Asia Pacific Journal of Molecular Biology and Biotechnology, 2006
Vol. 14 (1) : 11-17

Direct Regeneration and RAPD Assessment of Male Inflorescence Derived Plants of *Musa acuminata* cv. Berangan

Asnita Abu Harirah and Norzulaani Khalid

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Wilayah Persekutuan, Malaysia

Received 10 February 2006/ Accepted 25 March 2006

Abstract. Regenerated plants were established using male inflorescence of *Musa acuminata* cv. Berangan. Explants were cultured on Murashige and Skoog (MS) solid medium supplemented with three different concentrations of 6-benzylaminopurine (BAP). After 2 weeks, whitish bud-like structures (WBLS) were obtained and MS media supplemented with 70.0 μM BAP was observed as the best media for the growth of WBLS. After 3 months, shoot-like structures emerged and MS media supplemented with 31.0 μM BAP gave a large number of shoot formation. Normal looking plantlets were obtained within 4 - 6 months with an average of 80 - 130 shoots regenerated from each male inflorescence. Random amplified polymorphic DNA (RAPD) was carried out to determine the clonal fidelity on *in vitro Musa acuminata* cv. Berangan micropropagated from male inflorescence derived from the same mother plant. Eighteen arbitrary decamer primers were used to amplify DNA from *in vitro* plants materials. All RAPD profiles from regenerated plants were monomorphic thus no somaclonal variation was detected. A total of 81 bands were scored from PCR amplification of genomic DNA from 15 micropropagated plants. This was further confirmed by the value of similarity index which was equaled to 1. This result implied that male inflorescence could be used as an alternative explant for commercial planting

Keywords. *in vitro*, clonal propagation, male inflorescence, banana, similarity index, RAPD

IINTRODUCTION

In vitro propagation has played a key role in clonal propagation for obtaining a large number of homogenous regenerated plants and breeding of plaintains and bananas (*Musa* spp.) (Pierik, 1987). The application of micropropagation for clonal propagation of *Musa* spp. have been reported by several authors using different explant sources such as meristems (Ma and Shii 1972; 1974; Banerjee *et. al*., 1986), shoot tips (Cronauer and Krikorian, 1984; Wong, 1986; Vuylsteke, 1998; Kanchanapoom and Chanadang, 2001) and floral apices (Cronauer and Krikorian, 1985a; 1985b; Balakrishnamurthy and Sree Rangaswamy, 1988; Doreswamy and Sahijram, 1989). These methods were successfully performed on a wide range of banana cultivars.

RAPD technique has successfully been used for the assessment of clonal fidelity in chestnut (Carvalho *et al*., 2003), *Piper* spp. (Chaveerach *et al*., 2002) and turmeric (Neeta *et al*., 2001). The validation of RAPD bands obtained is crucial through repetitions of experiments performed (Innis *et al*., 1998). RAPD is also applicable for studies on diversity in *Ocimum* spp. (Singh *et al*., 2004), *Heliconia* spp. (Prakash *et al*., 1998), gingers (Rout *et al*., 1998) and *Musa* spp. (Bhat and Jarret, 1995, Howell *et al.,* 1994, Kaemmer *et al.,* 1992).

In this study, the method established for *in vitro* propagation from male inflorescence offers an efficient and relatively simple method for clonal and mass propagation of *Musa* spp. compared to previous reports. Moreover, no studies on the assessment of clonal fidelity on the male inflorescence derived regenerants were carried out. Based on this study, male inflorescence could be used as an alternative explant instead of meristems cultures currently used for commercial propagation. In addition, male inflorescence displayed low risk of latent contamination which is a major problem in banana micropropagation.

MATERIALS AND METHODS

Plant Material and Explants. Male inflorescence of *Musa acuminata* cv. Berangan (AAA) was used as plant material. The male inflorescence were shortened to 4 - 6 cm in length by removing the enveloping bracts, sterilized in 70% (v/v) ethanol for 15 minutes and rinsed with sterile distilled water in the laminar-air flow. Sterilised explants were then dissected

*Author for Correspondence.
Mailing address: Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
Tel: +603-79674380; Fax: +603-79-674-178; E-mail: eyta03@yahoo.com

longitudinally into six parts under aseptic condition measuring about 1 - 2 cm.

In Vitro MultiplicationA and Culture conditions. Ten male inflorescence consisting of 60 longitudinal sections of explants were cultured on MS (Murashige and Skoog, 1962) based initiation media containing 5.4 μM naphtaleneacetic acid (NAA), 1 mg l^{-1} ascorbic acid, 150 ml l^{-1} coconut water, 3% (w/v) sucrose, 2 g l^{-1} phytagel (MS1) and supplemented with three different concentrations of 6-benzylaminopurine (BAP) at 4.4 μM, 31.0 μM and 70 μM. The pH was adjusted to 5.8 before autoclaving at 121°C, 104kPa for 15 min. All cultures were incubated at 24 - 28°C under two fluorescent lamps (Philip TL 80W/55T) with a photoperiod of 16 hours.

Development of WBLS. After 4 - 6 weeks, the whitish bud-like structures (WBLS) occurred and the fresh weight of WBLS were recorded for each treatment at different concentrations of BAP. Two subcultures were made at every six weeks interval

Regeneration of plantlets. The shoots regenerated from male inflorescence were placed onto MS propagation medium consisting of 3% (w/v) sucrose, 2 g l^{-1} phytagel and supplemented with 13.3 μM BAP (MS2). The number of shoots were recorded and plantlets with fully-expanded leaves were formed after 4 - 6 weeks in culture vessels. Fresh leaves were collected for RAPD analysis

Experimental Design and Statistical Analysis. The experiment was conducted in a completely randomised design (CRD) with 20 explants for each treatment. Significance of treatment effects were determined using analysis of variance (ANOVA) procedure in PC-SAS programming language (Version 6.12) (SAS Institute, 1997). Differences among means were determined using the LSD test, $p \leq 0.05$.

Genomic DNA Extraction. Total genomic DNA was extracted using a modified CTAB method based on the protocol of Doyle and Doyle (1990). Fresh leaves 1 g were ground to a fine powder using liquid nitrogen and transferred into 15 ml polypropylene tube which contained 5 ml of extraction buffer (2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0) and 0.2% (v/v) β-mercaptoethanol. The mixture was incubated for 1 hour at 60°C in a water bath and mixed every 10 min. One volume of chloroform:isoamylalcohol (24:1 v/v) was added and mixed by gently inversion, then the mixture was centrifuged at 6,000 rpm, 25°C for 10 min (Hettich Zentrifugen, Germany). After centrifugation, the upper phase was collected and repeated chloroform:isoamylalcohol extraction. The supernatan was treated with RNase A to a final concentration of 100 μg ml^{-1} and incubated at room temperature for 30 min. The supernatant was subsequently added with 0.6 volume of isopropanol and mixed by inversion. DNA was spun down by centrifugation at 6,000 rpm, 4°C for 10 min. DNA pellet was washed with 5 ml washing solution (70% (v/v) ethanol, 10 mM NH_4 acetate) and collected by centrifugation at 6,000 rpm, 4°C for 10 min. Pellet was stored for 1 hour at -20°C, then added with 2 volume of 96% (v/v) ethanol, followed by centrifugation at 6,000 rpm, 4°C for 10 min. DNA pellet was washed once with 70% (v/v) ethanol and subsequently dried in DNA-mini (Heto, Denmark). DNA was dissolved in 500 μl of TE buffer (10 mM Tris-HCl pH 8.0, 0.5 mM EDTA pH 8.0) and kept at -20°C.

Polymerase Chain Reaction. Twenty decamer primers OPC01-OPC20 (Operon Technologies, USA) were used for PCR amplification in this study. PCR reactions were performed in reaction mixtures (Promega, USA) with a total volume of 25 μl containing 1X PCR buffer (without $MgCl_2$), 100 μM of dNTPs solution, 1.5 mM of $MgCl_2$, 0.5 U of *Taq* DNA polymerase, 4 ng μl^{-1} of DNA template, 2 pmol μl^{-1} of primers (1st Base, Malaysia) and deionized water. DNA amplification was performed in the Mastercycler Gradient 5331 Eppendorf Version 2.1 (Eppendorf, USA) programmed according to Williams *et al.* (1990) with minor modifications. DNA was predenatured at 94°C for 4 min followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 36°C for 1 min and primer extension at 94°C for 2 min. The last cycle was followed by final extension at 94°C for 1.5 min. PCR products were kept at 4°C and the amplifications were repeated twice to confirm the results. Electrophoresis was carried out on the amplified products using 2% (v/w) agarose gel, stained with ethidium bromide and visualised under UV illumination (Gel-Pro Imager, USA). The 100 bp DNA Ladder Plus (Fermentas, Germany) was used as a molecular weight marker.

Similarity Index. Evaluation of fragment patterns was carried out by Similarity Index. Reproducible bands were scored manually as '1' or '0' for presence or absence of the bands. Coefficients of similarity between samples were calculated by using the formula of Nei and Li (1979) as followed:

$$SI = \frac{2N_{ij}}{N_i + N_j}$$

where SI is the similarity index, N_{ij} is the number of common bands shared between genotypes i and j, N_i and N_j are the total number of DNA bands for genotypes i and j, respectively.

RESULTS AND DISCUSSION

Regeneration of Plantlets. Explants were initially cultured on MS1 media supplemented with three different

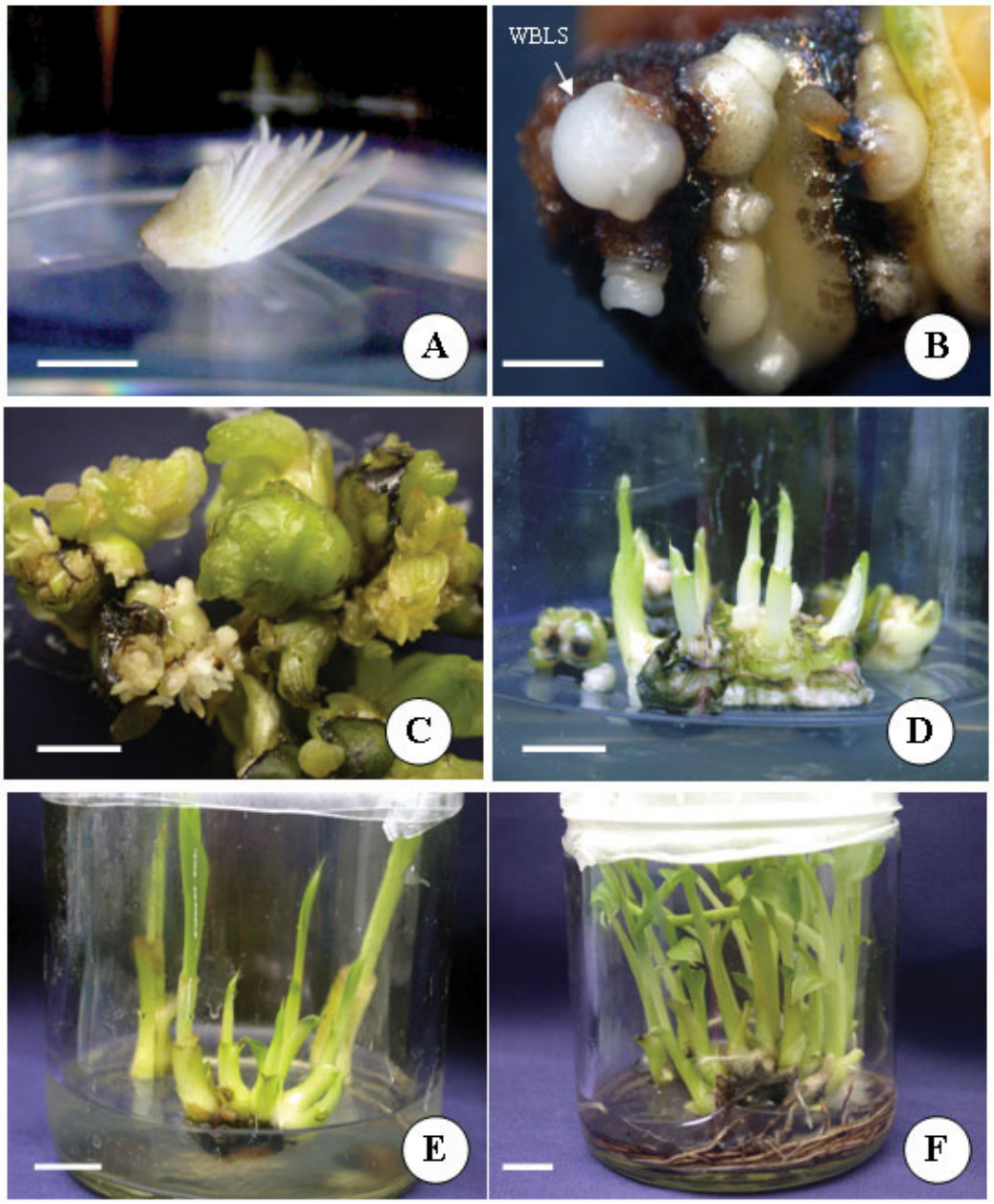

Figure 1: Direct regeneration from male inflorescence. (A) Longitudinal section of explant for initiation culture. Bar = 30 mm. (B) Formation of WBLS after 4 weeks in MS1 media supplemented with 31.0 µM BAP. Bar = 50 mm. (C) Multiplication of WBLS after first subculture. Bar = 15 mm. (D) Formation of shoots after 3 months in MS1 media supplemented with 31.0 µM BAP. Bar = 10 mm. (E) 15 weeks old shoot showing first green leaf. Bar = 10 mm. (F) Regenerated plants of *Musa acuminata* cv. Berangan. Bar = 10 mm.

concentrations of BAP at 4.4 µM, 31.0 µM and 70.0 µM (Figure 1A). After 3 to 5 days, explants showed greenish colouration at the tip which later spread to the base. Within 2 weeks of culture, the bracts opened to expose the immature male flowers and differentiation occurred to produce WBLS in less than 2 months (Figure 1B and C). The growth of WBLS are presented in Table 1, where the best growth of WBLS was obtained from media supplemented with 70.0 µM BAP. However, both MS1 media supplemented with 31.0 µM (T2) and 70.0 µM BAP (T3) did not show any significant difference (pd"0.05) for fresh weight of WBLS except for T1 treatment. Vuylsteke and De Langhe (1984) reported that MS media supplemented with 100 µM BA produced highly proliferative growth of adventitious buds,

associated with inhibition of apical shoot development for AA, AAA, AAB and ABB cultivars

After 3 months, shoot-like structures emerged followed by the growth of young leaf primordial (Figure 1D). Within 2 - 4 weeks the first green leaves appeared (Figure 1E) and unfurled their lamina. Once the shoots were formed, the concentration of BAP was reduced to 13.3 μM to increase the total number of plantlets (Figure F). Vuylsteke and De Langhe (1984) also reported that the regeneration of plantlets from multiple buds was achieved by subculturing on a medium with low cytokinin content. Furthermore, this protocol was more efficient and labour-saving to mass propagate tissue culture plantlets for commercial planting.

As shown in Table 1, although the highest mean number of shoots was obtained in T2 treatment, there was no significant difference between T2 and T3 treatments. However, the multiplication rate of WBLS is higher for T3 treatment in comparison to T2 treatment. Generally, it was found that high concentration of BAP, encouraged the multiplication rate of WBLS with less number of shoots produced subsequently. Similar observations were noted by Lee (2003) and Nathan *et. al.* (1992) in *Heliconia psittacorum*. Thus, this study suggested the use of high concentration of BAP (70.0 μM) for high multiplication rate of WBLS and a reduction of BAP concentration to 31.0 μM in order to obtain a large number of shoots. Generally, normal looking plantlets were obtained within 4 - 6 months, producing an average of 80 - 130 shoots from each male inflorescence.

Table 1. The fresh weight of WBLS at 6 weeks in initiation media

Treatments	Fresh weight of WBLS (g) (Mean ± S.E)	No. of shoots (Mean ± S.E)
T1 MS1 + 4.4 μM BAP	0.202 ± 0.050[a]	0.900 ± 0.315[a]
T2 MS1 + 31.0 μM BAP	1.330 ± 0.034[b]	19.700 ± 1.622[b]
T3 MS1 + 70.0 μM BAP	1.430 ± 0.036[b]	18.200 ± 1.439[b]

a,b: The mean values followed by the same letter are not significantly different at p≤0.05, LSD test

RAPD Analysis. The results were scored as patterns of bands obtained from *in vitro* micropropagated plants derived from male inflorescence of the same mother plant. Eighteen primers tested produced amplification products that were monomorphic across all micropropagated plants (Figure 2). The size of the monomorphic DNA fragments, produced by these primers were shown in Table 2. For each primer, 3 - 4 major bands were scored and the size of the amplification products ranged between 200 bp to 2.5 kb. A total of 81 bands were scored from PCR amplification of genomic DNA from 15 micropropagated plants of *Musa acuminata* cv. Berangan. No polymorphism or genetic changes in the

Table 2. Summary of the RAPD analysis on *in vitro Musa acuminata* cv. Berangan regenerated from male inflorescence.

Primers	Nucleotide sequences5' – 3'	Fragments range(bp)	No. of monomorphic bands	No. of polymorphic bands	Total no. of bands	Similarity index(SI)
OPC 01	TTCGAGCCAG	550-1500	5	0	5	1.0
OPC 02	GTGAGGCGTC	450-1031	5	0	5	1.0
OPC 03	GGGGGTCTTT	-	-	-	-	-
OPC 04	CCGCATCTAC	500-1000	4	0	4	1.0
OPC 05	GATGACCGCC	450-1200	6	0	6	1.0
OPC 06	GAACGGACTC	600-1500	4	0	4	1.0
OPC 07	GTCCCGACGA	-	-	-	-	-
OPC 08	TGGACCGGTG	200-1500	6	0	6	1.0
OPC 09	CTCACCGTCC	450-750	3	0	3	1.0
OPC 10	TGTCTGGGTG	600-2500	5	0	5	1.0
OPC 11	AAAGCTGCGG	500-2000	6	0	6	1.0
OPC 12	TGTCATCCCC	350-1500	4	0	4	1.0
OPC 13	AAGCCTCGTC	750-2000	3	0	3	1.0
OPC 14	TGCGTGCTTG	540-1550	4	0	4	1.0
OPC 15	GACGGATCAG	600-850	4	0	4	1.0
OPC 16	CACACTCCAG	400-2000	5	0	5	1.0
OPC 17	TTCCCCCCAG	380-1300	4	0	4	1.0
OPC 18	TGAGTGGGTG	350-2000	4	0	4	1.0
OPC 19	GTTGCCAGCC	540-1150	3	0	3	1.0
OPC 20	ACTTCGCCAC	450-2500	6	0	6	1.0
TOTAL			81	0	81	

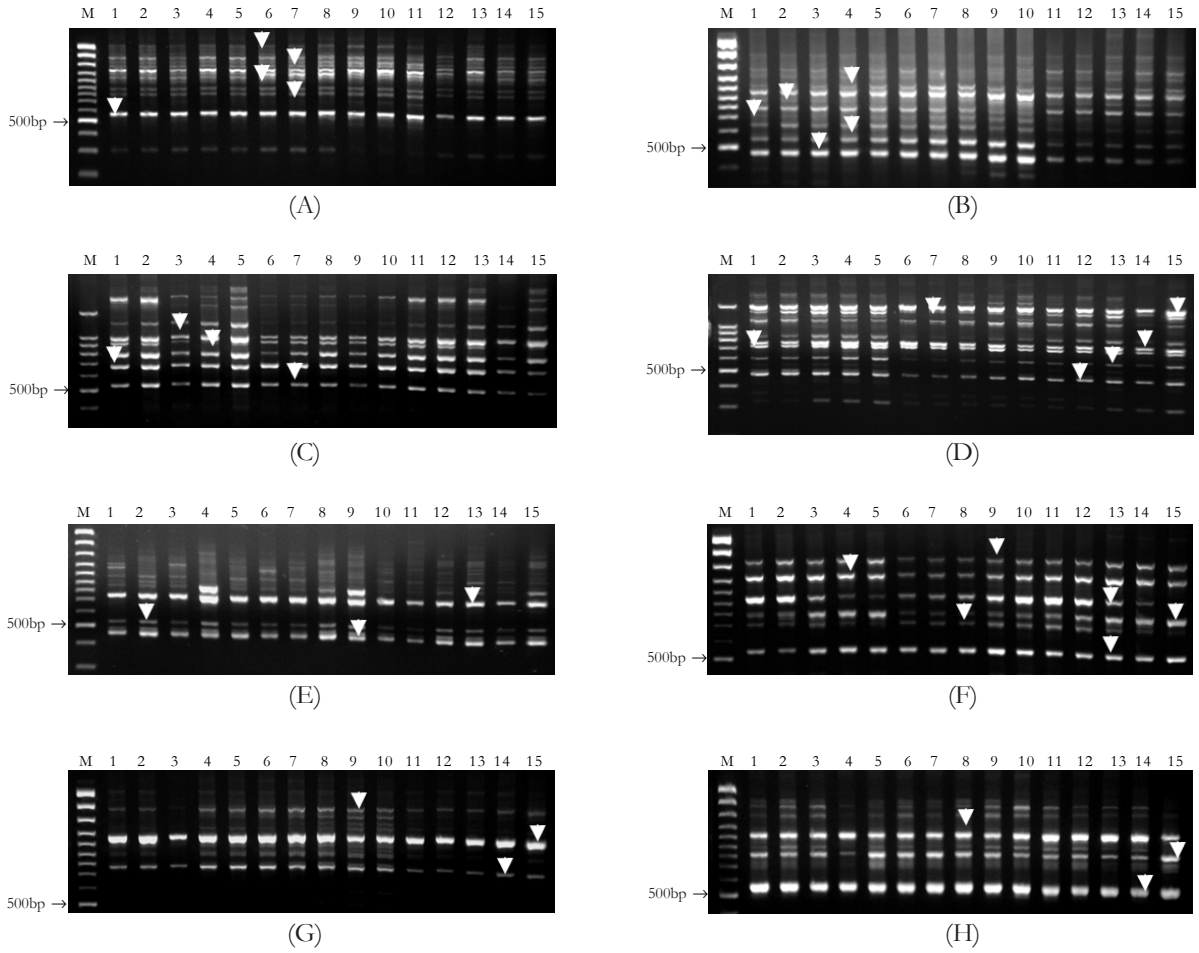

Figure 2. RAPD pattern bands generated by primers (A) OPC 01, (B) OPC 02, (C) OPC 04, (D) OPC 05, (E) OPC 09, (F) OPC 11, (G) OPC 13 and (H) OPC 19. Lane M = 100 bp DNA ladder plus (arrowhead with 500 bp), lane 1-15 regenerated plants from male inflorescence. Arrows indicate the reproducible bands in regenerated plants from male inflorescence *Musa acuminata* cv. Berangan.

amplified DNA were detected after amplification by PCR within micropropagated plants. Thus, all micropropogated *Musa acuminata* cv. Berangan showed 100% similarity where the similarity index was equaled to 1.0. Doreswamy and Sahijram (1989) also reported that no appreciable morphological differences were observed in regenerated plants from excised floral apices of 'Chandrabale', 'Rastali' and 'Robusta'. On the contrary, several researchers have reported somaclonal variation phenomena in banana micropropagation through meristems cultures. The rates of somaclonal variation in banana plants derived from meristems culture vary from 0 - 70% according to genotype (Smith, 1988; Vuylsteke *et al.*, 1991; Israeli *et al.*, 1995). The source of explants and mode of regeneration (somatic embryogenesis, organogenesis, axillary bud multiplication) plays an important role in determining the presence or absence of variation (Damasco *et al.*, 1996).

In this study, the RAPD results indicated that micropropagation through male inflorescence produced no somaclonal variation. Experiments were repeated three times to ensure reproducible scorable bands which seem to be the major flaw in this technique as described previously.

CONCLUSION

Direct regeneration from male inflorescence is a rapid and simple method for clonal and mass propagation of *Musa acuminata* cv. Berangan on a commercial scale. This method is cost effective and has an advantage of the absence of latent contamination occasionally faced by meristems cultures which is currently being used for commercial production of bananas. In this technique regenerated plants were obtained within 4 - 6 months at an average of 80 - 130 shoots from each male

inflorescence.RAPD analysis was adopted and has proven that these regenerated plants were clonal in nature.

ACKNOWLEDGEMENTS

This research was financially supported by Intensified Research Priority Areas Grant from the Malaysian Government through the Ministry of Science, Technology and Innovation and the University Malaya.

REFERENCES

Balakrishnamurthy G & Sree Rangaswamy SR (1988) Regeneration of banana plantlet from in vitro culture of floral apices. *Current Science*. 57: 270-272.

Banerjee N, Vuylsteke D & de Langhe E (1986) Meristem tip culture of Musa: histomorphological studies of shoot bud proliferation. In: Withers LA & Anderson PS (eds) *Plant Tissue Culture and its Agricultural Applications*. Butterworths, London. pp. 139-147.

Bhat KV & Jarret RL (1995) Random amplified polymorphic DNA and genetic diversity in India *Musa* germplasm. *Genetic Resources and Crop Evolution* 42: 107-118.

Carvalho LC, Goulão L, Oliveira C, Goncalves JC & Amâncio S (2003) RAPD assessment for identification of clonal identity and genetic stability of in vitro propagated chestnut hybrids. *Plant Cell, Tissue and Organ Culture* 77: 23-27

Chaveerach R, Kunitake H, Nuchadomrong S, Sattayasai N & Komatsu H (2002) RAPD patterns as a useful tool to differentiate Thai *Piper* from morphological alike Japanese *Piper*. *Sciense Asia* 28: 221-225.

Cronauer SS & Krikorian AD (1984) Multiplication of *Musa* from excised stem tips. *Annals of Botany* 53: 321-328.

Cronauer SS & Krikorian AD (1985a) Aseptic multiplication of banana from excised floral apices. *HortScience* 20:770-771.

Cronauer SS & Krikorian AD (1985b) Reinitiation of vegetatif growth from aseptically cultured terminal floral apex of banana. *American Journal of Botany* 72: 1598-1601.

Damasco OP, Graham GC, Henry RJ, Adkins SW, Smith MK. & Godwin ID (1996) Random amplified polymorphic DNA (RAPD) detection dwarf off-types in micropropagated Cavendish (*Musa* spp. AAA) bananas. *Plant Cell Reports* 16:118-123.

Doreswamy R & Sahijram L (1989) Micropropagation of banana from male floral apices cultured *in vitro*. *Sci. Horti.* 40:181-188.

Doyle JJ & Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12:13-15.

Howell EC & Newbury HJ (1994) The use of RAPD for identifying and classifying germplasm. *Musa* Genome 37: 328-332.

Israeli RL, Lahav E & Reuveni O (1995) *In vitro* culture of banana. In: *Bananas and Plaintains*. S. Gowen, London, Chapman & Hall. pp. 147-178.

Kaemmer D, Afza R, Weishing K, Kahl G & Novak FJ (1992) Oligonucleotide and amplification fingerprinting of wild species and cultivars of banana (*Musa* spp.). *BioTechnology* 10: 1030-1035.

Kanchanapoom K & Chanadang N (2001) *In vitro* culture of the banana *Musa* (AAA group, 'Gros Michel') 'Kluai Hom Thong' shoot tip. *J. ISSAAS*. 643-52.

Lee SW (2003) Micropropagation of Cavendish banana in Taiwan. Food & Fertilizer Technology Center.

Ma SS & Shii CT (1972) *In vitro* formation of adventitious buds in banana shoot apex following decapitation (In Chinese with English summary). *J. Chinese Soc. Hort. Sci.* 18:135-142.

Ma SS & Shii CT (1974) Growing banana plantlets from adventitious buds. (In Chinese with English summary). *J. Chinese Soc. Hort. Sci.* 20:6-12.

Murashige T & Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.

Nathan MJ, Goh CJ & Kumar PP (1992) *In vitro* propagation of *Heliconia psittacorum* by bud culture. *HortScience*. 27(5):450-452.

Nei M & Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Science USA*. 76: 5269-5273.

Neeta DS, Leela G & Susan E (2001) Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell, Tissue and Organ Culture* 66: 113-119.

Pierik RLM (1987) *In vitro* culture of higher plants. Martinus Nijhoff Publishers, Dordrecht. 345 pp.

Prakash PK, Janice CKY & Goh CJ (1998) Genetic Analyses of *Heliconia* species and cultivars with Randomly Amplifies Polymorphic DNA (RAPD) markers.*J. Amer. Soc. Hort. Sci.* 123: 91-97.

Rout GR, Das P, Goel S & Naina SN (1998) Genetic stability of micropropagated plants of ginger using Random Amplified Polymorphic DNA (RAPD) markers. *Bot. Bull. Acad. Sin.* 39: 23-27.

Singh AP, Dwivedi S, Bharti S, Srivastava A, Singh V & Khanuja SPS (2004) Phylogenetic relationships as in *Ocimum* revealed by RAPD markers. *Euphytica* 136: 11-20.

Smith MK (1988) A review of factors influencing the genetic stability of micropropagated bananas. *Fruit* 43: 219-223.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA & Tingey SV (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.

Wong WC (1986) *In vitro* propagation of banana (*Musa* spp.): initiation, proliferation and development of shoot-tip cultures on defined media. *Plant Cell Tiss. Org. Cult.* 6: 159-166.

Vuylsteke D & De Langhe E (1984) Feasibility of in vitro propagation of bananas and plaintains. *Trop. Agric. (Trinidad).* 62: 323-328.

Vuylsteke D, Swennen R & De Langhe E (1991) Somaclonal variation in plaintains (Musa spp, AAB group) derived from shoot-tip culture. *Fruits* 46: 429-439.

Vuylsteke D (1998) Shoot-tip culture for the propagation, conservation and exchange of *Musa* Germplasm. International Institute of Tropical Agriculture, Ibadan, Nigeria. 82 pp.