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Plant regeneration from embryogenic suspension cultures of *Musa acuminata* cv. Mas (AA)

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Abstract

Embryogenic callus was established using immature male flower of *Musa acuminata* cv. Mas. After 5–6 months of culture, embryogenic callus was obtained at 21.75%±11.9 from 750 immature male flower clusters with translucent somatic embryos proliferated from the whitish friable callus. It was observed that flower clusters ranging from 4 to 11 responded to form embryogenic callus and out of which 3–10 somatic embryos were formed per flower cluster. Embryogenic callus were obtained at a percentage of 10.00%±0.3 on M1 medium initially supplemented with 18 μM 2,4-dichlorophenoxyacetic acid (2,4-D) for 3 months and subsequently transferred to the same media with reduced 2,4-D (9 μM) for the next 2–3 months. Embryos developed into translucent spheres and slightly torpedo shaped embryos in suspension cultures. Plantlets were obtained on medium M4 supplemented with 0.8 μM BA, at an average regeneration rate of 13.00%±0.58.

Abbreviations: BA – benzylaminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – indoleacetic acid; NAA – naphthaleneacetic acid

Introduction

Banana is one of the most important food crops in the world with global sales of 5 billion (US) per annum. Cavendish type bananas dominate the world's market for dessert bananas. Malaysia has 50 types of banana cultivars to be exploited as export crops. However the quality of the fruit must be enhanced to meet the market and export standards. Conventional improvement programmes have been hampered due to its sterile and polyploidy nature. Due to these problems, new strategies combining tissue culture and genetic engineering techniques to complement breeding programmes must be developed.

Somatic embryogenesis is one of the important prerequisite for genetic engineering. It has been successful only in a few banana cultivars such as Bluggoe, ABB (Novak et al., 1989; Dhed'a et al., 1991; Panis et al., 1993); Grande Nain, AAA (Novak et al., 1989; Escalant et al., 1994; Côte et al., 1996; Becker et al., 2000) and Mysore, Rastali (AAB) (Escalant et al., 1994; Ganapathi et al., 2001). The banana explants that have been used in somatic embryogenesis studies are proliferating meristems (Cronauer-Mitra and Krikorian, 1983); zygotic embryos (Cronauer-Mitra and Krikorian, 1988; Escalant and Teisson, 1989; Marroquin et al., 1993); rhizome and leaf sheaths (Novak et al., 1989); immature male flowers (Ma, 1991; Shii et al., 1992; Escalant et al., 1994; Côte et al., 1996; Grapin et al., 1996) and female flowers (Grapin et al., 2000). Among the explants that have been used, immature male flowers are the most responsive starting material for initiating embryogenic cultures of Grande Nain (Escalant et al., 1994; Côte et al., 1996; Navarro et al., 1997; Sagi et al., 1998; Becker et al., 2000). To date, there are not many published reports on somatic embryogenesis from immature male flower of dessert banana belonging to the AA group. Genotypic dependence might further explain why success was limited to certain

cultivars and the induction of somatic embryos is not yet routine or efficient for the majority of the banana cultivars.

Besides using somatic embryos as target materials for genetic engineering, they could also be used for large-scale propagation of plants and the production of synthetic seeds. For mass propagation of somatic embryos, cell suspensions should be established. This work is aimed at establishing a regenerable embryogenic cell suspensions from immature male flowers of *Musa acuminata* cv. Mas (AA), a popular commercial variety in the South East Asian region.

Materials and methods

Explant source and sterilization

Inflorescence male buds of *Musa acuminata* cv. Mas (AA) were used as explant materials. First dissection was carried out in the general laboratory where the male bud was shortened to 6–8 cm in length by removing the enveloping bracts. For second dissection the male bud was then transferred to a laminar-air flow cabinet. Explants were sterilized in 70% ethanol for about 15 min and rinsed three times with sterile distilled water. The male bud was further shortened to 1–1.5 cm in length for culture and immature male flower clusters from position 1 to 15 were removed under stereo-microscope until the meristem was exposed.

Initiation of embryogenic callus

A total of 50 male buds consisting of 750 immature male flowers were cultured on initiation medium, M1 (Escalant et al., 1994; Côte et al., 1996) which contained Murashige and Skoog (1962) (MS) basal medium supplemented with 4.1 μM biotin, 5.7 μM indoleacetic acid (IAA), 5.4 μM naphtaleneacetic acid (NAA), 87 μM sucrose, 2 g l^{-1} gelrite and combinations of two different concentrations of 2,4-D (Table 1). The cultures were maintained on each 2,4-D concentration for a period of 3 months.

Suspension cultures

Two culture media were tested to determine the best media for embryo development of *Musa acuminata* cv. Mas. Friable embryogenic callus was transferred to two different liquid media (M2a and b). M2a media (Côte et al., 1996) contained MS basal medium supplemented with 4.1 μM d-biotin, 680 μM glutamine, 100 mg l^{-1} malt extract (Sigma) and 130 mM sucrose. M2b media (Dhed'a et al., 1991) consisted of half strength MS macronutrients, MS micronutrients, half strength MS FeEDTA supplemented with Dhed'a vitamins, 5 μM 2,4-D, 10 mg l^{-1} ascorbic acid, 1 μM zeatin and 20 g l^{-1} sucrose.

Initiation of suspension culture was done by transferring 0.3–1.0% packed cell volume (PCV) embryogenic callus into 10 ml fresh suspension media and subculture was done every 10 days. A small sample of the suspension was taken out for morphological observations. The viability of the cells was estimated by flourescein diacetete (FDA) (Widhlom, 1972). Embryogenic characteristic were confirmed through histochemical stains described by Gupta and Durzan (1987). Growth of cell suspensions were measured by determining the settled cell volumes (SCV) every 3 days over a period of 1 month.

Table 1. Percentage (%) of embryogenic callus obtained from immature male flower of *Musa acuminata* cv. Mas (AA) with different combination of 2,4-D

Concentration of 2,4-D (μM)		No. of male bud	No. of immature male flower cluster	No. of explant giving embryogenic callus	Percentage of embryogenic callus (%)
Subculture 1 (3 months)	Subculture 2 (3 months)				
18	18	9	135	8	5.92±0.12
18	9	8	120	12	10.00±0.30
9	9	8	120	4	3.33±0.10
9	4.5	8	120	2	1.67±0.02
4.5	4.5	8	120	1	0.83±0.04
4.5	2.25	9	135	–	–
Total		50	750	27	21.75±11.9

Development of somatic embryos

Embryos developed from suspension culture were transferred to media M3 (Côte et al., 1996) for further development. Media M3 consisted of Schenk and Hildebrant (1972) (SH) macronutrients, SH micronutrients, SH FeEDTA, MS vitamins supplemented with 4.5 μM d-biotin, 680 μM L-glutamine, 2 mM proline, 100 mg l^{-1} malt extract (Sigma), 1.1 μM NAA, 0.2 μM zeatin, 0.5 μM kinetin, 0.7 μM N^6-(2-isopentenyl) adenine, 29 mM lactose, 130 mM sucrose and solidified with 3 g l^{-1} gelrite.

Regeneration of somatic embryos

Somatic embryos were regenerated on M4 media (Côte et al., 1996) consisting of MS macronutrients, MS micronutrients, MS Fe EDTA, Morel and Wetmore vitamin (Morel and Wetmore, 1951) supplemented with 1.1 μM indoleacetic acid (IAA), 87 μM sucrose and solidified with 2 g l^{-1} gelrite. Three different concentrations of benzylaminopurine BA (0.2, 0.4 and 0.8 μM) were also added to the media separately. In comparison, MS media without any plant growth regulators (MSO) was also used. Five replicates were done for each media tested. The cultures were incubated in dark conditions until adventitious buds appeared and subsequently transferred to light (16-h photoperiod) for shoot development. Percentage of regeneration was scored by counting the number of clumps giving rise to shoots.

Rooting of plantlets

MSO with 1% (w/v) activated charcoal was used as rooting media. Elongated plantlets were transferred individually into the rooting media.

Culture conditions

The pH of the media was adjusted to 5.7 prior to autoclaving. All culture media were autoclaved at 121 °C for 20 min. Cultural conditions for initiation of embryos, maintenance of suspension culture and development of embryos were maintained at 28 °C under a 16-h photoperiod with light intensity of 31.4 μmol m^{-2} s^{-1}. Cell suspensions were maintained at 70 rpm continuously on a rotary shaker. For plant regeneration, cultures were initially placed in the dark until adventitious buds appeared and subsequently transferred to light conditions.

Results and discussion

In this study, immature male flower of *Musa acuminata* cv. Mas (AA) was used to induce embryogenic callus. The original explants became brown at the base within a week of culture and began to swell and increased in size after 2–3 weeks. The brown color was due to the release of phenolic compounds from the original explants and the increasing size was a result of cell divisions. After 2–3 months, yellow globular calluses appeared and later formed white friable calluses. About 5–6 months of culture, 21.75%$\pm$11.9 of the flowers produced embryogenic callus which later formed whitish translucent somatic embryos (Figure 1A). The feature of the somatic embryos obtained was translucent spheres which were similar to those obtained by Côte et al. (1996) and Escalant et al. (1994). However, there was a difference in the time taken for somatic embryos formation in this work which was probably due to the cultivar differences. On the average 3–5 somatic embryos were formed but a maximum of 10 could be obtained per flower cluster. Initiation of embryogenic callus was done on M1 medium supplemented with 2,4-D because this auxin has been proven to be superior compared to other plant growth regulators (Evans et al., 1981). M1 media with an initial concentration of 18 μM 2,4-D for the first 3 months and later reduced to half the concentration for another 3 months was shown to be the best media for callus initiation (Table 1). Even though a high level of 2,4-D is needed to induce embryogenic callus, prolong exposure will reduce the embryogenic nature of the callus. Only flower clusters ranging between 4 and 11 responded to form embryogenic callus at a percentage of 54%. The highest percentage (18%) of embryogenic callus obtained is from immature male flower cluster number 9 (result not shown).

Upon transfer to suspension medium, friable embryogenic callus released embryogenic cells with dense cytoplasm. Suspension cultures obtained consisted of heterogenous cells where the embryogenic cells were smaller, more spherical in shape and had dense yellow cytoplasm with very few small vacuoles (Figure 1B). Cells with dense cytoplasm were observed in 1-month-old suspension cultures. When the suspensions were 3–4 months old, fine embryogenic

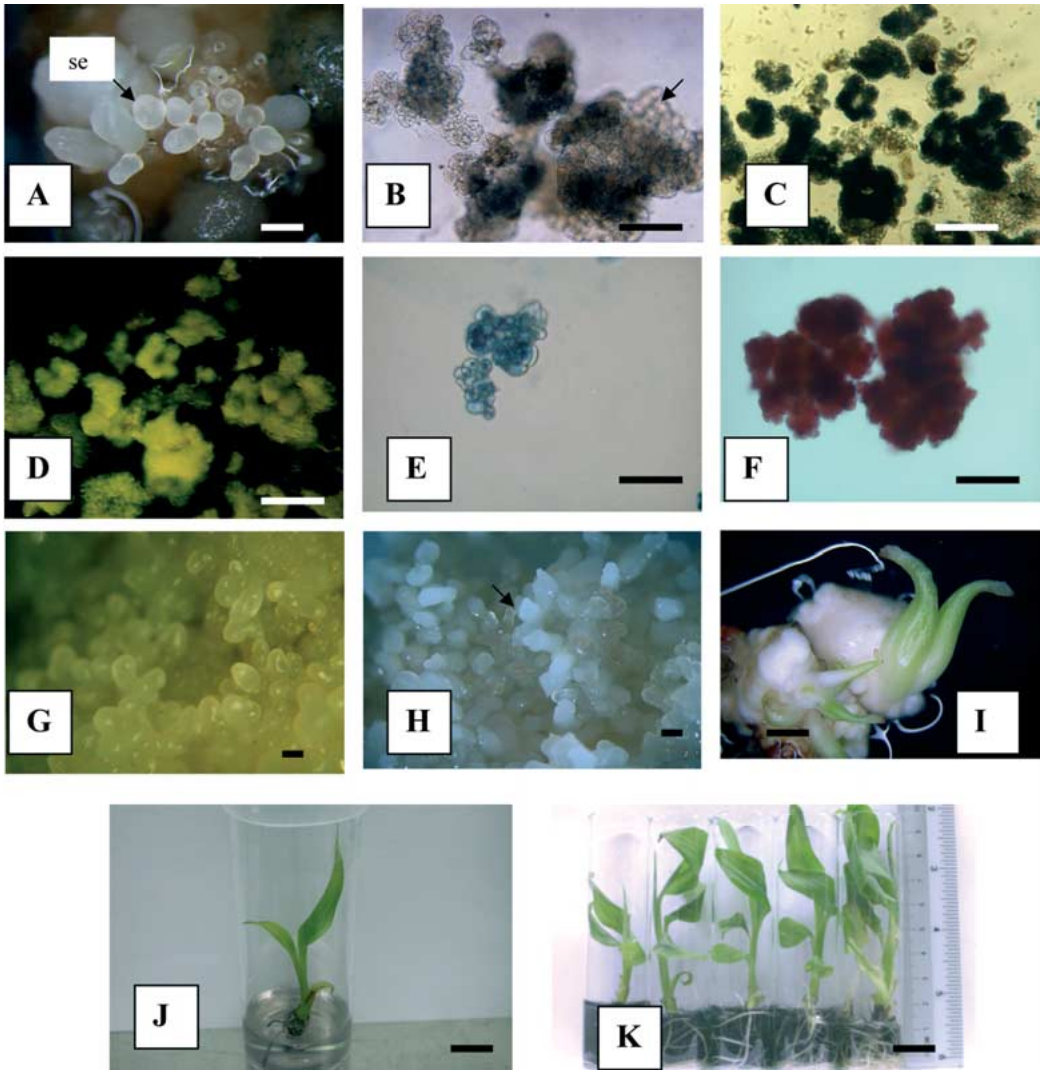

Figure 1. (A–K) Plant regeneration from somatic embryos of *Musa acuminata* cv. Mas (AA). (A) Embryogenic callus with somatic embryos from immature male flower of *Musa acuminata* cv. Mas (AA). se: somatic embryos. Bar=200 μm. (B) Cell suspension of *Musa acuminata* cv. Mas (AA) with dense yellow cytoplasm. Bar=100 μm. (C) Control – Cell suspension without staining of FDA. Bar=150 μm. (D) Cell suspension stained green fluorescence with FDA staining. Bar=150 μm. (E) Control – representing double-stained non-embryogenic callus cells derived from initial explant. Bar=125 μm. (F) Cell cluster from suspension culture showing embryogenic characteristic by stained red by double-staining. Bar=100 μm. (G) Translucent spheres and slightly torpedo shaped embryos upon plating on M3 medium after 1–2 months of culture. Bar=0.8 mm. (H) Mature somatic embryos on M3 medium after 2–3 months of culture. Bar=1 mm. (I) Germinating embryos on M4 medium. Bar=0.5 cm. (J) Normal plants germinated from somatic embryos Bar=1 cm. (K) Rooted plant in 1% charcoal. Bar=1 cm.

cells were observed as yellowish white in colour. The yellow pigmentation of cytoplasm indicated the embryogenic character of the suspension cells (Becker – pers. comm.). The same result was also reported by Ganapathi et al. (2001). Cells stained with flourescein diacetate showed more than 90% were green flourescence which indicated the viability of cells (Figure 1D). The embryogenic characteristic was proven with the double staining of cells using aceto-carmine and Evans Blue which exhibited positive results (Figure 1F).

Initiation of suspension culture was critical especially at the initial stage due to the presence of phenolic exudates and if left to accumulate, it will make viable cells necrotic. In this study, comparison between two established suspension medium namely

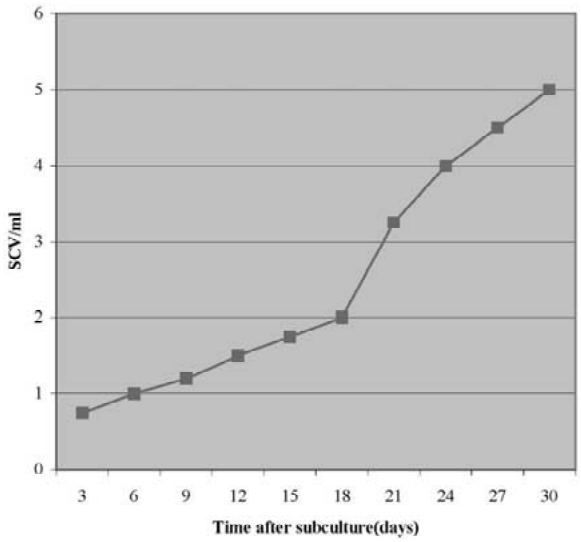

Figure 2. Growth (Settled cell volume) of *Musa acuminata* cv. Mas (AA) suspension culture.

M2a (Côte et al., 1996) and M2b (Dhed'a et al., 1991) was carried out to determine the best medium for establishing *Musa acuminata* cv. Mas (AA) suspension culture. The choice was based on a medium that displayed less phenolic exudates. Medium M2b selected for the initiation of the suspension culture of *Musa acuminata* cv. Mas (AA) showed only 10% phenolic compounds compared to 40% in medium M2a after 7 days of initiation. This result agreed with Becker et al. (2000) who observed that Dhed'a et al. (1991) suspension medium produced more embryos and had less necrosis compared to Côte et al. (1996) medium. The growth curve of the suspension culture was measured using settled cell volume where the cell volume was seen to increase and the volume doubled every 9 days (Figure 2). A typical sigmoidal curve could be observed probably over a longer period.

Upon transfer to M3 semisolid medium translucent spheres and slightly torpedo shaped embryos were formed (Figure 1G, H). Comparatively the best media

Table 2. Percentage of regeneration from somatic embryos of *Musa acuminata* cv. Mas (AA)

Treatment	Replicate	No. of clumps (2–3 mm)	No. of clumps forming shoot and root	Percentage of regeneration (%)	Min. of percentage regeneration (%)	Normal plant obtained	% of normal plant
T1	r1	20	–	–	1.00±0.09	1	100.00±0.00
(M4+0.2 μM BA)	r2	20	–	–			
	r3	20	–	–			
	r4	20	1	5			
	r5	20	–	–			
T2	r1	20	–	–	4.00±0.31	4	100.00±0.00
(M4+0.4 μM BA)	r2	20	1	5			
	r3	20	1	5			
	r4	20	–	–			
	r5	20	2	10			
T3	r1	20	2	10	13.00±0.58	12	92.00±0.02
(M4+0.8 μM BA)	r2	20	3	15			
	r3	20	2	10			
	r4	20	3	15			
	r5	20	3	15			
T4	r1	20	–	–	3.00±0.36	2	66.67±0.19
(MSO)	r2	20	1	5			
	r3	20	–	–			
	r4	20	2	10			
	r5	20	–	–			

% of regeneration – clumps giving shoots.

to obtain regeneration of somatic embryos was on M4 medium supplemented with 0.8 μM BA with 10–15% regeneration rate (Table 2). For plant regeneration, somatic embryos were transferred to dark conditions to induce adventitious buds. When adventitious bud emerged, the cultures were transferred to light condition to allow formation of chloroplast and development of shoots (Figure 1I). Normal plants were subsequently obtained on the same media after 3 weeks (Figure 1J) and rooted on MSO media supplemented with activated charcoal for complete plant formation (Figure 1K).

Conclusions

In this study, somatic embryos were successfully induced from male inflorescence of *Musa acuminata* cv. Mas (AA). A protocol for suspension cultures was successfully established and complete plant regeneration from the embryogenic cell suspension was also obtained.

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