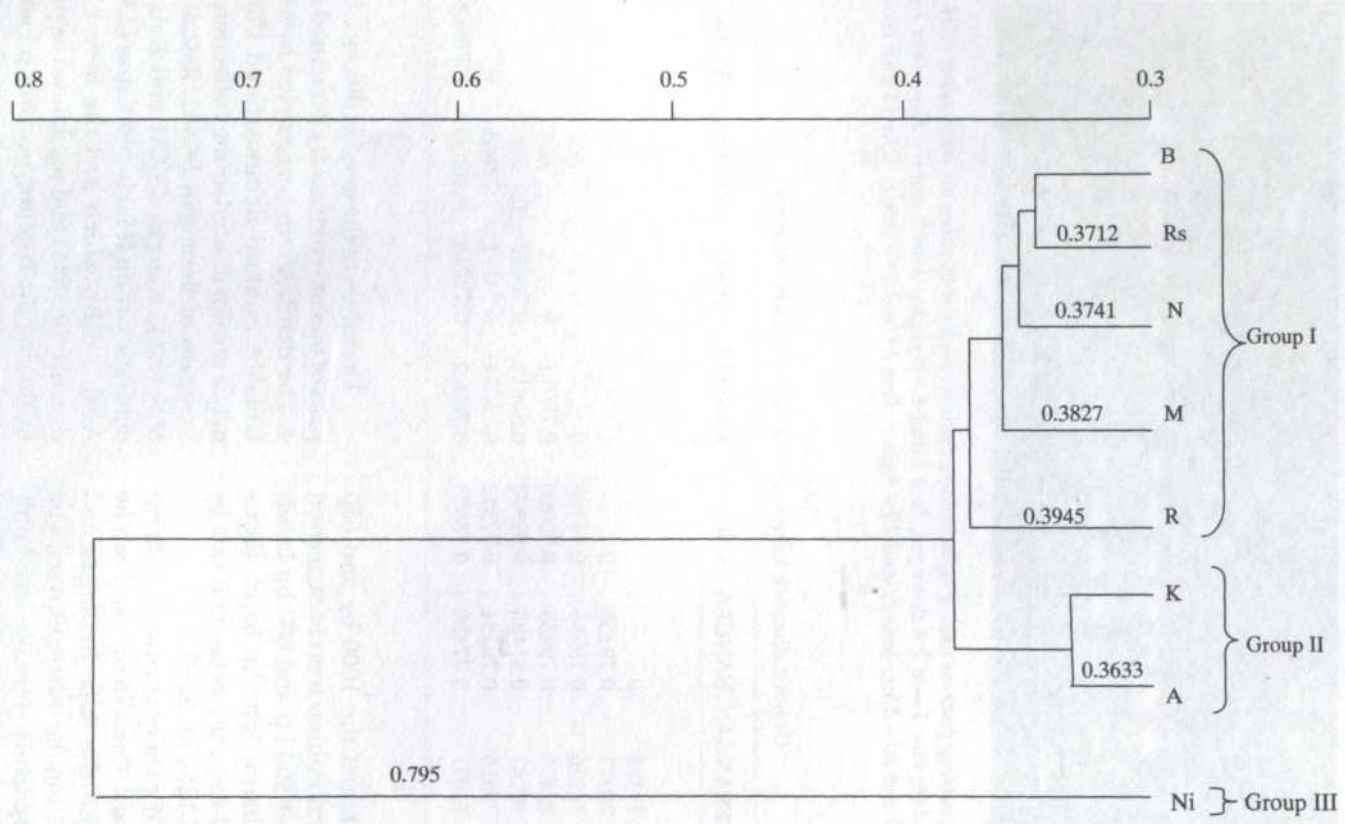

Fig. 1: Dendrogram showing the clustering of eight banana cultivars constructed by using the UPGMA method based on genetic distance values. (B = Berangan, Rs = Rastali, N = Nangka, M = Mas, R = Raja, K = Kapas, A = Awak, Ni = Nipah)

DISCUSSION

In this study, genetic distances among the common eight local banana cultivars were evaluated. Using the genetic distance data, a dendrogram was constructed to demonstrate the genetic relationships among the eight local banana cultivars. All cultivars sharing at least an A genotype were separated from the cultivars having at least a B genotype, as shown in *Fig. 1*. A similar observation was also made by Rekha *et al.* (2001). In general, low genetic distance was demonstrated between cultivars sharing the A genotype and within Group II itself. All the cultivars are different morphologically, especially the pseudostem colour, height and fruit shape as reported by Jamaluddin (1990) and also fruit size, leaf shape and flavour (Valmayor *et al.*, 2000). However, the results revealed that they are genetically closer and share at least an A genotype.

The Nipah cultivar which has a triploid BBB genotype was distantly separated from the other two groups although some of them shared at least a B or BB genotypes. This showed that the contribution of the B or BB genotypes in the triploid bananas did not significantly affect the genetic relatedness among the eight banana cultivars.

The LP-RAPD technique allowed the detection of polymorphisms by only using five long primers. The amplification products were generally reproducible and reliable. Some aspects need further investigations, as there was a confusion regarding the grouping of two popular bananas, Berangan (AAA) and Mas (AA), in the AAB group. Some variations might occur in both cultivars due to human selection or geographical factors as reported by Sagredo *et al.* (1998).

CONCLUSIONS

The results of this study have proven that LP-RAPD is an effective molecular technique to be used in assessing the genetic variation and relationships among the eight banana cultivars. Low genetic distance was demonstrated between cultivars sharing the A genotype. All cultivars sharing at least an A genotype were separated from cultivars having at least a B genotype. Among the eight cultivars, Kapas and Awak had the lowest genetic distance while Nipah was distantly separated from the other cultivars.

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Statistical Mapping of Quantitative Trait Loci Controlling the Time to First Callusing in Oil Palm (*Elaeis guineensis* Jacq.) Tissue Culture

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Keywords: Quantitative Trait Loci (QTL), Time to First Callusing (TFC), oil palm, genomic loci

ABSTRAK

Sebanyak 400 penanda genetik (126 RFLP, 274 AFLP) telah berjaya dipetakan daripada 87 progeni F_1 dari kacukan Deli dura X Yangambi pisifera. Pemetaan tersebut telah menghasilkan peta genetik yang lebih padat dan meliputi genom yang berukuran 1,714cM dan 1,225cM bagi pisifera dan dura. Menggunakan Kosambi interval mapping, perisian MapQTL Versi 4.0, kajian selanjutnya telah dijalankan untuk mencari 'quantitative trait loci' (QTL) yang mempengaruhi tempoh pembentukan kalus (TFC) bagi kultur tisu sawit. Data kultur tisu menunjukkan taburan yang berterusan. Dalam kajian ini, tiga QTLs telah dikesan pada pisifera manakala dua untuk dura pada nilai signifikan 99% dan 95%. Ini menunjukkan lokasi QTL tersebut adalah signifikan secara statistik dalam mempengaruhi variasi bagi TFC. Oleh yang demikian, maklumat ini menunjukkan bahawa loci genomik memberi kesan terhadap kemampuan sawit untuk dikulturkan.

ABSTRACT

An additional 400 genetic markers (126 RFLPs, 274 AFLPs) were successfully mapped on the earlier developed linkage maps using 87 F_1 progenies derived from Deli dura X Yangambi pisifera cross. This resulted in a denser map with coverage length of 1,714cM and 1,225cM for pisifera and dura, respectively. Further exploration to search for quantitative trait loci (QTL) associated with time to first callusing (TFC) was carried out by Kosambi Interval Mapping using the computer program MapQTL Version 4.0. The tissue culture trait data showed a continuous distribution. In this paper, three likelihood QTLs were detected in pisifera and two QTLs in dura at 99% and 95% significant thresholds. These QTL locations can be designated as statistically significant for contributing to the variation of TFC. Therefore, the information points to a genomic loci affecting tissue culturability in oil palm.

INTRODUCTION

In the current tissue culture production of oil palm planting material, some genotypes are observed to be more amenable to tissue culture than others. The rate of callus formation has been reported to be variable between clones (Ginting and Fatmawati, 1995; Rival *et al.*, 1997; Wooi, 1995). This variability also extends to time to first callusing (TFC). This was clearly observed in TFC data collected in the current study where, some of the palms easily formed callus as early as 3 weeks and others took up to 33 weeks to form callus.

The development of molecular markers has made it possible to generate a linkage map for individual palm and consequently allows identification of QTLs based on the framework map. QTL mapping for tissue culture traits

have been reported in other crops such as rice (Taguchi-Shiobara *et al.*, 1997) and barley (Mano and Kotmatsuda, 2002). This has clearly showed that tissue culture traits can be mapped as QTLs in order to find out significant regions within the genome affecting the variation. Detection of QTLs opens up a fine view of quantitative genetic architecture and provides potential tools in marker-assisted selection.

The methodology of QTL detection is somehow laborious and involves complex mathematical calculations. However, computer software packages such as JoinMap and MapQTL have made mapping of QTLs possible for almost every species. Several QTL mapping approaches have been developed such as, Interval Mapping (Lander and Botstein, 1989) and MQM mapping (Jansen, 1993, 1994) which can be used together to improve the mapping resolution. A simple calculation for determining the significant LOD threshold for declaring a QTL was developed by Van Ooijen (1999) based on the result of stochastic simulation of a diploid species with a map density of one marker every 1cM. This helps to provide confidence in detection of QTLs and avoid false positives.

The objective of this study was to generate additional RFLP and AFLP markers to improve the dura (ENL48) and the pisifera (ML161) linkage maps developed by Chua *et al.* (2006). The dura linkage map constructed by Chua *et al.* (2006) consisted of 42 RFLPs and 36 AFLPs while the pisifera linkage map, had 65 RFLPs and 68 AFLPs. The QTL analysis was then carried out by using the updated framework maps to identify QTLs associated with TFC with the eventual aim to develop diagnostic tools for selection of ortets that are amenable to tissue culture.

MATERIALS AND METHODS

Plant Materials

The mapping material used in this study consisted of 87 F_1 palms produced by a cross between Ulu Remis Deli dura (ENL48) and Yangambi pisifera (ML161). The oil palm material was supplied by FELDA Agricultural Services Sdn. Bhd.

Tissue Culture Response: Time to First Callusing (TFC)

The tissue culture of the F_1 palms was carried out by the collaboration of seven major laboratories of the oil palm industry namely: Guthrie Biotech Laboratory Sdn. Bhd., IOI Corporation Bhd, United Plantation Bhd, Golden Hope Plantation Bhd., Ebor Laboratories, Applied Agriculture Research Sdn. Bhd. and Malaysian Palm Oil Board (MPOB). The cultures were observed closely for the first callus formation via TFC. The TFC, which is a phenotypic data, was transformed to $\arctan\sqrt{(x+1)}$ for improving the normality of variance prior to QTL analysis.

RFLP Analysis

RFLP procedures which included DNA extraction (Doyle and Doyle, 1990), digestion using various restriction enzymes (*Bam*HI, *Bcl*I, *Bgl*II, *Dra*I, *Eco*RI, *Hind*III, *Hin*dI, *Bst*NI, *Rsa*I, *Hae*III, *Taq*I and *Xba*I), electrophoresis, Southern blot and Southern hybridization were performed as described by Cheah *et al.* (1993). The oil palm cDNA probes used in the RFLP analysis were generated from different tissues and stages of development such as, young etiolated seedling, mesocarp, kernel, inflorescence, callus and embryoids which were kindly supplied by the MPOB Biological Research Centre (MBRC).

AFLP Analysis

Two restriction enzyme-combinations: *Eco*RI/*Mse*I and *Pst*I/*Mse*I were used for generating AFLP markers. The experimental assay for *Eco*RI/*Mse*I was carried out as described by the manufacturer in the manual (cat no. 10717-015 and 10719-011, Invitrogen™ Life Technologies) with some minor modifications as described by Rajinder *et al.* (1998). For *Pst*I/*Mse*I enzyme-combination, adapters and primers were synthesized by Invitrogen™ Life Technologies.

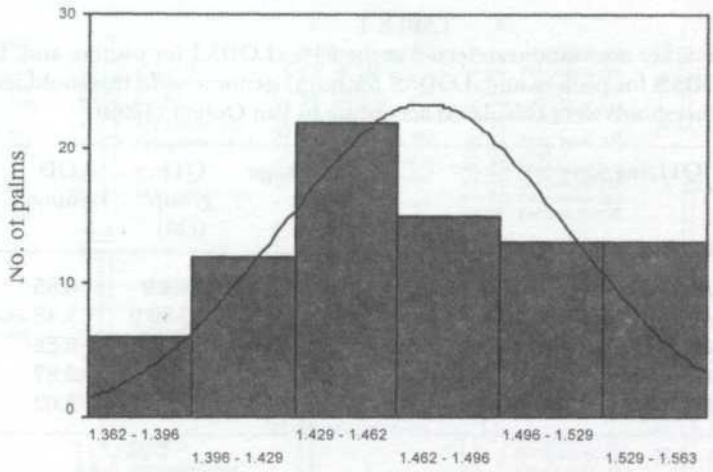

Fig. 1: Distribution of the time to first callusing trait (TFC) in F_1 mapping progenies

RFLP and AFLP Linkage Mapping

The RFLP and AFLP segregating data were expected to meet the Mendelian ratio in a pseudo-testcross strategy (Grattapaglia and Senderoff, 1994) and scored according to Lespinasse *et al.* (2000). The deviation from the hypothesis was tested using goodness of fit (X^2 test) at $p<0.05$. The linkage maps of dura and pisifera were constructed using JoinMap™ version 3.0 (Van Ooijen and Voorrips, 2001). Kosambi mapping function was applied for calculating the map distances in centiMorgan (cM). Linkage groups were determined at LOD score of 4.0 and recombination frequency 0.499. The order of markers arrangement was examined across the range from minimum LOD 1.0 to LOD 7.0.

QTL Mapping

QTL analysis was performed using the Interval Mapping function, MapQTL version 4.0 (Van Ooijen, 2002). In addition, a multiple-QTL model (MQM) mapping was applied that included the significant markers to control for genetic background effects. Associations between TFC and marker were determined at both 95% and 99% genome-wide empirical thresholds. The significant thresholds were calculated based on the formula:

$$1-\alpha_c = \sqrt[n]{(1-\alpha_g)}$$

where, α_g is the genome-wide significance level; α_c is the chromosome-wide significant level and n is the number of linkage groups. The LOD score at $1-\alpha_c$ value was then obtained in the cumulative distribution function Table 1. The method is described in detail by Van Ooijen (1999).

RESULTS

Mapping Family

The mapping family used in this study was obtained from a dura X pisifera cross. It was reported by Chin and Suhaimi (1996) that the Ulu Remis origin dura combines well with Yangambi pisifera and the progenies gave good oil yield. As such the cross is of importance in the breeding programme and will be well maintained.

Distribution of TFC Data

The $\arctan\sqrt{(x+1)}$ transformed TFC data was found to be normally distributed at $p<0.05$ (Kolmogorov-Sminov test). The frequency distribution of transformed TFC trait of the 87 F_1 palms is shown in *Fig. 1*. The TFC data showed a continuous distribution, covering a

TABLE 1

Significant TFC-marker associations detected at the 95% (LOD3.1 for pisifera and, LOD3.0 for dura) and 99% (LOD3.9 for pisifera and, LOD3.8 for dura) genome-wide threshold level. Empirical thresholds were calculated according to Van Ooijen (1999)

Linkage map	QTL-markers	Linkage group	QTL (cM)	LOD Position	Variation (%)
ML161	FDB112/EACG/MCTA>330/FDB120	4	0.0-24.9	6.85	62.1
ML161	CB6F/TAGG/HCTA-550	5	78.0-80.9	5.48	70.2
ML161	TAAC/HCAA-650	8	71.7	3.22	17.4
ENL48	EACA/MCAG-99	8	0.0	3.87	78.1
ENL48	G142	19	16.3	3.02	18.6

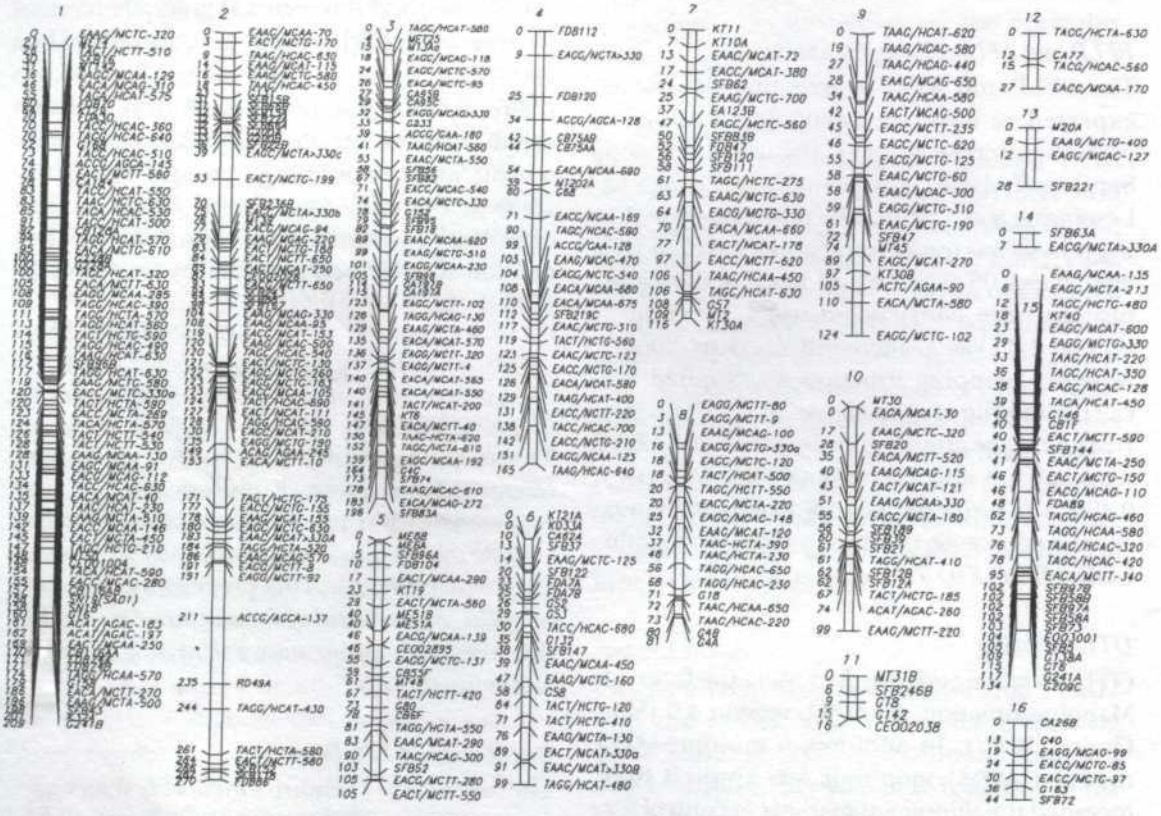

Fig. 2: Genetic linkage map of the pisifera (ML161) parental palm. Distances in Kosambi centimorgans (cM) are labeled on the left side of linkage groups

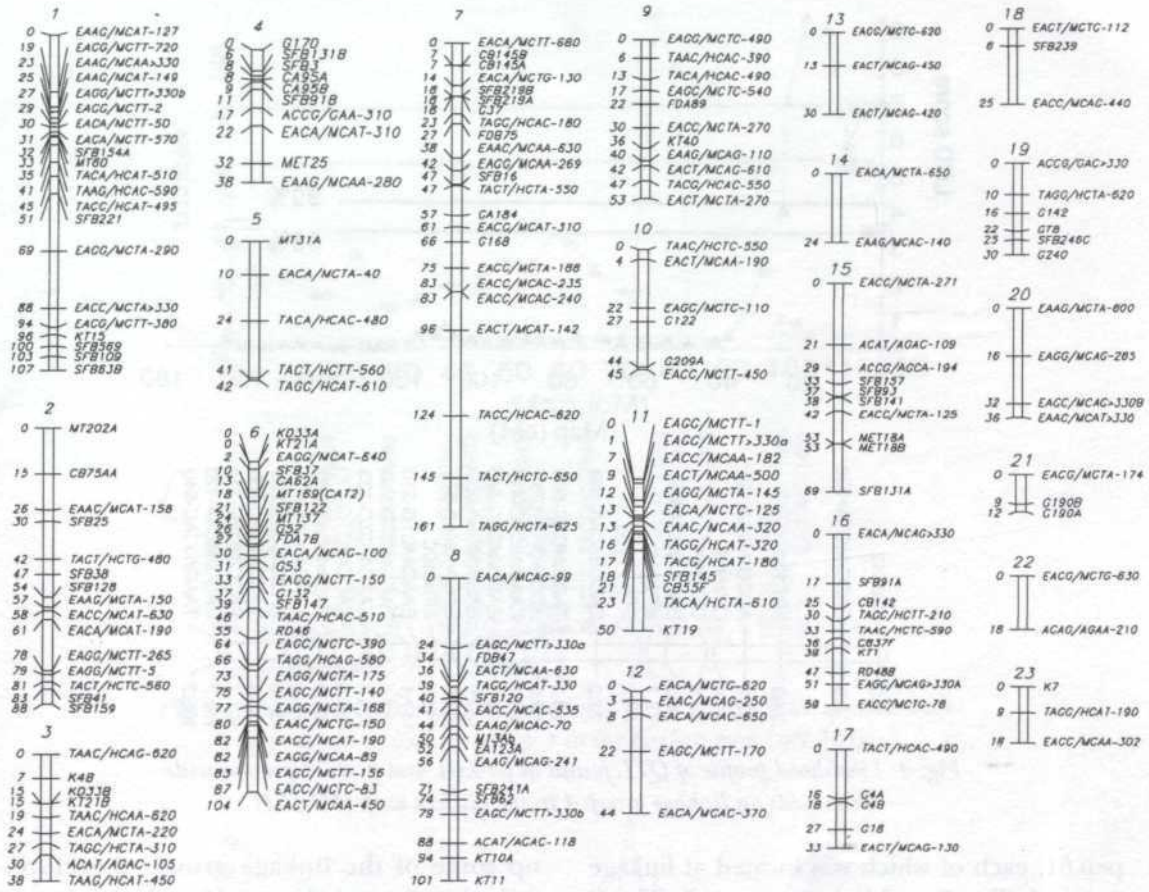

Fig. 3: Genetic linkage map of the dura (ENL48) parental palm. Distances in Kosambi centimorgans (cM) are labeled on the left side of linkage groups

wide range, 4 to 33 weeks with a mean of 12.10 ± 7.75.

RFLP and AFLP Linkage Mapping

A total of 299 and 173 segregating bands were scored from RFLP and AFLP analysis for the pisifera and dura parent, respectively. Linkage maps were constructed with density 395 loci/1,714cM for pisifera and 214 loci/1,225cM for dura. Correspondingly, the map distances between adjacent markers were 4.34cM and 5.72cM for the pisifera and dura maps, respectively. For pisifera, all the mapped loci were assigned into 16 linkage groups with size ranging from 7cM to 270cM *(Fig. 2)*. For dura, a map comprising of 23 linkage groups was constructed ranging from12cM to 161cM *(Fig. 3)*.

QTLs Associated to TFC

Using the Interval mapping analysis, three associated QTLs were identified in pisifera (ML161) and two in dura (ENL48) at the genome-wide significant threshold level calculated according to Van Ooijen (1999) (Table 1). Two highly significant QTLs, FDB112+EACG/MCTA>330+FDB120 and CB6F+TAGG/HCTA-550 were identified at

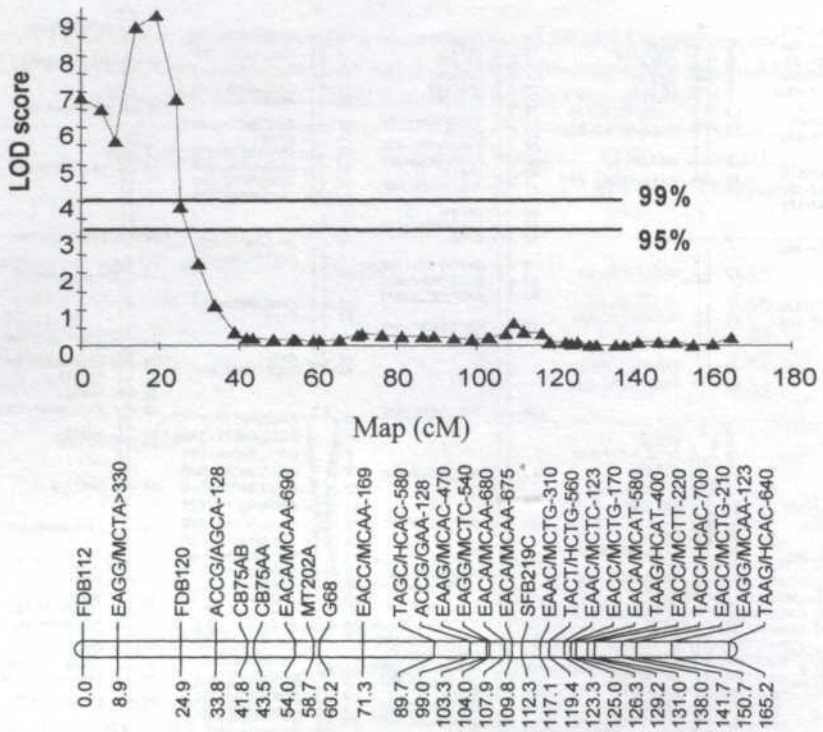

Fig. 4: Likelihood profile of QTL found at p<0.01 and p<0.05 genome-wide thresholds on linkage group 4 in the pisifera map (ML161)

p<0.01, each of which was located at linkage group 4 *(Fig. 4)* and linkage group 5 *(Fig. 5)* explaining 62.1% and 70.2% of the phenotypic variances, respectively. Another locus, TAAC/HCAA-650, located at linkage group 8 *(Fig. 6)* was found to be associated with TFC at p<0.05. In dura, two QTLs (EACA/MCAG-99 and G142) were detected at the end of linkage group 8 *(Fig. 7)* and at 16.3cM on linkage group 19 *(Fig. 8)*, respectively. Each of these QTLs accounted for 78.1% and 18.6% of the phenotypic variances of TFC, respectively.

DISCUSSION

A denser linkage map of dura and pisifera were produced by mapping of additional markers compared to the earlier map constructed by Chua *et al.* (2006). Additional 82 RFLP loci and 181 AFLP loci were successfully mapped and they produced a higher density pisifera linkage map. The additional markers have also linked up some of the linkage groups therefore, reducing the number to 16 linkage groups representing the basic chromosome number of oil palm (n=16). For example, linkage groups P2, P7, P15 and P17 from Chua *et al.* (2006) were linked together to form linkage group 2 in this study. Group 9 and group 15 were also formed by combination of groups P5 and P10 and; P6 and P8, respectively from the earlier map constructed. At the same time, the additional markers also formed new linkage groups such as groups 4, 14 and 16. The additional markers have widened the map coverage from 1,099.3cM to 1,714cM in this study. The total map length for pisifera reported here is close to the estimated genome size of 1,561cM calculated by Moretzsohn *et al.* (2000) for pisifera. The differences in map length may be due to the different marker systems, mapping programs and mapping functions used for constructing the linkage maps.

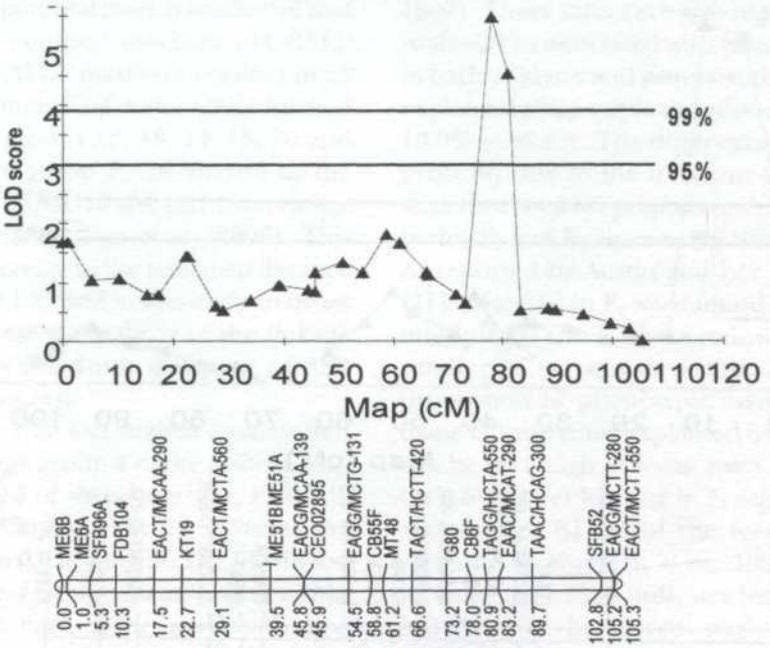

Fig. 5: Likelihood profile of QTL found at p<0.01 and p<0.05 genome-wide thresholds on linkage group 5 in the pisifera map (ML161)

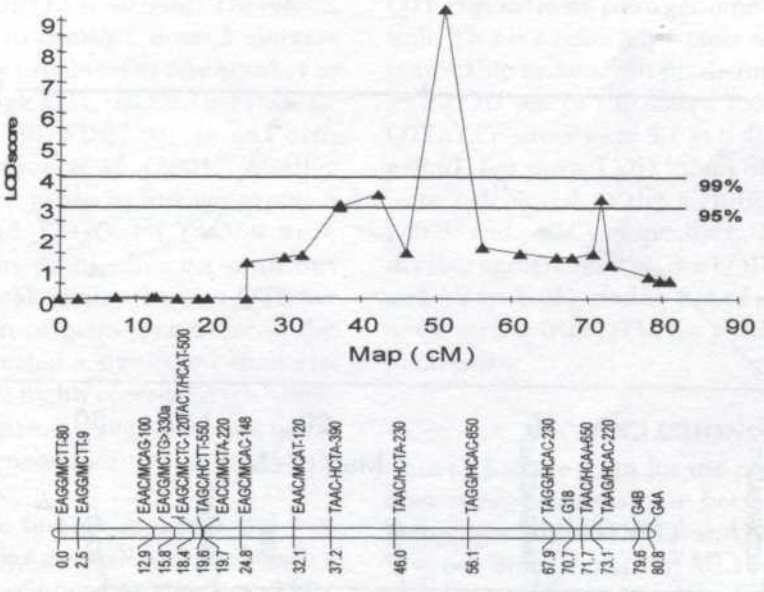

Fig. 6: Likelihood profile of QTL found at p<0.01 and p<0.05 genome-wide thresholds on linkage group 8 in the pisifera map (ML161)

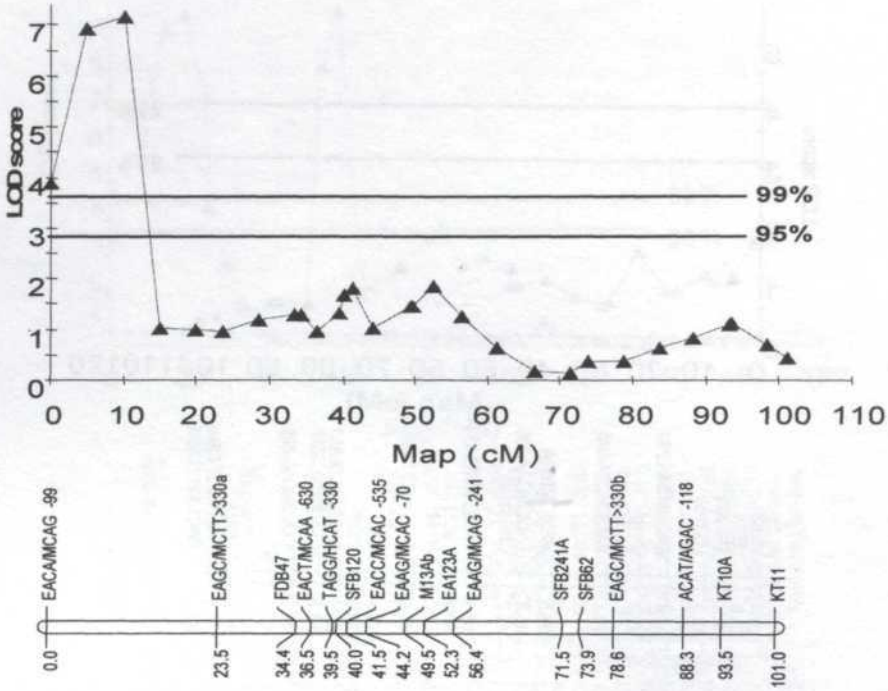

Fig. 7: Likelihood profile of QTL found at p<0.01 and p<0.05 genome-wide thresholds on linkage group 8 in the dura map (ENL48)

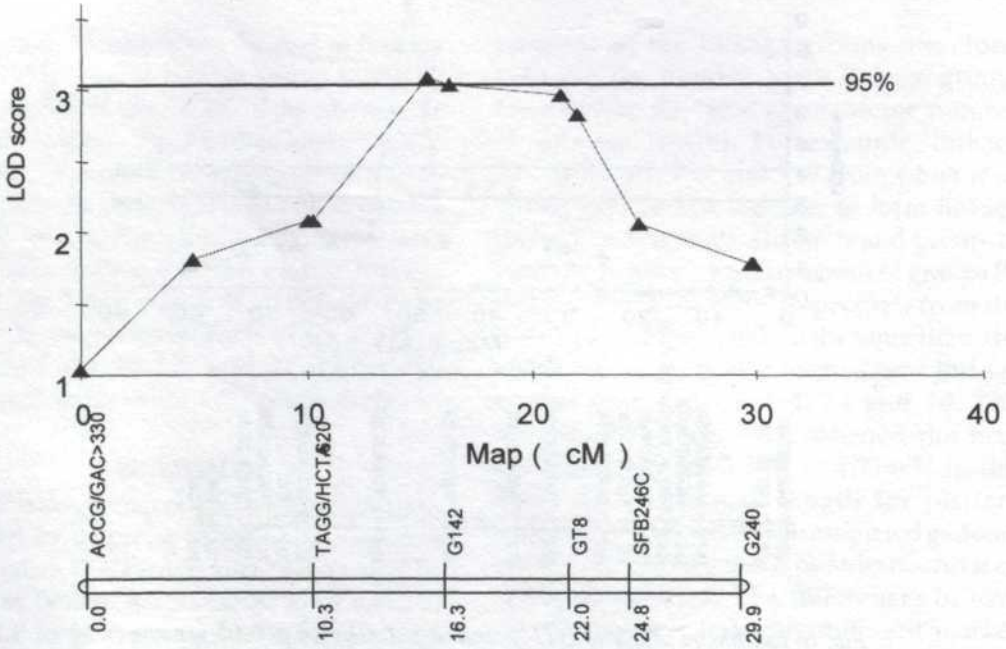

Fig. 8: Likelihood profile of QTL found at p<0.05 genome-wide threshold on linkage group 19 in the dura map (ENL48)

In the dura parental map, it was found that the additional mapped markers (44 RFLP markers and 93 AFLP markers) resulted in 23 linkage groups including some newly formed groups such as groups 12, 13, 14, 18, 20 and 22. Only linkage group 2 was formed by the integration of groups D10 and D11 from earlier map constructed by Chua *et al.* (2006). This resulted in an increase in the total map distance from 584.1cM to 1,225cM in this study. In order to further increase the density of the linkage maps especially for dura, mapping of SSR markers is in progress.

Quantitative trait loci analysis has detected a region at linkage group 4 of the pisifera map which, comprised of three markers, FDB112, EACG/MCTA>330 and FDB120, covering the distance between 0.0 to 24.9cM. The likelihood QTL profile *(Fig. 4)* of these markers fall within a narrow region. Each of the markers showed highly significant linkage to TFC trait by sharing an average LOD score of 6.9. In addition, correlation analysis (SPSS version 11.0) found that FDB120 was significantly ($p<0.05$) correlated to EACG/MCTA>330A ($r=0.337$) and FDB112 ($r=-0.548$). Therefore, it is appropriate to consider these 3 markers which are in close proximity to one another as representing a single QTL (labeled as FDB112/EACG/MCTA>330/FDB120) as was also described by Rance *et al.* (2001). Similar observations were made in linkage group 5 where CB6F and TAGG/HCTA-550 were detected close to each other on positions 78.0cM and 80.9cM, giving rise to a QTL for TFC. Comparison of genotypes between the two markers, revealed a significant similarity at $p<0.05$ and were highly correlated ($r=-0.905$) to each other. Hence, it is suggested that single QTL position is encompassing the two closely linked markers.

The QTLs on linkage groups 4 and 5 of pisifera and, linkage group 8 of dura showed a large proportion of phenotypic variance. The values are slightly higher than that for rice which is 38.5% and 32.6% for number of regenerated shoots per callus and regeneration rate, respectively (Taguchi-Shiobara *et al.*, 1997). These values are also higher compared to the QTLs associated with tissue culture traits in barley (Mano and Kotmatsuda, 2002) which explained phenotypic variations ranging from 10.0% to 42.8%. The differences observed are probably due to the different types of family structure used for mapping, where in rice and barley F_5 and F_{10} lines were used, respectively. As reported by Austin and Lee (1996), single QTL detected in F_2 were found dissected into multiple QTL in F_6 where individual QTL with smaller effect was detected. However, the proportion of phenotypic variance of none-tissue culture traits explained by certain QTLs can be very high in some cases. For example, QTL for flower bearing in F_2 sugi was found to account for 81.2% of the total phenotypic variance (Yoshimaru *et al.*, 1998). The high variance explained indicates that the markers identified in the current study have a major effect on TFC.

The LOD genome-wide significant thresholds calculated from the method presented by Van Ooijen (1999) were used as a guide to search for statistically significant QTL regions in oil palm genome affecting TFC trait. This is a relatively simple approach with reasonable accuracy in predicting true QTLs. The LOD scores calculated for detection of QTLs in pisifera were 3.1 at $p<0.05$ and 3.9 at $p<0.01$. For dura, LOD values of 3.0 and 3.75 were calculated as the significant levels at $p<0.05$ and $p<0.01$, respectively. The values are in close agreement with the LOD 3.4 ($p<0.05$) and 4.2 ($p<0.01$) used by Rance *et al.* (2001) in determining true QTLs for yield components in oil palm.

CONCLUSION

Genetic linkage maps for the pisifera and the dura parental palms have been improved by mapping additional RFLP and AFLP markers. The two linkage maps of ML161 and ENL48 were constructed with map densities of 395 loci/1,714cM and 214 loci/1,225cM, respectively. Using the framework maps, quantitative trait loci (QTLs) associated with time to first callusing (TFC) have been

identified for oil palm tissue culture. Three statistically significant QTLs contributing to TFC were detected in the pisifera map and two QTLs in the dura map. The QTL regions marked by RFLP markers (which essentially are cDNA clones) will prove useful for future efforts at map based cloning of the genes influencing the trait concerned. The marker(s)-QTL detected in this study are currently being verified in other crosses of oil palm.

ACKNOWLEDGEMENT

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Mitochondrial DNA Diversity of *Tor douronensis* Valenciennes (Cyprinidae) in Malaysian Borneo

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ABSTRAK

Kajian ini telah dijalankan untuk mengkaji struktur populasi dan taksonomi Tor douronensis, *sejenis ikan air tawar tempatan yang penting di Malaysia Borneo, menggunakan analisis penjujukan 466 pasangan bes gen mitokondria sitokrom c oksides I (COI). Sejumlah 62 ekor sampel ikan telah diperoleh dari lima lokasi di Sarawak (N=53) dan Sabah (N=8). Analisis filogenetik menggunakan kaedah "Neighbour-Joining" (NJ) menyokong status monofiletik di antara "*T. douronensis *dan* Tor tambroides; *seterusnya mengukuhkan lagi status taksonomi kedua-duanya sebagai spesies yang berlainan. Haplotaip* T. douronensis *seterusnya boleh dibahagikan kepada tiga kumpulan yang utama, dengan ikan Pelian dari Sabah membentuk kumpulannya sendiri (Kluster III) dengan sokongan (bootstrap) statistik yang kuat. Perbezaan genetik yang tinggi di antara haplotaip-haplotaip dari Sabah dengan Sarawak menunjukkan bahawa ikan Pelian Sabah mungkin merupakan sejenis spesies kriptik. Kajian ini menunjukkan variasi-variasi intra dan inter-populasi yang tinggi dalam* T. douronensis. *Variasi dalam kalangan sampel dalam populasi dijumpai di dalam kesemua populasi* T. douronensis *kecuali di dalam populasi Bario. Kehadiran perbezaan-perbezaan haplotaip yang tetap (unik) bersamaan dengan nilai* F_{ST} *yang tinggi antara populasi-populasi* T. douronensis, *menyokong kesimpulan bahawa sedikit atau tiada migrasi berlaku di antara populasi-populasi yang dipisahkan oleh jarak geografi yang jauh atau sistem sungai yang berlainan. Walau bagaimanapun, perkongsian beberapa haplotaip antara populasi-populasi tersebut, contohnya antara Batang Ai dan Bario (HS6) dan antara Batang Ai dan Ulu Limbang/Ba Kelalan (HS2) memberi bukti yang* T. douronensis *mempunyai taburan yang meluas di kawasan tersebut di masa lalu, terutama semasa zaman Quaternary. Keseluruhannya, kajian ini berjaya memberikan maklumat-makluat yang berguna tentang struktur populasi dan taksonomi ikan* T. douronensis *di Malaysia Borneo.*

ABSTRACT

This study examines the population structure and taxonomy of Tor douronensis, *an important indigenous freshwater fish species in Malaysian Borneo, by using sequence analysis of 466 base pairs of the mitochondrial cytochrome c oxidase I (COI) gene. A total of 62 fish samples were collected from five locations in Sarawak (N=54) and Sabah (N=8). The phylogenetic analysis using the Neighbour-Joining (NJ) method supported the monophyletic status between* T. douronensis *and* Tor tambroides, *which further reinforced their taxonomic status as distinct species. The* T. douronensis *haplotypes were further divided into three major groups, with the Pelian fish from Sabah forming its own group (Cluster III) with strong bootstrap support. The large genetic differences separating the Sabah haplotypes from its Sarawak congeners suggested that the Pelian fish might represent a cryptic species. The current study showed high levels of intra and inter-population variations in* T. douronensis. *Within all population variations,* T. douronensis *populations were found, except in Bario. The presence of fixed haplotype differences along with high* F_{ST} *values among the populations of* T. douronensis, *support the conclusion that little or no migration occurred among the extant*

populations separated by large geographic distances or river systems. However, the sharing of haplotypes between some such populations, for example between Batang Ai and Bario (HS6), and between Batang Ai and Ulu Limbang/Ba Kelalan (HS2) provided support that T. douronensis *had a historically widespread natural distribution in the region probably during the Quaternary period. Overall, the present study was able to shed light on the taxonomy and population structure of* T. douronensis *in Malaysian Borneo.*

INTRODUCTION

Freshwater fishes of the genus *Tor* Gray, commonly known as mahseers, belong to the family Cyprinidae (subfamily Cyprininae) (Mohsin and Ambak, 1983; Roberts, 1989; Kotellat *et al.*, 1993). They are distributed throughout the Indian subcontinent, Southeast Asia and Southern China and inhabit the upper streams and headwaters of most major river systems (Kottelat *et al.*, 1993; Rainboth, 1996). Environmental degradation such as river pollution, deforestration and watershed erosion had led to the rapid destruction of Tor natural habitat. Uncontrolled fish harvest (overfishing) has also greatly reduced their population size (Ng, 2004). Their distributions in Malaysian Borneo are now limited to the upper streams and protected areas (natural parks) of Sarawak and Sabah (Litis *et al.*, 1997; Nyanti *et al.*, 1999; Ng, 2004). There are currently three described *Tor* species in Malaysia: *Tor tambroides* Bleeker, *Tor tambra* Valenciennes, and *Tor douronensis* Valenciennes (Kottelat and Whitten, 1996; Roberts, 1989; Rainboth 1996; Ng, 2004).

T. douronensis is presumably the most widespread mahseer species recorded in East Malaysia, apart from the less abundant *T. tambroides* found in Sarawak, and it is the only mahseer currently described from Sabah (Inger and Chin, 2002). *T. douronensis*, locally known as "ikan semah" in Sarawak and "ikan pelian" in Sabah has been named""the state fish of Sarawak" due to its importance as a high value food fish as well as for eco-tourism and recreational fishing (Litis *et al.*, 1997; Ng, 2004).

Nevertheless, the taxonomic differentiation of *T. douronensis* and its related species, *T. tambroides* is still unresolved, with many conflicting descriptions among different researchers (Roberts, 1989; Kottelat *et al.*, 1993; Rainboth, 1996; Zhou and Chu, 1996; Ng, 2004). Roberts (1999) classified them to be a single species, and a junior synonym to *T. tambra.*

Thus, the application of molecular techniques (such as DNA sequencing) should offer better insights into the unresolved taxonomy and population size of *T. douronensis* (Nguyeń *et al.*, 2006). Molecular markers can give more reliable and consistent results for rapid species identification (Ryan and Esa, 2006), levels of genetic variability, levels of gene flow and population subdivisions and for understanding factors contributing to fitness in freshwater fishes (Vrijenhoek, 1998). Analysis of DNA sequence polymorphism utilizing the existing "universal primers" for mitochondrial DNA (mtDNA) (Palumbi *et al.*, 1991) provides the highest resolution of genetic variation which had been widely applied in molecular systematic studies (Arnason *et al.*, 2002; Liu and Chen, 2003; Nguyen *et al.*, 2006). Thus, this study was conducted to clarify aspects of the systematics and population structure of *T. douronensis* in various populations in Malaysian Borneo by analysing the cytochrome oxidase I (COI) nucleotide sequences of the mitochondrial DNA.

MATERIALS AND METHODS

Sample Description and Collection Location

Samples of *T. douronensis* were collected from five locations in Sarawak and three locations in Sabah (*Fig. 1*). However, samples from Sabah (Keningau, Liwagu and Tawau) were pooled together into a single population (Sabah population) due to the small number of individuals obtained for this study. The fish were sampled using a variety of fishing methods, including seine, gill and cast nets and fishing rod. Some samples from Sarawak were

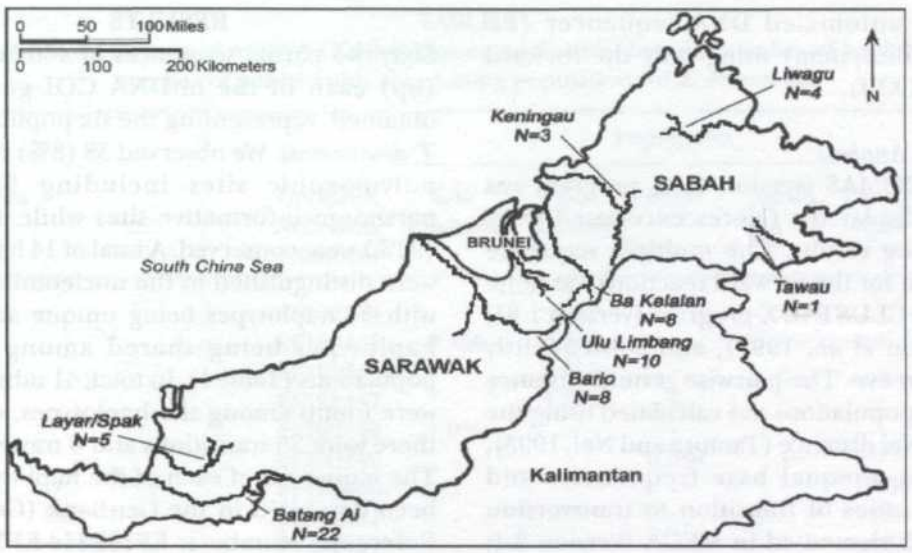

Fig. 1: Map showing sampling locations and sample sizes of T. douronensis collected for this study. N= sample size.

provided by the Indigenous Fish Research and Production Center (IFRPC), Tarat, Sarawak. The weight and standard length of the fish samples used in the study ranged from 50-500 g and 5-100 cm, respectively. Whole samples were morphologically identified by using the keys provided by Inger and Chin (2002), Mohsin and Ambak (1983), and Kottelat *et al.* (1993). Fresh samples in the form of full specimens, muscle tissues, scales or fin clips were placed in a -80°C freezer for long-term storage. However, in most cases, samples collected in the field were preserved in 95% ethanol and stored at -20°C prior to genetic analyses.

DNA Extraction and Polymerase Chain Reaction (PCR)

Total DNA was isolated using the modified CTAB method (Grewe *et al.*, 1993) in the presence of Proteinase K. The pelleted DNA was redissolved in 100μL of sterilized distilled water. The DNA quality and approximate yield were determined by electrophoresis in a 1% agarose gel containing ethidium bromide at 90 V for 30 min. The isolated genomic DNA was used for the mtDNA analysis.

A 500 bp segment of the *cytochrome c oxidase* I gene was amplified with the oligonucleotide primers COIf (5' CCTGCAGGAGGAGGAG AYCC 3', forward) and COIe (5' CCAGAGATT AGAGGGAATCAGTG 3', reverse) (Palumbi *et al.*, 1991). Approximately, 50-100 ng of the template DNA was amplified in a 25 ml reaction mixture containing 50 mM 10X buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP (Promega), 0.1 mM of each primer, and 0.5 units of *Taq* DNA Polymerase (Promega). The cycle parameters consisted of 35 cycles of denaturation (95°C, 30 seconds), annealing (45°C, 30 seconds), and extension (72°C, 60 seconds. The amplified products were visualized on a 1% agarose gel containing ethidium bromide for approximately 30 min at 90 V and photographed under UV light. A digested lambda DNA ladder (GeneRuler™ 1 kb DNA Ladder) was used as a size standard marker (Promega). The PCR products were further purified using a DNA purification kit (Invitrogen) according to the manufacturer's instructions. The purified PCR products were then directly sequenced using the'BigDye® Terminator v3.0 Cycle Sequencing kit on an

ABI 377 automated DNA sequencer (PE Applied Biosystem) using only the forward primer (COIf).

Statistical Analysis

The CHROMAS (version 1.45) program was used to display the fluorescence-based DNA sequencing results. The multiple sequence alignment for the forward reactions was done using the CLUSTAL X program (version 1.81; Thompson *et al.*, 1997), and subsequently aligned by eye. The pairwise genetic distance between populations was calculated using the Tamura-Nei distance (Tamura and Nei, 1993), based on unequal base frequencies and unequal ratios of transition to transversion (Ti:Tv) implemented in MEGA (version 3.1; Kumar *et al.*, 2004). The MEGA program was also used to construct a neighbour-joining (NJ) tree (Saitou and Nei, 1987) using two indigenous cyprinids, (*Barbonymus gonionotus* (Genbank accession number: DQ532806) and *Barbonymus schwanenfeldii* (Genbank accession number: DQ532805)) obtained from the Jempol River, Negeri Sembilan as outgroup species. Four haplotypes of *T. tambroides* (Genbank accession number: DQ532827, EF192458, EF192460, EF192461) were also included in the analysis to demonstrate the reciprocally monophyletic status between the two mahseers. The phylogenetic confidence was estimated by bootstrapping with 1000 replicate data sets (Felsenstein, 1985).

The levels of mtDNA COI variation within the *T. douronensis* population were examined by computing the nucleotide (with the Jukes-Cantor correction; Jukes and Cantor, 1969) and haplotype diversity indices implemented in the DnaSP (version 4.0) program (Rozas *et al.*, 2003). The level of population subdivision (F_{ST}) (Hudson *et al.*, 1992) between populations and the Chi-square probability test for population differentiation using 1000 permutations of the data sets were also estimated using the DnaSP program.

RESULTS

Sixty-two partial sequences of 466 base pairs (bp) each of the mtDNA COI gene were obtained, representing the six populations of *T. douronensis*. We observed 38 (8%) variable/polymorphic sites including 33 (7%) parsimony-informative sites while 430 sites (92%) were conserved. A total of 14 haplotypes were distinguished in the nucleotide data set with 11 haplotypes being unique and three haplotypes being shared among the six populations (Table 1). In total, 41 substitutions were found among the haplotypes, of which there were 35 transitions and 6 transversions. The sequences of each of the haplotype have been deposited in the GenBank (GeneBank Reference Numbers: EF192444-EF192457). The mean total nucleotide composition was A=25.7%, T=32.3%, C=22.7% and G=19.3%.

The *T. douronensis* samples from Sabah harboured four unique haplotypes (HS11P to HS14P) which were not shared with *T. douronensis* populations from Sarawak. On the other hand, HS6 was the only haplotype found in the *Bario* population although it was also found in the *Batang Ai* population. The *Layar/Spak* population also had four haplotypes, three being unique haplotypes while the fourth one (HS10L) was also found in the *Batang Ai* population (Table 1).

Overall, the nucleotide diversity was low with the Sabah population showing the highest value (0.016). The haplotype diversity varied, ranging from 0 to 0.900 (*Layar/Spak*) (Table 1). The pairwise F_{ST} (Hudson *et al.*, 1992) and the results of the Chi-square tests for genetic differentiation among the populations are presented in Table 2. Significant levels of genetic differentiation were found in all comparisons among the *T. douronensis* populations except between the *Ulu Limbang* and the *Ba Kelalan* populations (F_{ST}= 0.075), and between the *Layar/Spak* and the Sabah populations, although their pairwise F_{ST} value was high (0.726) (Table 2).

Phylogenetic analysis of the haplotypes using the NJ method strongly supported the reciprocally monophyletic status between *T.*

TABLE 1
Distribution of 14 observed mtDNA COI haplotypes, nucleotide diversity, number of haplotypes and number of polymorphic sites among populations of *T. douronensis*

Haplotypes	GenBank Accession Numbers	Population					
		Ulu Limbang	Ba Kelalan	Bario	Sabah	Layar/ Spak	Batang Ai
HS1BA	EF192444	-	-	-	-	-	1.000
HS2	EF192445	0.350	0.210	-	-	-	0.440
HS3	EF192446	-	1.000	-	-	-	-
HS4	EF192447	-	1.000	-	-	-	-
HS5	EF192448	1.000	-	-	-	-	-
HS6	EF192449	-	-	0.530	-	-	0.470
HS7L	EF192450	-	-	-	-	1.000	-
HS8L	EF192451	-	-	-	-	1.000	-
HS9L	EF192452	-	-	-	-	1.00 0	-
HS10L	EF192453	-	-	-	-	0.400	0.600
HS11P	EF192454	-	-	-	1.000	-	-
HS12P	EF192455	-	-	-	1.000	-	-
HS13P	EF192456	-	-	-	1.000	-	-
HS14P	EF192457	-	-	-	1.000	-	-
Nucleotide diversity (P_i JC)		0.001	0.001	0.000	0.016	0.006	0.008
Number of haplotypes		2	3	1	4	4	4
Haplotype diversity (Hd)		0.200	0.607	0.000	0.750	0.900	0.697
Number of polymorphic sites		1	2	0	15	4	13

Numbers under each population indicate the frequencies of individuals with that haplotype in each population.

TABLE 2
Lower diagonal: pairwise Tamura-Nei genetic distances among the six populations of *T. douronensis*

	1. Ulu Limbang	2. Ba Kelalan	3. Bario	4. Sabah	5. Layar/Spak	6. Batang Ai
1		0.075^{ns}	0.947^{***}	0.811^{**}	0.880^{*}	0.147^{*}
2	0.001		0.857^{**}	0.804^{*}	0.865^{*}	0.175^{*}
3	0.004	0.005		0.817^{*}	0.890^{*}	0.327^{*}
4	0.046	0.047	0.047		0.726^{ns}	0.724^{***}
5	0.026	0.027	0.027	0.042		0.706^{**}
6	0.005	0.006	0.006	0.046	0.024	

Upper diagonal: population subdivision (F_{ST}) values and probability test (Chi-square) for population differentiation based on 1000 permutations of the sequence data set, significance levels (ns=not significant, P<0.05=*, P<0.01=**, P<0.001=***)

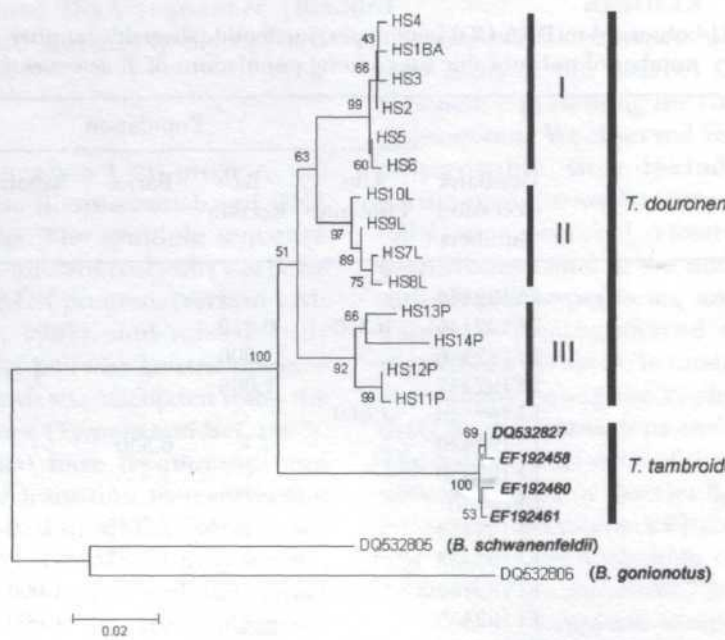

Fig. 2: Neighbour-joining (NJ) phylogram showing the relationships among COI haplotypes of T. douronensis, T. tambroides and two outgroups analysed in the present study.
The number at each node represents the bootstrap percentage value based on 1000 pseudoreplications for NJ analysis

douronensis and *T. tambroides* (*Fig. 2*) and further divided the former mahseer into three major groups (Cluster I to III) with strong bootstrap supports. Cluster I grouped haplotypes from the *Ulu Limbang, Ba Kelalan* and *Bario* populations (Northern Sarawak) with those from the *Batang Ai* population (Southern Sarawak). Cluster II grouped all the Southern Sarawak haplotypes consisting of three unique haplotypes from the *Layar/Spak* population and one shared haplotype (HS10L) with the *Batang Ai* population. Interestingly, Cluster III grouped all the four unique haplotypes from Sabah (North Borneo).

The pairwise genetic distances (number of nucleotide substitutions per site) calculated using the Tamura-Nei model (Tamura and Nei, 1993) among the *T. douronensis* populations are shown in Table 2. The highest genetic distance was observed between the Sabah population and both the *Bario* and *Ba Kelalan* populations of Sarawak (4.7%) while the lowest value was between the *Ulu Limbang* population and the *Ba Kelalan* population (0.1%). Within the Sarawak populations, the *Layar/Spak* population had genetic distances of 2.4% to 2.7% separating it from the other four Sarawak populations. Interestingly, the *Batang Ai* population had a closer genetic distance (0.5% to 0.6%) with the Northern Sarawak populations (*Ulu Limbang, Ba Kelalan* and *Bario*) than with the Southern *Layar/Spak* population (2.4%).

DISCUSSION

The results of the mtDNA analysis in this study enabled us to shed light on the taxonomic status of *T. douronensis* in the Malaysian part of Borneo Island. The phylogenetic analysis of the COI gene confirmed the reciprocally monophyletic status between *T. douronensis* and *T. tambroides*, thus further reinforcing their taxonomic status as distinct species (Roberts,

1989; Kottelat *et al.*, 1993; Rainboth, 1996; Zhou and Chu, 1996; Ng, 2004). The current mtDNA results also did not show any mixing of haplotypes between *T. douronensis* and *T. tambroides* as was observed by Nguyen *et al.* (2006). The major finding of this study is the bifurcation of the *T. douronensis* haplotypes into three highly differentiated groups, with the Sabah (North Borneo) haplotypes forming its own subgroup (Cluster III). The bootstrap support among the three clusters was high although the consensus positioning of Cluster I and Cluster II with regards to Cluster III was moderately supported. A plausible explanation for this is that the *Pelian* fish from Sabah might represent a cryptic species.

This study found high levels of intra and inter-population variations in *T. douronensis*. Within population variations were found in all the *T. douronensis* populations except in the *Bario* population. The large mtDNA differences currently found among the *T. douronensis* populations could be explained by one or several factors including small population sizes, past bottleneck events, or the presence of physical barriers to gene flow among the populations (Nguyen *et al.*, 2006). The presence of fixed haplotype differences among the populations, along with high F_{ST} values among populations of *T. douronensis*, supported the conclusion that little or no migration occurred among the extant populations separated by large geographic distances, or river systems (Nguyen *et al.*, 2006). Nevertheless, the sharing of haplotypes between such populations does occur, for example between *Batang Ai* and *Bario* (HS6), and between *Batang Ai* and *Ulu Limbang/Ba Kelalan* (HS2) and this provided support that, in the past, *T. douronensis* had a widespread natural distribution in the region. Geological evidence suggested that the river systems of the Northern and the Southern parts of Sarawak were historically interconnected, most probably during the Tertiary and Quaternary periods (Inger and Chin, 2002).

The large genetic differences between the *T. douronensis* population from Sabah with its congeners from Sarawak, and the presence of fixed haplotypes supported the hypothesis that the North Borneo region was the most isolated region and probably had no connection with the other Borneo regions during the Pleistocene glaciation periods (Inger and Chin, 2002). Thus, the *Pelian* fish from Sabah could possibly have evolved through allopatric speciation and formed new or cryptic mtDNA lineages. The lack of a clear geographical structuring of haplotype distributions between the *Semah* fish from the Northern and the Southern parts of Sarawak is also demonstrated in other indigenous freshwater fish species with a widespread natural distribution such as in *Hampala macrolepidota* (Ryan and Esa, 2006).

This study demonstrated the usefulness of genetic studies in assessing the taxonomy and population structures of Malaysia's indigenous freshwater fish taxa for appropriate conservation and management strategies. However, further studies are required using larger sample sizes per population, samples from other areas of their geographical distributions, sequence data from other mtDNA regions and information based on nuclear DNA (i.e. single locus microsatellite) markers.

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Paraquat (Methyl viologen) Toxicity in *Centella asiatica* Callus Cultures

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ABSTRAK

Kajian telah dijalankan untuk mengkaji kesan rawatan paraquat (PQ) ke atas pertumbuhan, viabiliti, kandungan hidrogen peroksida dan malondialdehida di dalam kultur kalus Centella asiatica (CA03 dan CA09). Kalus dirawat dengan 50 µM PQ selama 5 hari di dalam medium cecair Murashige dan Skoog (MS). Pertumbuhan, viabiliti kalus dan juga kandungan hidrogen peroksida (H_2O_2) dan malondialdehida (MDA) ditentukan pada hari 0, 1, 2, 3 dan 5 rawatan. Berat basah dan berat kering kalus CA03 yang diberi rawatan adalah lebih rendah berbanding kalus kawalan. Bagi kalus CA09 pula, pertumbuhan kalus rawatan adalah lebih rendah berbanding kalus kawalan pada peringkat akhir tempoh rawatan. Walaupun terdapat perencatan pertumbuhan bagi kedua-dua CA03 dan CA09 yang diberi rawatan, perencatan berat basah sebanyak 36% bagi CA09 berbanding dengan kawalannya pada akhir tempoh rawatan adalah lebih tinggi dari CA03 di mana terdapat perencatan pertumbuhan berat basah sebanyak 18.2%. Penurunan peratus viabiliti sel juga adalah sangat ketara terutama pada kalus CA09 selepas dirawat dengan PQ. Walaupun kandungan MDA adalah lebih tinggi di dalam kalus CA03 berbanding CA09 yang diberi rawatan pada peringkat awal, ia menunjukkan corak perubahan yang menurun mengikut masa manakala kandungan MDA pada CA09 pula menunjukkan corak perubahan yang meningkat. Pada akhir tempoh rawatan, MDA pada CA09 adalah lebih tinggi berbanding CA03. Tambahan lagi, kandungan H_2O_2 pada amnya adalah lebih tinggi pada CA09 yang diberi rawatan berbanding CA03 kecuali pada hari ke 3. Kajian ini menunjukkan bahawa rawatan dengan PQ boleh meransang penghasilan MDA dan H_2O_2 dan juga merencatkan pertumbuhan dan juga viabiliti kalus. Kajian juga menunjukkan kalus CA03 adalah lebih toleran kepada PQ berbanding CA09.

ABSTRACT

The effect of paraquat (PQ) treatment on growth, cell viability, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels were investigated in the callus of two Centella asiatica accessions (CA03 and CA09). Callus of C. asiatica were treated with 50µM PQ for five days in Murashige and Skoog (MS) liquid medium. Callus growth, viability of the callus as well as H_2O_2 content and MDA levels were evaluated at 0, 1, 2, 3 and 5 days of treatment periods. Fresh weight and dry weight of treated calli were significantly lower in CA03 as compared to the untreated calli. In CA09, the growth of treated calli was significantly lower compared to controls at the later stages of the treatment period. Although decreases in growth were observed for both treated CA03 and CA09, the final reduction in fresh weight at 36% for CA09 compared to its control was much higher compared to CA03 with an 18.2% final reduction in fresh weight. PQ treatment also resulted in a marked decrease in the viability of the callus especially in CA09. Although MDA levels were significantly higher in treated CA03 as compared to treated CA09 at the early treatment stages, they showed a decreasing trend, while MDA levels in CA09 showed an increasing trend, which was significantly higher than that of CA03 at the end of the treatment period. In addition, H_2O_2 concentrations were generally higher in treated CA09 compared to treated CA03 except at day 3. This study indicated that PQ treatment can induce increases in levels of MDA and H_2O_2 associated with the decrease in growth and viability of the callus. Results also suggested that CA03 was more tolerant to PQ treatment as compared to CA09.

INTRODUCTION

Oxidative stress is very frequent in nature and is due to an increase of the reactive oxygen species (ROS). It is induced by several biotic and abiotic factors including phytotoxic chemical agents including non selective herbicides (e.g paraquat, PQ). The bipyridyl herbicides such as paraquat, also known as methyl viologen, with its active compound 1,1'-dimethyl-4,4'-bipyridylium dichloride and diquat are non-selective, quick acting herbicides, effective against grasses as well as most broad-leaved weed species (Calderbank and Slade, 1976). The major target of the bipyridyl compounds in PQ seems to be the chloroplast; PQ can accept one electron from photosystem I and the formed PQ radicals is rapidly oxidized under the catalyzation of metal ions, leading to the formation of superoxide radicals. In further reactions, various ROS e.g H_2O_2 and hydroxyl radicals are generated (Lorenzini *et. al.*, 2002). The hydroxyl radical generated will rapidly react with membrane unsaturated fatty acid leading to membrane damage, reduction in CO_2 uptake and degradation of chloroplast and pigments (Kirtikara and Talbot, 1996). These ROS are efficiently scavenged by a series of enzymes and quenching systems such as superoxide dismutase, enzymes in ascorbate glutathione cycle, ascorbate, glutathione and membrane bound α-tocopherol (Suntres, 2002).

Oxidative stress is also involved in loss of viability of plants exposed to a variety of environmental stress. The 2,3,5-triphenyl tetrazolium chloride (TTC) assay was used as a viability assay for callus exposed to various concentrations of PQ. The production of MDA and changes in cell conductivity have frequently been used as sensitive markers for herbicides' action in plants (Peleg' *et al.*, 1992).

Centella asiatica is commonly used as a vegetable or eaten raw as an 'ulam' (Malay salad). Apart from being a nutritious plant,' *C. asiatica* is also believed to have many healing properties, conferring a wide range of beneficial effects and is treated as a valuable medicinal plant in Chinese traditional medicine and classical Indian Ayurvedic medicine. Research has demonstrated that *C. asiatica* is a rich source of natural antioxidants. These antioxidants are scavengers of ROS and inhibitors of lipid peroxidation and thus, can protect and defend cells against damage by the ROS (Subramaniam *et al.*, 1998).

The main objectives of this study were to determine the effect of PQ treatments on growth, cell viability, H_2O_2 and MDA levels of two *C. asiatica* callus cultures i.e CA03 and CA09.

MATERIALS AND METHODS

Callus Initiation and Maintainance

Sterile leaf explants of *C. asiatica* were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) and kinetin. The cultures were maintained by regular subculturing at 10 day intervals onto fresh medium. All cultures were incubated in 12h/12h (light/dark) photoperiod under cool, white fluorescent lamps at 27 ± 2^{o}C.

PQ (1,1'-dimethyl-4,4'-bipyridylium dichloride) Treatment

Callus pieces were transferred to MS medium containing 50 μM PQ. PQ is heat-stable and for all experiments, was added to the medium prior to autoclaving. Callus growth, cell viability, H_2O_2 content and MDA levels were assayed at 0, 1, 2, 3 and 5 days of treatment periods.

Callus Growth and Viability

Treated callus were washed with distilled water and weighed immediately for fresh weight. For dry weight, callus were dried in an oven at 50°C for 2 days. The 2,3,5-triphenyltetrazolium chloride (TTC) assay was used to estimate the proportion of viable cells after PQ treatments. The absorbance of the supernatant was determined at 485 nm (Towill and Mazur, 1974).

MDA Assay

MDA concentration was determined by the thiobarbituric acid (TBA) reaction, based on the method by Heath and Packer (1968) with slight modification by Hodges *et al.* (1999).

H_2O_2 Determination

H_2O_2 assay was done following the method of Velikova *et al.* (2000).

RESULTS AND DISCUSSION

The changes in growth of *C. asiatica* callus cultures treated with 50 μM PQ are shown in *Figs. 1* and *2*. Decreases in fresh weight and dry weight were observed 24 hours after treatment with PQ in both accessions. The reduction in growth was greater in CA09 as compared to CA03 especially after 2 days of treatment (*Figs. 1C* and *2C*). In CA03, the growth of treated callus were significantly lower ($p<0.05$) than the control (*Figs. 1A* and *2A*), but in CA09 the growth of treated callus were only significantly lower than its control ($p<0.05$) at the later stages of treatment periods (*Figs. 1B* and *2B*). This however was due to the sharp decrease in fresh weight of the control CA09 callus at day 2 which increased slightly thereafter. Therefore, although significant differences in fresh weight for CA09 only occurred at the later stages of treatment period, this was due to the low fresh weight values of its control. A comparison of the actual final reduction in growth between CA03 and CA09 showed a 36% reduction in CA09 while CA03 showed 18.2% reduction of growth compared to their respective controls (*Figs. 1A* and *1B*). The similar dry weight values of treated CA03 and CA09 showed that there were no differences in terms of actual organic matter content in the two accessions (*Fig. 2C*). However, the lower reduction in growth of CA09 is probably due to the significantly lower percentage of viability compared to CA03 (*Fig. 3C*). *Fig. 3* demonstrates that PQ treatment resulted in a marked decrease in the viability of the callus especially in CA09. After 24 hours treatment with PQ, only 5% of CA09 callus were still viable while only 1.5% of the cells were viable after 5 days of treatment (*Fig. 3C*). The viable proportion of the callus were significantly higher ($p<0.05$) in controls as compared to the treated callus in both accessions (*Figs. 3A* and *3B*). The results of Wong (2000) also suggest that PQ at a low concentration (0.02 mg/l) can significantly inhibit the growth, photosynthetic rates and chlorophyll content of *Scenedesmus quadricauda* Berb 614. Clearly, PQ is more effective in decreasing the growth and cell viability of CA09 callus than CA03. Results thus indicate that PQ could induce oxidative stress in *C. asiatica* callus cultures.

The condition of oxidative stress induced by PQ resulting in peroxidation of membrane lipids in the *C. asiatica* cultures is clearly indicated by the increased MDA levels in both accessions compared to their respective controls except for days 1 and 3 in CA09 callus (*Figs. 4A* and *4B*). MDA levels were initially significantly higher ($p<0.05$) in CA03 as compared to CA09 especially up to 2 days of treatment periods. However, longer treatment period decreased the MDA levels in CA03 and increased the MDA levels in CA09 (*Fig. 4C*). At the end of the treatment period, MDA levels in CA09 were significantly higher ($p<0.05$) than that of CA03.

H_2O_2 concentrations between control and treated cultures of CA03 and CA09 were not significantly different except for day 3 for CA03 where the H_2O_2 concentrations in the treated callus were significantly higher ($p<0.05$) than its control (*Fig. 5A*). The treated CA09 callus did not show any increases in H_2O_2 concentrations after day 2 (*Fig. 5B*). This could be due to a compromised antioxidative defense in CA09 which although may have increased levels of superoxide radicals ($O_2^{\cdot-}$) production due to the PQ-induced oxidative stress, was not able to dismutate these radicals to H_2O_2. CA03 on the other hand was probably still capable of modulating its antioxidative defense resulting in the sudden burst of H_2O_2 production which was subsequently neutralized to less harmful forms. This was reflected in the decreased levels of H_2O_2 after day 3 (*Fig.*

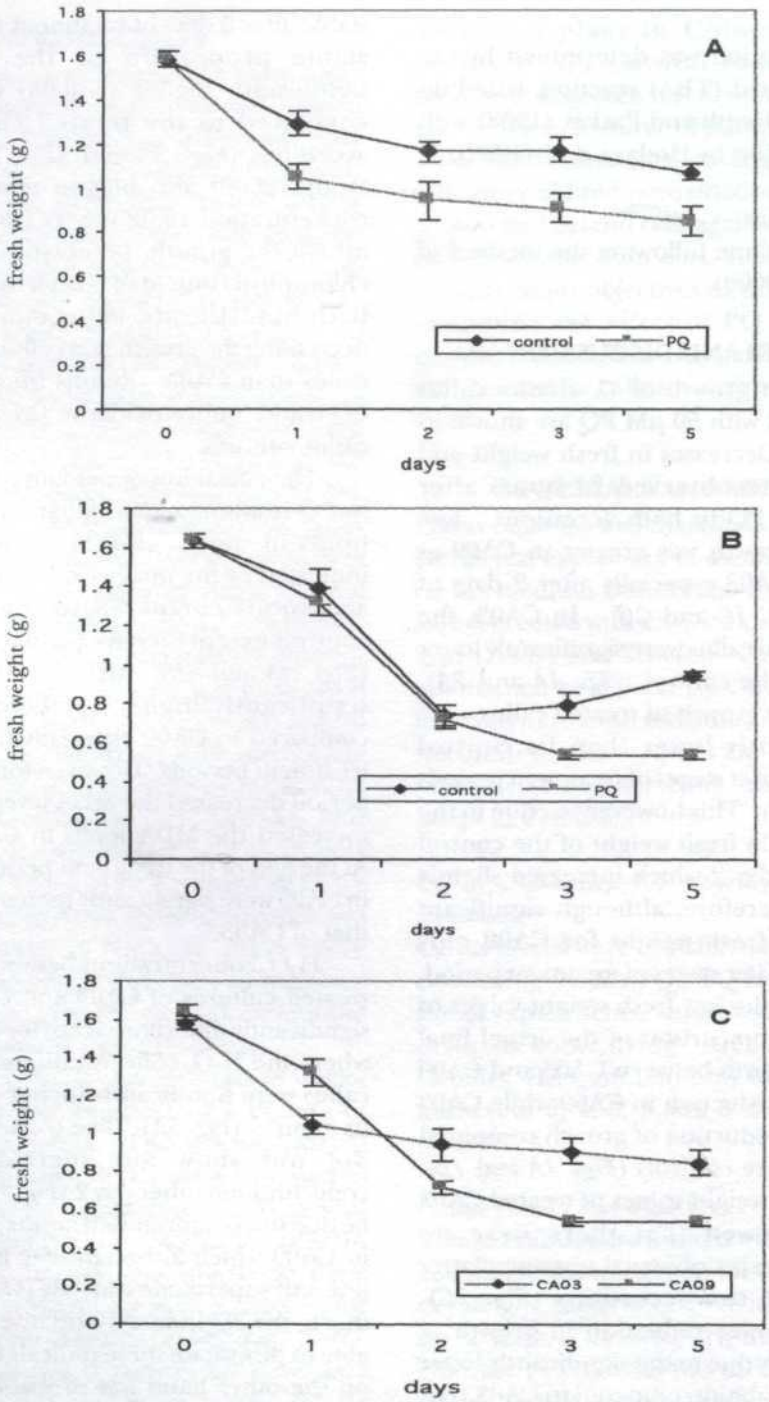

Fig. 1: Fresh weight (g) of C. asiatica *callus culture:*
A) CA03 callus
B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)

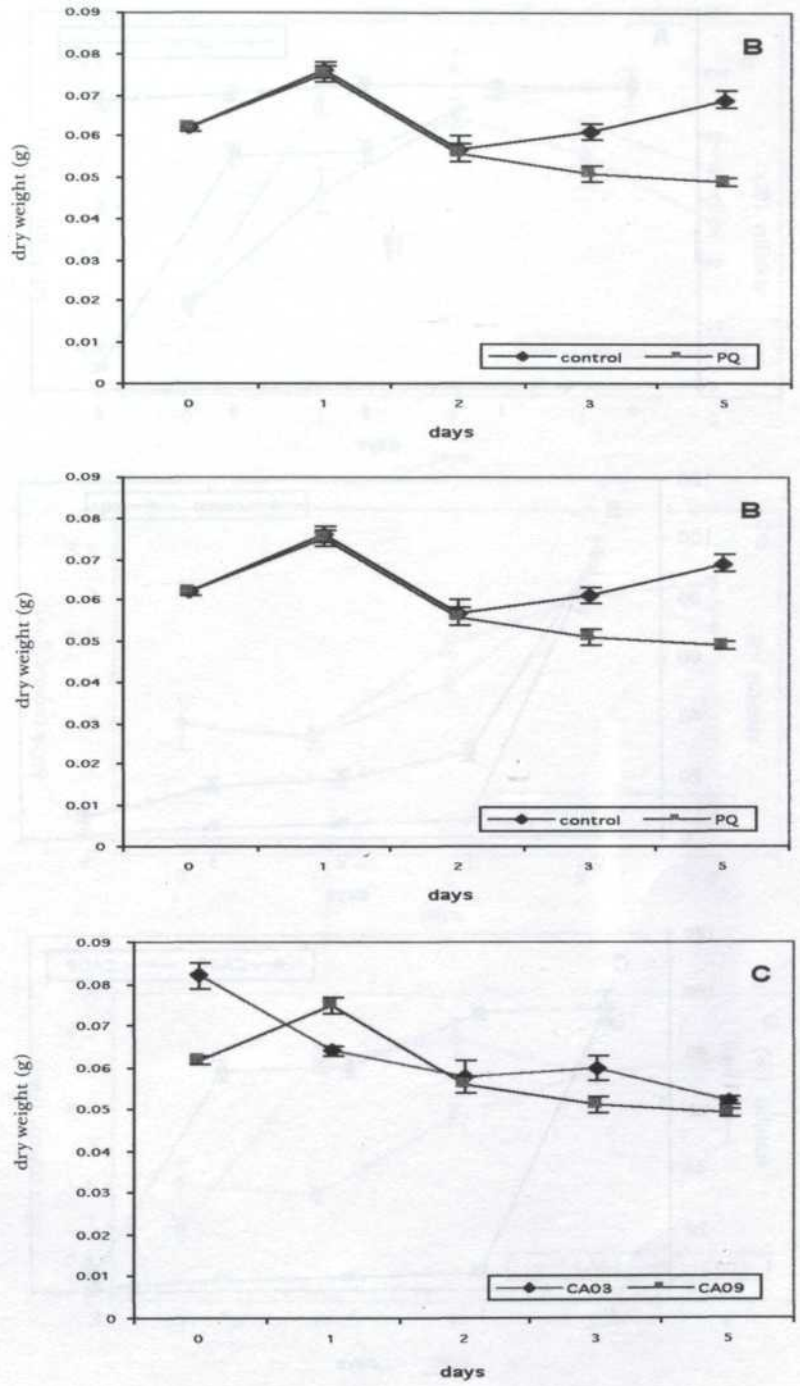

Fig. 2: Dry weight (g) of C. asiatica *callus culture:*
A) CA03 callus
B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)

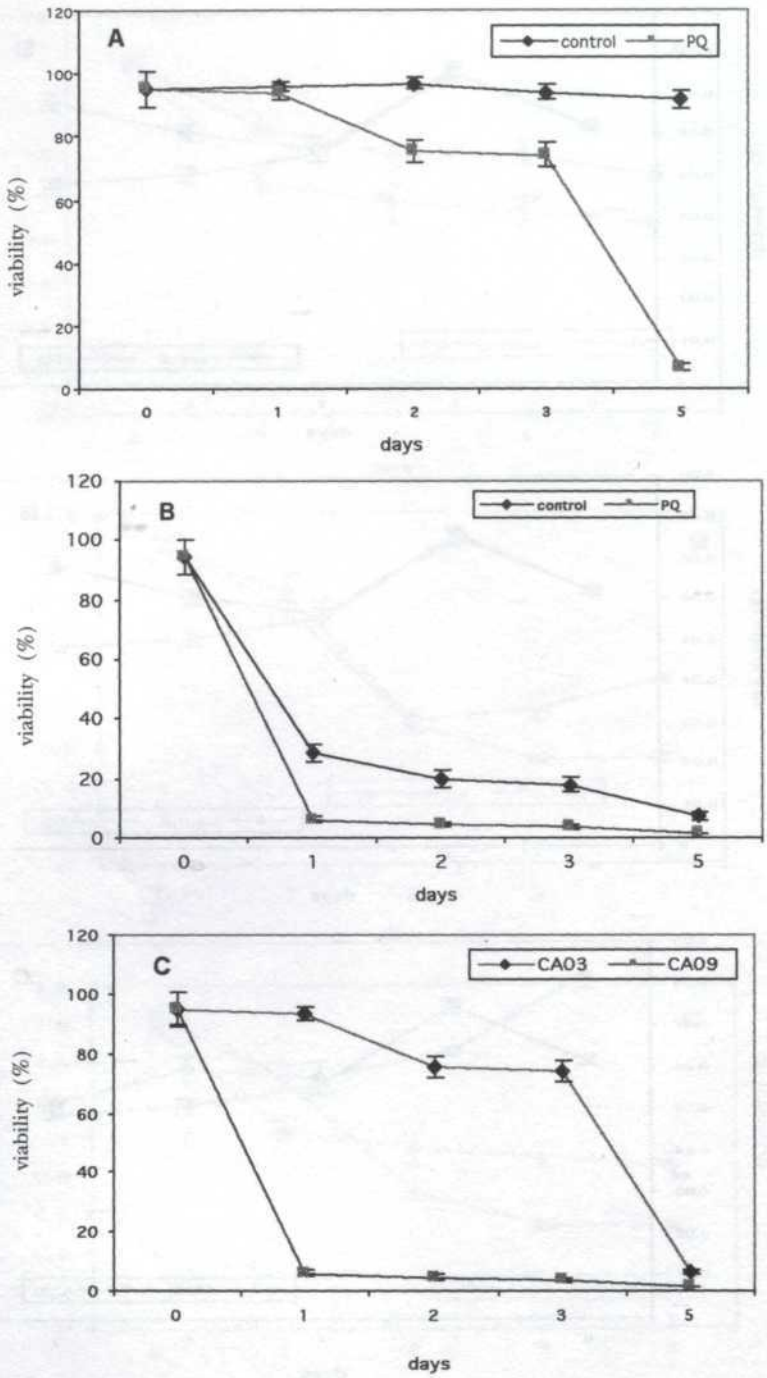

Fig. 3: Viability (%) of C. asiatica *callus culture:*
A) CA03 callus
B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)

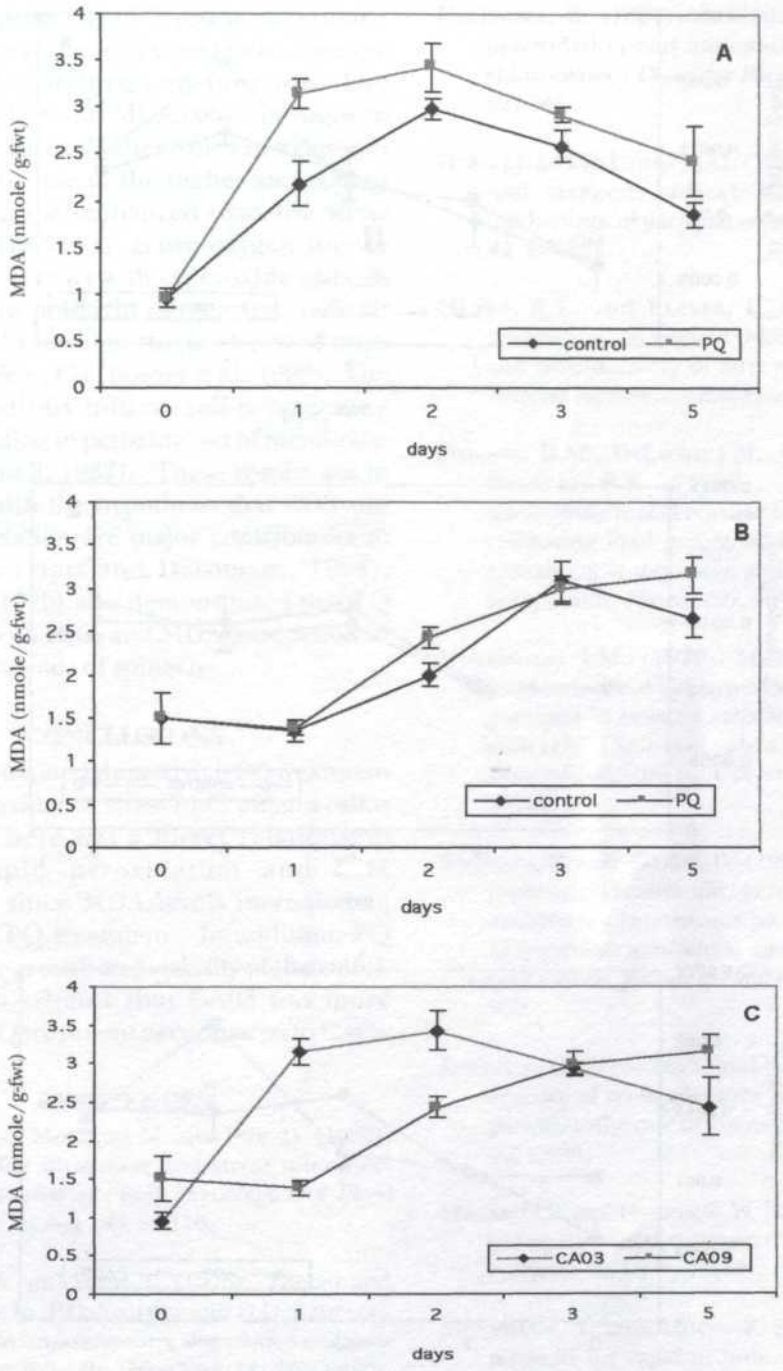

Fig. 4: Malondialdehyde (MDA) concentrations of C. asiatica *callus culture:*
A) CA03 callus B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)

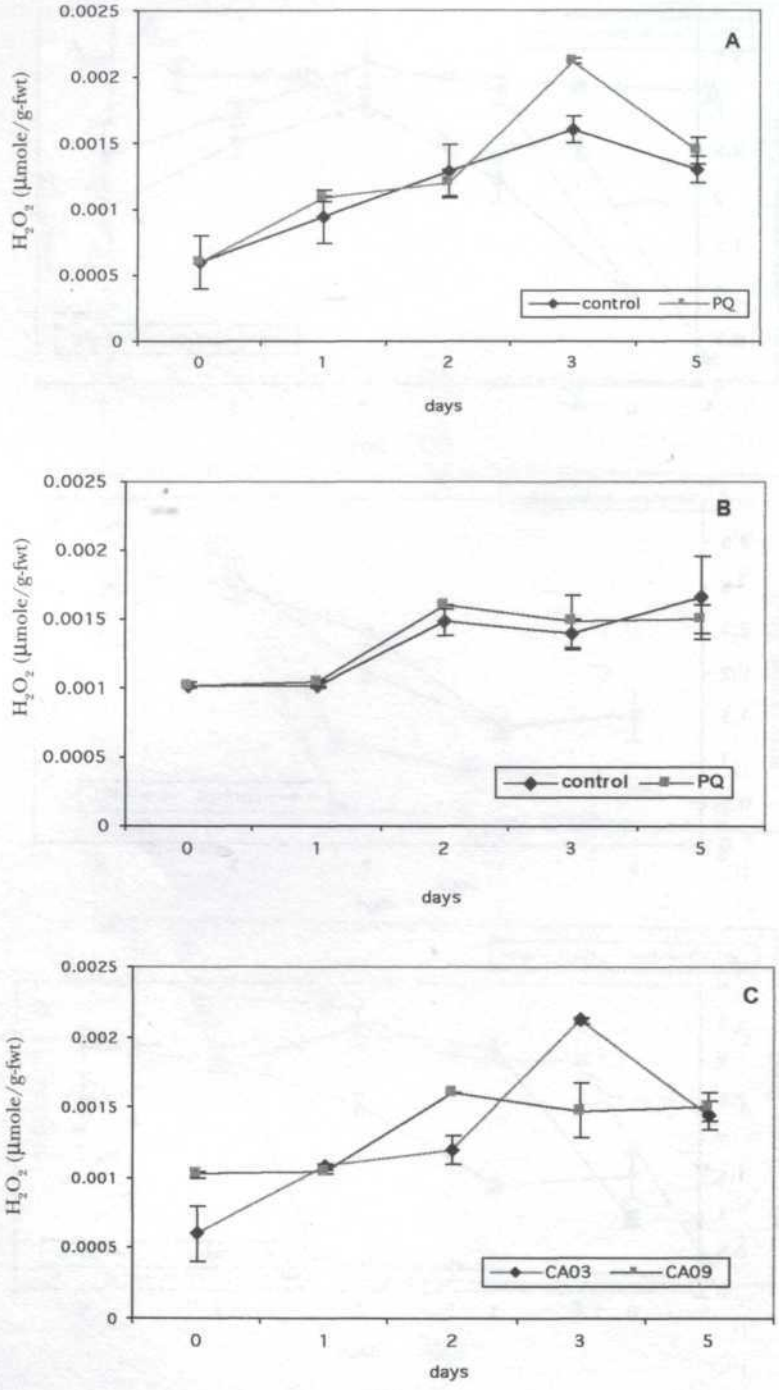

Fig. 5: Hydrogen peroxide (H_2O_2) concentrations of C. asiatica *callus culture:*
A) CA03 callus B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)

5C). Accumulation of H_2O_2 is potentially harmful since it can lead to oxidative damage and loss of structure and function. The decrease in H_2O_2 and MDA concentrations in CA03 callus especially after 3 days of treatment period may be due to the higher antioxidant activity which had enhanced oxidative stress tolerance. H_2O_2 is an active oxygen species which can also react with superoxide radicals to form more powerful oxygen free radicals and hydroxyl radical in the presence of trace amounts of Fe or Cu (Bowler *et al.*, 1992). The hydroxyl radicals initiate self-propagating reactions leading to peroxidation of membrane lipids (Halliwell, 1987). These results are in agreement with the hypothesis that ROS and lipid peroxidation are major contributors to PQ toxicity (Hart and DiTomaso, 1994). Hutchison (1979) also demonstrated that PQ stimulated both H_2O_2 and MDA production in leaf and thylakoids of spinach.

CONCLUSIONS

The results obtained showed that PQ treatment can induce oxidative stress in *C. asiatica* callus cultures. There was a direct relationship between lipid peroxidation and ROS production since MDA levels increased in response to PQ treatment. In addition, PQ inhibited the growth and viability of the callus. Results also suggest that CA03 was more tolerant to PQ treatment as compared to CA09.

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SHORT COMMUNICATION

A Survey of Water Consumption and Product Output from Ten Sago Factories in India

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ABSTRAK

Kebanyakan unit pemprosesan ubi kayu India mengasingkan kanji dan sluri dengan menggunakan kaedah enapan graviti. Pengenapan yang berlaku dalam tangki enap membolehkan kontak antara kanji dan air. Proses ini menyebabkan penapaian berlaku dengan alkohol dan asid organik menjadikan persekitaran tercemar. Sisa air dari kilang pemprosesan ubi kayu mengandungi keperluan oksigen kimia yang tinggi (11,077-19,083 mgl^{-1}), pH yang rendah (4.33-5.60) dan menyebabkan pencemaran. Efluen daripada industri ubi kayu adalah berasid dan berorganik secara semula jadi, membawa kepada keperluan oksigen biologi pada kadar 1500 hingga 2000gm^{-1}). Juzuk-juzuk tak organik seperti fosfat, sulfat, klorida, dan beberapa jenis logam terdapat dalam kuantiti surih. Kajian ini menerangkan penggunaan air, output produk, dan penjanaan efluen dalam industri pemprosesan ubi kayu. Kadar yang perlu untuk air adalah 4.512 m^3 untuk memproses 1000 kg ubi. Apabilá ubi-ubi tersebut digunakan untuk pengilangan kanji, 16.7% daripada hasil produk adalah kanji, 1.6% kanji kotor dan 7.0% 'thippi' terhasil dan 18.6% sagu, 1.8% kanji kotor, 19.1% kupasan dan 3.9% 'thippi' terhasil apabila ubi digunakan untuk pengilangan sagu. Sebanyak 95% air yang digunakan terhasil sebagai efluen.

ABSTRACT

Most of the tapioca processing units in India separate starch from slurry by employing the gravity settling method. Sedimentation in settling tanks allows the contact of starch with water. This process leads to fermentation in which alcohols and organic acids are formed and polluting the environment. Wastewater from tapioca processing factories contain high chemical oxygen demand (11,077-19,083 mg l^{-1}), low pH (4.33-5.60) and causes pollution. The effluent from tapioca industries is acidic and organic in nature, contributing biological oxygen demand in the range of 1500 to 2000 g m^{-3}. Inorganic constituents like phosphate, sulphate, chloride, and several metals are also found in trace quantities. This paper explains the water consumption, product output and effluent generation in tapioca processing industries. The average water requirement was 4.512 m^3 to process 1000 kg of cassava tubers. When the tubers are used for starch manufacture, a product yield of 16.7% starch, 1.6% dirty starch and 7.0% thippi were obtained, and 18.6% sago, 1.8% dirty starch, 19.1% peel and 3.9% thippi were obtained when the tubers are used for sago manufacture. About 95% of the consumed water is leaving the factory as effluent.

INTRODUCTION

Tapioca is a productive crop in poor soils and requires the least labour in cultivation, and can tolerate drought, but the labour requirement in processing after harvest is high (Radhakrishnan, 1996). Dry tapioca root consists of 80 to 90% carbohydrate out of

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which the most important is starch. Starch content in tapioca ranges from 78.1 to 90.1% on dry basis. Tapioca industry is an agro based seasonal industry with huge employment potential in India. Tapioca is mainly processed into starch and sago. There are more than 1000 tapioca processing units in India producing starch and sago in cottage and small scale sectors. The major unit operations involved in sago production are Peeling and washing, Rasping and pulping, Screening, Settling and purification of starch, Pulverization, Globulation, Sizing, Roasting, Sun drying, Polishing and Packing. The starch and sago factories in India use age-old technology, involving longer duration of extraction and unhygienic handling of the material leading to poor quality end products (Rangaswami, 1993). Padmaja *et al.* (1990) reported that the starch or sago factories were becoming obsolete and use of labour intensive indigenous technologies, which often imparted off colours, off odour and microbial contamination to starch. Since the enzymatic process are likely to develop as soon as the roots were dug up and during manufacture, which ultimately reduces the quality of the end product, it is necessary to process the roots immediately after harvesting (Grace, 1977). Almost all tapioca processing industries in India have two major problems. The first problem is the huge requirement of water for better extraction of starch from tubers. Second is the generation of large volumes of effluent. Many factories are being closed due to the unavailability of water. Hence there is a need for suitable methods or equipment and technology to reduce the water consumption in tapioca starch production without sacrificing the starch extraction efficiency. It is necessary to have a thorough knowledge on the different unit operations involved in the starch production, water requirement, product output and effluent generation to develop technology to solve the water problem. The objective of this paper is to quantify the water requirement, product output and effluent production in the tapioca starch processing industries.

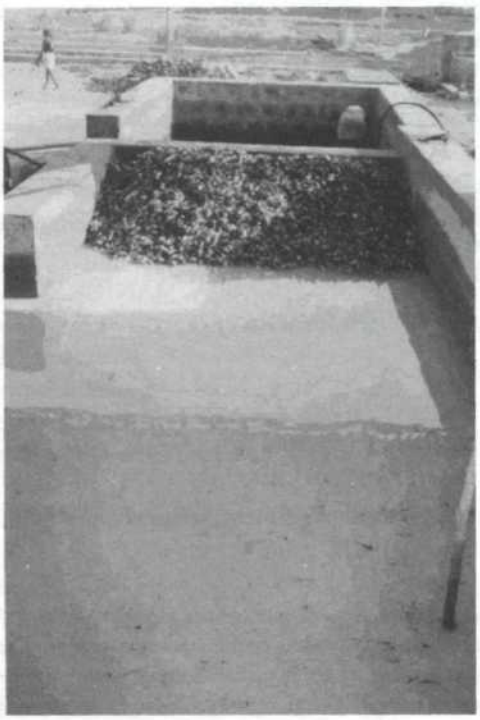

Fig.1: Addition of water for washing the tapioca roots

Conventional Method of Starch Production

(i) Washing and Peeling

Root tubers are washed manually to remove adhering dirt. The tubers are then cut longitudinally and transversely to a depth corresponding to the thickness of the peel, which can be easily removed. Any dirt remaining on the smooth surface of the core of the tuber is then washed off and the peeled tubers are put in a concrete tank, where they remain immersed in water until taken out for rasping. The conventional method of washing is shown in *Fig. 1.*

(ii) Rasping

The peeled roots are subjected to high-pressure water jets during conveying for rasping. It is necessary to rupture cell walls in order to release the starch granules. Rasping

Fig. 2: Addition of water while rasping the roots

facilitates rupture of cell walls and release of starch granules (Ahmed, 1978). This is carried out by pressing the roots against a swiftly moving surface provided with sharp protrusions. A large quantity of water is added to the roots (*Fig. 2*) during this process. The cell walls get ruptured during rasping and the whole root is turned into a mass in which substantial portions of the starch granules are released. It is difficult to remove all the starch in a single operation even with efficient rasping devices. Therefore, the pulp is subjected to a second rasping process after straining. Rasping is usually done in machines using a wooden roller over which the sheet metal rasping surfaces are nailed with the burrs facing outward.

(iii) Straining

After rasping, the pulp is screened in the shaking screens (*Fig. 3*). In separating the pulp from the free starch, a liberal amount of water is added to the pulp as it is delivered by the rasper and the resulting dispersion is stirred vigorously before screening. The fresh pulp after mixing with water in distribution tanks is transferred by pipes to the higher end of the screen. During screening, the dispersion passes through a set of screens with increasing fineness, the first one retains the coarse pulp, the others the fine particles. The 'overs' from the first screen are returned to the fine rasp and then returned for re-screening.

Fig. 3: Addition of water when straining the tapioca pulp

(iv) Settling and Purification of Starch After the separation of starch by screening, the starch milk is subjected to a settling process. The starch milk is pumped to a tank fitted with effluent outlets at varying heights. Settling takes about 6 to 20 hours depending on the quantity as well as the size of the settling tank (Radhakrishnan, 1996). Settling is an important unit operation in cassava processing where the extracted starch is separated from its aqueous dispersion under gravity (Sajeev *et al.*, 2002). The upper layer of sediment flour, which has a yellowish green tint, contains many impurities and is generally scraped off and discarded. The remaining moist flour is then stirred up with water and transferred to another tank where starch is settled. The final settled moist floor is removed by using a

crowbar. Sreenarayana *et al.* (1990) reported that a rapid separation of starch from the milk and the removal of impurities from the colloidal material could be achieved by centrifugation. Hydraulic jack may be used as dewatering technique by putting cassava starch pulp in a bag of woven cloth and subjected to high compressive pressure (Igbeka *et al.*, 1992).

(iv) Drying

Drying of starch is generally done by sun drying in the drying yards and then sent for further processing for the production of sago.

(v) Hydrogen Cyanide (HCN) in Tapioca

According to Mkpong *et al.* (1990) cited by Zoe *et al.* (1998), cyanagenic glucosides are synthesized in the cassava leaves and stored in the roots. The problems of inherent nutritional hazard (fresh roots contain 50 to 400 mg cyanide kg^{-1} root) and high perishability of the roots call for elaborative processing prior to consumption (Chinyere *et al.*, 1997). The major factor which limits or affects the utilization of cassava as a food for man is its content of the toxic hydrogen cyanide in both free and bound forms (Edijala *et al.*, 1999). Effective processing can reduce all cyanogens in cassava products to below the safe level of 10 mg HCN equivalent per kg dry weight set by FAO in 1988 (Mlingi *et al.*, 1995).

(vi) Effluent from Cassava Industries

Because of the rapid chemical changes occurring in the solution of starch dispersion, fermentation takes place resulting in the production of alcohol and organic acids especially butyric acid. Hence the process of separation of pure starch from starch milk should be done without time delay. Wastewater from tapioca processing factories containing high chemical oxygen demand (COD) (11,077-19,083 mg l^{-1}), and low pH (4.33-5.60) causes water pollution (Hien *et al.*, 1999). Belliappa (1990) found that the effluent from tapioca industries was acidic and organic in nature, contributing biological oxygen demand (BOD) in the range of 1500 to 2000 g m^{-3}. He also stated that inorganic constituents like phosphate, sulphate, chloride, and several metals were also found in trace quantities. Most factories dispose of the effluent into the nearby rivers, streams or lakes. It releases undesirable odour, pollutes the environment and surface ground water. The volatile solid content estimated for this industry effluent was 1.2 (Periasamy, 1996).

MATERIALS AND METHODS

The study was conducted in the tapioca processing units at Namakkal taluk of Rajaji district, Tamilnadu, India, where most of the tapioca processing industries in India are present. Ten factories of different production capacities (15 to 45 ton of root per day) were selected. The water requirement, product output and effluent production were measured in each factory for three days continuously and the average value recorded for analysis. For ease and clarity, all values were expressed per ton of cassava root.

RESULTS AND DISCUSSION

The process of extraction of starch from cassava tubers requires a huge volume of water. The wastewater arising out of washing of the roots and the supernatant from the starch settling tanks constitute the effluent. The average water requirement was 4.512 m^3 to process one ton of cassava root. Out of this, 4.31 m^3 and 4.27 m^3 of water was released as effluent, when the tubers were used for starch production and sago manufacture respectively. The product output during manufacture of starch and sago are shown in the Table 1. When the tubers were used for starch manufacture, a product yield of 16.7% starch, 1.6% dirty starch and 7.0% *thippi* were obtained and 18.6% sago, 1.8% dirty starch, 19.1% peel and 3.9% *thippi* were obtained when the tubers were used for sago manufacture. In both processes, about 95% of

TABLE 1
Water consumption and product output in sago factory (mean of 10 factories)

Raw Material		
Product	Quantity	CV
Cassava root	1 ton	
Water	4.512 m^3	7.3

Output – During Manufacture of Starch		
Product	Quantity	CV
Starch	167.40 kg	9.7
Dirty starch	16.32 kg	9.9
Thippi (Dried)	69.65 kg	13.7
Effluent	4.310 m^3	6.9

Output – During Manufacture of Sago		
Product	Quantity	CV
Sago	186.14 kg	13.6
Dirty starch	18.32 kg	13.2
Peel	190.53 kg	8.5
Thippi (Dried)	38.60 kg	8.5
Effluent	4.272 m^3	8.3

the consumed water is coming out as effluent. Since each factory is using different types of machinery, there was a variation in product output between the factories. The coefficient of variation (CV) in the product output ranged between 6.9 and 13.7 in starch the manufacturing process and between 8.3 and 13.6 in the sago manufacturing process. In both cases, the CV was less for effluent generation. The pattern of effluent production was almost similar in all factories when compared to other products. Since the quality of waste water released from the starch settling tank is not suitable for reuse in the production process, almost all factories are facing severe problems in treating the huge volume of effluent. Quick separation of water from starch milk in the settling tank will retard the deterioration in water and hence the released water may be reused in the production process. The dual problems (high water requirement and effluent production) in the starch factories may be solved by the quick separation of water from the starch milk with the help of equipment and reusing the water in the production. A solid-liquid separation equipment such as, hydrocyclone may be used for the quick separation in order to replace the conventional gravity settling method.

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