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ORIGINAL PAPER

Plant regeneration from protoplasts of *Musa acuminata* cv. Mas (AA) via somatic embryogenesis

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Abstract A protocol for plant regeneration from protoplasts of *Musa acuminata* cv. Mas (AA) via somatic embryogenesis was developed. Viable protoplasts were isolated from embryogenic cell suspensions at a yield of 1.2×10^7 protoplasts/ml packed cell volume (PCV). Liquid and feeder layer culture systems with medium-A and medium-B were used for protoplast culture. In liquid culture system, medium-B was more efficient for inducing cell division (17.5% at 14 days) and colony formation (6.7% at 28 days) than medium-A. However, all protoplast-derived cell colonies (PDCC) obtained from liquid culture system could not develop further. In feeder layer culture system, there was no significant difference between medium-A and medium-B on cell division and colony formation of the cultured protoplasts, and the cell division frequency at 14 days and colony formation frequency at 28 days were 24.5% and 11.2%, respectively, in medium-B. Comparative study on the effects of BAP (2.2 μM, 4.4 μM, 8.8 μM), zeatin (0.4 μM, 0.8 μM, 1.2 μM) and TDZ (0.2 μM, 0.4 μM, 0.6 μM) on embryo formation of PDCC from feeder-layer culture indicated that TDZ was best. TDZ at 0.4 μM induced 7906 mature embryos per ml PCV PDCC, which was 4-fold the frequency as with BAP at 4.4 μM, 7.5-fold as with zeatin at 0.8 μM and 150-fold as control medium (no mentioned cytokinins) after 45 days on M3 medium. About 44% of the mature embryos were converted into plantlets with poor root system after subculture on M4 medium. Root further development of regenerated plantlets was promoted by addition of activated charcoal (AC) to MS basal medium.

Keywords *Musa acuminata* cv. Mas (AA) · Protoplast culture · Somatic embryogenesis · Cytokinin · Plant regeneration

Abbreviations

AC	Activated charcoal
BAP	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
ECS	Embryogenic cell suspensions
FDA	Fluorescein diacetate
Gln	Glutamine
IAA	Indolacetic-3-acid
MES	2-*N*-morpholino ethanesulfonic acid
NAA	1-naphthaleneacetic acid
PCV	Packed cell volume

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PDCC Protoplast-derived cell colonies
TDZ Thidiazuron

Introduction

Banana (*Musa* spp.) is the most important fruit crop in tropical and subtropical countries. The plants are currently being threatened by the spread of viral and fungal disease, which cause a significant reduction in yield and quality. Classical breeding methods have limited success due to the high sterility and triploidy of most cultivated bananas. Somatic hybridization through protoplast fusion to improve disease resistance has been applied with several crops, e.g. *Solanum tuberosum* L. (Fock et al. 2000) and *Oryza sativa* L. ssp. *japonica* (Yan et al. 2004), and could be used to develop new varieties of banana (Matsumoto et al. 2002; Assani et al. 2005). A number of studies on banana protoplast culture have been reported since Bakry (1984) reported the first successful isolation of viable banana protoplasts. It has been shown that besides cultivars the successful protoplast culture of banana and plantain would be dependent on cultural technologies such as application of feeder-layer (Panis et al. 1993; Assani et al. 2001), and the preferred donor material for protoplast isolation has been shown to be embryogenic cell suspensions (ECS) (Assani et al. 2002). The effects of factors such as protoplast isolation, plating density, culture method, nurse cells, medium composition etc. on cell division and microcolony formation, have already been evaluated by Panis et al. (1993) and Assani et al. (2001, 2006). Until now, the cultivars where successful plant regeneration from protoplasts was reported include cooking banana Bluggoe (*Musa* spp., ABB group) (Megia et al. 1993; Panis et al. 1993), Brazilian dessert banana (*Musa* spp., AAB group) (Matsumoto and Oka 1998), Grande Naine (*Musa* spp., Cavendish AAA group) (Assani et al. 2001; Assani et al. 2002), Gros Michel (*Musa* spp., Cavendish AAA group), Currare Enano and Dominico (*Musa* spp., Plantain AAB group) (Assani et al. 2002). However, successes with protoplast culture of banana have still been limited and plant regeneration from protoplast is not yet routine or efficient for the majority of banana cultivars. Thus, a study on the establishment of a reproducible regeneration protocol of protoplasts, applicable to various banana varieties, is required.

Musa acuminata cv. Mas (AA) is a popular commercial dessert variety planted in South East Asia. It is favored by consumers for its special sweet taste and thin peel, but this cultivar is highly susceptible to *Fusarium oxysporun f.* sp.*Cubense* race 4. It is possible to improve its resistance to this disease through protoplast culture and somatic hybridization. In previous study, we established the ECS of this cultivar from male inflorescence buds (Wei et al. 2005). To our knowledge, there are no reports on protoplast culture of dessert diploid banana though Megia et al. (1992) reported the protoplast sustained divisions leading to callus formation of wild diploid AA banana *Musa acuminata* ssp. *burmannica* cv long Tavoy without plantlet regeneration, and Assani et al. (2002, 2005) reported plants regeneration from protoplasts of cooking diploid AA banana SF265, Col49 and IRFA 903. The main objectives of this research were to establish a protocol for plant regeneration from protoplasts of this cultivar, and to study some factors which influence the efficiency of plant regeneration through somatic embryogenesis. In this study we report for the first time that TDZ showed a strong effect on promotion of embryo formation of the protoplast-derived cell colonies (PDCC), and we also showed that activated charcoal (AC) significantly stimulated the root development of regenerated plantlets.

Materials and methods

Plant materials and cultural media

The donor plant, *Musa acuminata* cv. Mas (AA), a popular commercial dessert variety planted in the South East Asian region, was kindly provided by the Pomology Fruit Research Institute of Guangdong Academy of Agricultural Sciences (China). All media used in this study were listed in Table 1.

Initiation and maintenance of embryogenic cell suspensions

ECS of *Musa acuminata* cv. Mas (AA), which composed of several cells to more than hundreds

Table 1 The culture media for protoplast culture used in this experiment

Medium	Basic medium ingredients	PGR	Carbohydrates
M1	MS + 4.1 μM biotin + 2 g l^{-1} gelrite	18 μM 2,4-D; 5.4 μM NAA; 5.7 μM IAA	87 mM sucrose
M2	MS + 100 mg l^{-1} malt extract + 680 μM Gln + 4.1 μM biotin	4.5 μM 2,4-D	130 mM sucrose
Medium -A	N_6 salts + KM vitamins, organic acids, sugar alcohols + Morel vitamins + 1.9 mM KH_2PO_4 + 0.5 mM MES	0.9 μM 2,4-D; 5.4 μM NAA; 2.3 μM zeatin	117 mM sucrose; 0.4 M glucose
Medium -B	MS + 100 mg l^{-1} malt extract + 680 μM Gln + 4.1 μM biotin + 0.5 mM MES	4.5 μM 2,4-D	117 mM sucrose; 0.4 M glucose
PCM	MS salts + Morel vitamins + 2.5 mM myo-inositol + 10% (v/v) nurse cells + 0.6% agarose	9 μM 2,4-D	2.8 mM glucose; 116 mM sucrose; 278 mM maltose
M3	MS + 100 mg l^{-1} malt extract + 680 μM Gln + 4.1 μM biotin + 2 g l^{-1} gelrite	2.3 μM IAA; different cytokinins	130 mM sucrose
M4	MS + 100 mg l^{-1} malt extract + 680 μM Gln + 4.1 μM biotin + 2 g l^{-1} gelrite	2.3 μM IAA; 2.2 μM BAP	130 mM sucrose
RM	MS + 0.1% AC + 2 g l^{-1} gelrite		87 mM sucrose

M1: (Escalant et al. 1994; Côte et al. 1996); M2: (Côte et al. 1996); MS: (Murashige and Skoog 1962); N_6: (Chu et al. 1975); KM: (Kao and Michayluk 1975); Morel: (Morel and Wetmore 1951); Medium-A: (Assani et al. 2001; Assani et al. 2006); PCM: (Assani et al. 2001); PGR: plant growth regulators

cells (Wei et al. 2005), were initiated from male inflorescence buds according to our previous protocol (Wei et al. 2005). Briefly, immature male flowers were excised and cultured on M1 medium (Table 1) (Escalant et al. 1994; Côte et al. 1996) for callus induction. After 5–6 months of culture, friable embryogenic calli were selected and subsequently cultured in liquid M2 medium (Table 1) (Côte et al. 1996) to initiate cell suspensions. After sieving selection of the cultures using a stainless mesh with pore sizes of 154 μm at 15 day-intervals for 3 months, homogeneous and yellow ECS were obtained. The stable ECS routinely used in this study were subcultured every 2 weeks at 1.5% (v/v) dilution in 100-ml Erlenmeyer flasks containing 30 ml of M2 medium on a rotary shaker (100 rpm) at 27°C in the dark.

Protoplast isolation

ECS after 3–5 days of subculture were used for protoplast isolation. The cells were sieved through a 154 μm stainless mesh to select the small cell aggregates. 2 ml packed cell volume (PCV) of this ECS were subsequently incubated in 10 ml protoplast enzyme solution containing 3.5% (w/v) cellulase R-10 (Yakult Pharma., Tokyo, Japan), 1% (w/v) macerozyme R-10 (Kinki Yakult, Mishino., Japan), 0.15% (w/v) pectolyase Y-23 (Seishin Pharma., Japan), 204 mM KCl and 67 mM $CaCl_2$ (pH 5.7). Enzymatic digestion was carried out on a gyratory shaker (30 rpm) at 27°C in the dark for 8–10 h.

Following digestion, the mixture was sieved through a 74/25 μm stainless mesh combination and then collected by centrifugation at 50*g* for 5 min. The pellet was resuspended in washing solution [204 mM KCl, 67 mM $CaCl_2$, 0.5 mM 2-*N*-morpholino ethane-sulfonic acid (MES) (Sigma) and 10% (w/v) mannitol, pH5.7] and centrifuged twice at 50*g* for 5 min each. Protoplast viability was determined by fluorescein diacetate (FDA) staining (Widholm 1972). Protoplast yield was estimated using a Nageotte hematocytometer immediately after purification, only viable protoplasts were counted. The presence or absence/regeneration of cell wall was examined after isolation and during culture with Calcofluor White under UV microscope (Nikon) (Nagata and Takebe 1970).

Protoplast culture

Protocol for plant regeneration from protoplast culture was shown in Fig. 1. We used two culture systems (liquid and feeder-layer culture) and two media (medium-A and medium-B) (Table 1) in each

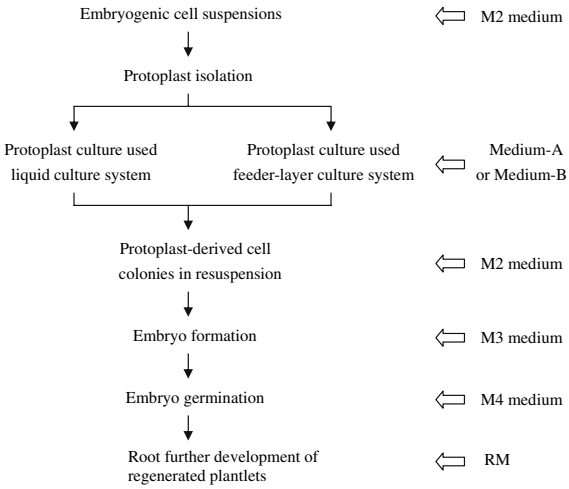

Fig. 1 Protocol for plant regeneration from protoplasts of *Musa acuminata* cv. Mas (AA) via somatic embryogenesis. The media used here were showed in Table 1

culture system to study the optimal protocol for protoplast culture. The pH of the media was adjusted to 5.7 with HCl/NaOH before filter-sterilization.

Feeder-layer culture system

Preparation of feeder-layer was based on the protocol reported by Assani et al. (2001) slightly modified. ECS of cv. Mas (AA) were used as nurse cells. The nurse cultures were prepared (1 day before protoplast isolation) as follows: (1) The ECS were initially sieved through a 154 μm stainless mesh in order to select the small cell aggregates. The feeder-layer medium (PCM) (Table 1) was sterilized by filtration. Nurse cell suspensions were mixed with 100 ml of double-concentrated PCM to obtain a final cell concentration of 10% (v/v). (2) Sea-plaque Agarose (1.2 g) (Sigma) was dissolved in 100 ml water and then autoclaved (pH 5.7); when the temperature of agarose solution decreased to 30–35°C, it was gently mixed with 100 ml double-concentrated PCM containing nurse cells. (3) Aliquots of 20 ml of this mixture were poured into Petri dishes (9.5 cm in diameter). After solidification, the medium was covered with a sterilized filter, and 1 ml of the protoplast suspension in medium-A or medium-B at density of 1×10^6 protoplasts/ml was transferred onto the filter. The Petri dishes were sealed with Parafilm.

Liquid culture system

Protoplasts were suspended in 2 ml of liquid "medium-A" or "medium-B" at a density of 1×10^6 protoplasts/ml and then transferred into small Petri dishes (5.5 cm in diameter), which were sealed with Parafilm.

All cultures were maintained at 27°C in the dark. Division frequency of cultured protoplasts was recorded on day 14 after culture and colony formation frequency on day 28.

Initiation of cell suspensions of protoplast-derived cell colonies

After 45 days of culture, cell colonies derived from feeder-layer and liquid culture system in medium-B were collected and resuspended in liquid M2 medium (Table 2). Aliquots of 0.3 ml PCV of those cell colonies were cultured in 50-ml Erlenmeyer flasks containing 10 ml liquid M2 medium. The cultures were kept on a gyratory shaker at 100 rpm at 27°C in the dark.

Somatic embryogenesis

After culture in liquid M2 medium for 1 month, 10% ml PCV of cell suspensions derived from liquid and feeder-layer culture system in medium-B were used for somatic embryogenesis. One milliliter of these cell suspensions was transferred into Petri

Table 2 Effects of culture methods on cell division and colony formation of cultured protoplasts of *Musa acuminata* cv. Mas (AA)

Culture systems	Culture media	Division frequency (%) after 14 days of culture	Colony formation frequency (%) after 28 days of culture
Feeder layer culture	Medium-A	23.7 ± 3.7 a	10.9 ± 0.9 a
	Medium-B	24.5 ± 2.0 a	11.2 ± 0.9 a
Liquid culture	Medium-A	8.8 ± 1.7 b	0.6 ± 0.1 b
	Medium-B	17.5 ± 1.9 a	6.7 ± 0.4 a

Data represent average ± S.E of three replicates. Means followed by the same letters are not significantly different by Duncan's multiple-range test ($P = 0.05$)

dishes (9.5 cm in diameter) with 20 ml of M3 medium (Table 1). The M3 medium without cytokinins (control) or supplemented with BAP (2.2 μM, 4.4 μM, 8.8 μM), zeatin (0.4 μM, 0.8 μM, 1.2 μM) or TDZ (0.2 μM, 0.4 μM, 0.6 μM) were used for studying the effects of cytokinins on the somatic embryogenesis. TDZ and zeatin were added by filter sterilization to the autoclaved medium. All cultures were maintained at 27°C in the dark, and subcultured every 15–20 days. The frequency of embryo formation was calculated 45 days after plating of PDCC on M3 medium. The bipolar embryos (mature embryos) were counted under dissecting microscope and the frequency of embryo formation was recorded as number of mature embryos per ml PCV PDCC.

Mature bipolar embryos were picked out from M3 medium with 0.4 μM TDZ and subcultured every 15–20 days on M4 medium (Table 1) for germination until embryos developed into plantlets with shoot of 2–3 cm long. Such regenerated plantlets were transferred onto rooting medium (RM) (Table 1) which consisted of basal MS medium with or without 0.1% AC to promote root further development. All cultures were maintained at 27°C under 16 h photoperiod (65 μmol m^{-2} s^{-1}). The conversion frequency of embryos into plantlets was calculated 30 days after subculturing of mature embryos on M4 medium and was recorded as the number of plantlets/mature embryos initially incubated × 100%. Rooting responses were recorded 25 days after transferring regenerated plantlets in RM, including the number of roots (roots longer than 1 cm), the average length of roots and height of plantlets.

Statistical analysis

All experiments were repeated at least three times. The data collected was analyzed using Duncan's multiple range test. Least significant differences were calculated at the 5% level of probability.

Results

Protoplast isolation

Protoplasts were released from ECS after 8–10 h of incubation in enzyme solution. Protoplast yield was 1.2×10^7 protoplasts/ml PCV ECS, and they showed over 85%-90% viability as assessed with FDA staining. The freshly isolated protoplasts were rich in cytoplasm and relatively uniform in size (Fig. 2a). These protoplasts showed no fluorescence after staining with Calcoflour White (Fig. 2a, 2b).

Protoplast culture

Cell division and colony formation in feeder-layer culture system

Protoplasts cultured on feeder-layer in medium-A and medium-B gave rise to no significant difference with regard to cell division and colony formation (Table 2). Most protoplasts in either media became large and oval in shape 2 days after plating on feeder-layer (Fig. 2c-2, d-2, e-1). Fluorescence of protoplasts within 48 h of culture, using the fluorescent dye Calcofluor White, indicated cell wall regeneration (Fig. 2d-2). First divisions of protoplasts were observed at 5 days (Fig. 2e-2). After 25–30 days of culture, cell colonies were visible by the naked eyes (Fig. 2g). Microscopic observations showed that cells in the colonies were tightly packed and with a dense cytoplasm, suggesting that they were embryogenic (Fig. 2g). Cell division frequency at 14 days of culture on medium-A and medium-B was 23.7% and 24.5% respectively and colony formation frequency at 28 days of culture on medium-A and medium-B was10.9% and 11.2% respectively (Table 2).

Cell division and colony formation in liquid culture system

In general, protoplasts became elliptical and started to divide by the fifth day of culture. Cell division and colony formation were affected by medium composition. Medium-B was found to be more efficient for inducing cell divisions and colony formation than medium-A (Table 2).

In comparison with feeder-layer culture system, it was worth noting that all cell colonies derived from liquid culture system either in medium A or B were composed of large, highly vacuolated cells that appeared to be non-embryogenic (Fig. 2f), growth of those colonies was stopped at stage of 6 to 10-cells.

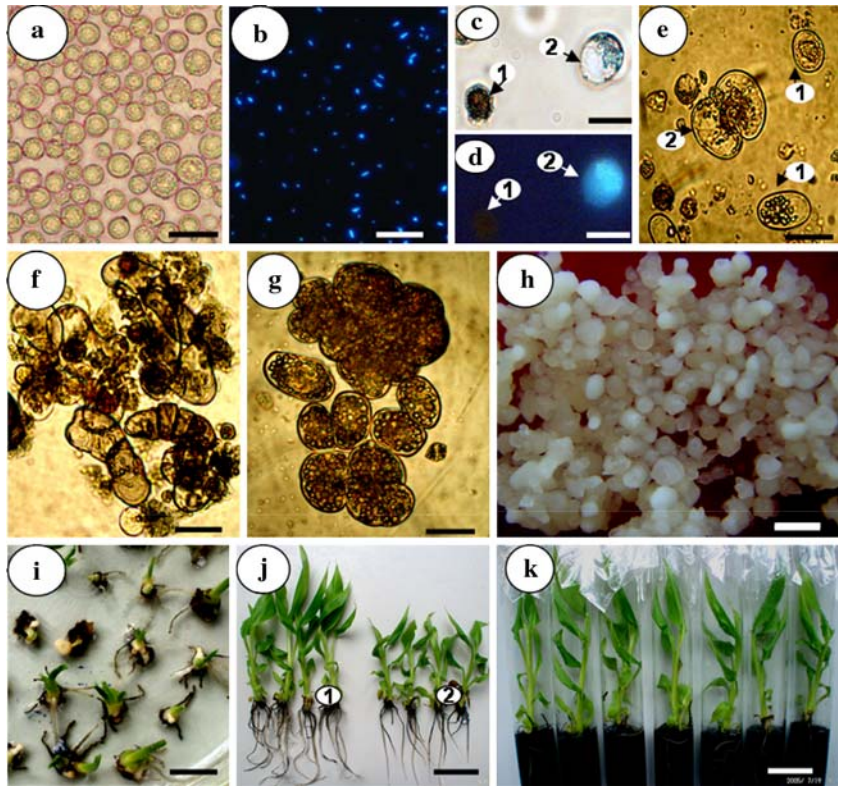

Fig. 2 Plant regeneration from protoplasts of *Musa acuminata* cv. Mas (AA) via somatic embryogenesis (**a**) Freshly isolated protoplasts, bar 50 μm. (**b**) Freshly isolated protoplasts stained with Calcofluor White showed no fluorescence, cell wall debris showed fluorescence, bar 50 μm. (**c**) Arrow 1: dead protoplast; Arrow 2: elongated protoplast after 2 d of culture; bar 50 μm. (**d**) Arrow 1: dead protoplast stained with Calcofluor White showed no fluorescence; Arrow 2: elongated protoplast stained with Calcofluor White showed fluorescence; bar 50 μm. (**e**) Protoplast became large and oval in shape (arrow 1) and the first division of the protoplasts (arrow 2) in feeder-layer culture system, bar 50 μm. (**f**) Non-embryogenic cell colonies derived from liquid culture system 20 d after incubated in medium-B, bar 50 μm. (**g**) Embryogenic cell colonies derived from feeder-layer culture system 20 d after incubated in medium-B, bar 50 μm. (**h**) Cultures containing somatic embryos at different development stages, bar 4 mm. (**i**) Germinating embryos, bar 2 cm. (**j**) 1: Plantlets regenerated on MS medium with activated charcoal; 2: Plantlets regenerated on MS medium; bar 5 cm. (**k**) Plantlets regenerated via somatic embryogenesis from protoplasts by feeder-layer culture; bar 4 cm

Re-initiation of cell suspensions from protoplast-derived cell colonies

Cell colonies derived from feeder-layer and liquid culture systems in medium-B were transferred into M2 liquid medium at a concentration of 3% (v/v). Following 1–2 months of culture, embryogenic and stable cell suspensions were established from PDCC derived from feeder-layer culture system. Morphological studies of these new suspension cultures showed no significant difference from the cv. Mas (AA) original ECS. PDCC derived from liquid culture in medium-B could remain alive in liquid M2 medium for more than 2 months, but the growth of those colonies was very slow and they showed non-embryogenic characteristics.

Effects of BAP, zeatin and TDZ on the formation of somatic embryo

After suspension in liquid M2 medium for 1 month, PDCC from both liquid and feeder-layer culture system in medium-B were transferred onto M3 medium with different concentrations of BAP, zeatin and TDZ for somatic embryogenesis. The M3 medium without cytokinin was used as control. PDCC derived from liquid culture in medium-B died after 10–15 days of culture, while PDCC derived

from feeder-layer culture system in medium-B were increased in size, turned pale yellow after 7 days of culture and developed into mature bipolar embryo after 30 days of culture (Fig. 2h). Somatic embryos were formed within 1 month in all treatments with different frequencies. The frequency of embryo formation increased with BAP, zeatin and TDZ in the M3 medium. BAP at 4.4 μM, zeatin at 0.8 μM and TDZ at 0.4 μM were most effective compared to other concentrations tested (Table 3). While only 70 somatic embryos were obtained from 1 ml PCV PDCC in control medium, a maximum frequency of 7906 embryos/ml PCV PDCC was obtained with 0.4 μM TDZ, which was about 4-fold the frequency as with the best BAP concentration and 7.5-fold that with the best zeatin content and 150-fold the frequency as observed in control medium.

Plant regeneration

Mature bipolar embryos (Fig. 2h) derived from M3 medium with 0.4 μM TDZ were transferred onto M4 medium for germination, 44% of mature embryos were germinated after 30 days of culture (Fig. 2i). Poor root formation was observed from the base of plantlets regenerated in M4 medium. The regenerated plantlets with 2–3 cm shoots and poor root system were then transferred on RM for root further development. Addition of AC to the RM significantly promoted development of the root and shoot, and increased root number (9.5), root length (5.4 cm) and the height of plants (11.2 cm) compared to those cultured in control medium (5.7, 3.6 cm and 7.7 cm) (Table 4). The regenerated plants on MS medium with AC were more vigorous (Fig. 2j-1) than those on control MS medium (Fig. 2j-2) and they were easier to acclimatize.

Table 3 Effects of BAP, zeatin and TDZ on embryo formation of protoplast-derived cell colonies (PDCC)

PGR (μM)	No. embryos per ml PCV PDCC
Control	70 ± 12
Zeatin	
0.4	673 ± 53 b
0.8	1061 ± 99 a
1.2	922 ± 97 a
BAP	
2.2	813 ± 45 c
4.4	2020 ± 97 a
8.8	1281 ± 79 b
TDZ	
0.2	6589 ± 204 b
0.4	7906 ± 188 a
0.6	3864 ± 167 c

Results were obtained in three independent experiments, each with four replicates. Data represent average ± S.E. Data followed by the same letters are not significantly different according to Duncan's multiple range test at $P = 0.05$ (PGR: plant growth regulators)

Discussion

Effects of culture systems and media on cell division and colony formation from protoplasts

In this study we reported an efficient plant regeneration protocol from cell suspension-derived protoplasts of the dessert banana cv. Mas (AA) via somatic embryogenesis. We also showed that protoplasts of cv. Mas (AA) could divide and form cell colonies in liquid culture system (Table 2, Fig. 2g), contrasting previous reports that protoplasts of banana could not divide and form cell colonies when cultured in liquid medium (Panis et al. 1993; Assani et al. 2001). However, the cell colonies derived from liquid culture system did not develop further, while those derived from feeder-layer culture system could develop through a somatic embryogenesis pathway. It has been shown that the feeder-layer technique is always required for the protoplast culture of banana (Assani et al. 2002). One function of nurse cells, ECS of cv. Mas (AA), used in this study might be to secrete some soluble signal molecules that could

Table 4 Effects of AC on root development of regenerated plantlets

Medium	Number of roots*	Average length of roots*	Plant height (cm) *
MS	5.7 ± 1.5 b	3.6 ± 0.9 b	7.7 ± 1.1 b
MS + 0.1% AC	9.5 ± 1.7 a	5.4 ± 0.7 a	11.3 ± 1.5 a

Data represent means ± S.E of three independent experiments. Means followed by the same letters are not significantly different by Duncan's multiple-range test ($P = 0.05$) (*per regenerated plantlet)

stimulate the cell colonies derived from the protoplasts to become embryogenic cultures. ECS have been reported to secrete signal molecules such as endochitinase (De Jong et al. 1992; Egertsdotter and von Arnold 1998), and arabinogalactan proteins (AGPs) (Thompson and Knox 1998; Chapman et al. 2000), which were necessary for the development of somatic embryogenesis of *Daucus carota, Picea abies* and *Cichorium* hybrid '474'. It also has been showed that addition of carrot-seed AGPs to non-embryogenic cell suspensions of carrot resulted in the re-induction of embryogenic potential (Kreuger and van Holst 1993). The cell colonies derived from protoplasts of banana in liquid culture system were non-embryogenic and did not develop further, this problem might be resulted in no such signal molecules from nurse cells during liquid culture.

Our results showed that M2 liquid medium used for donor ECS material of cv. Mas (AA), with glucose as osmotic regulator, was suitable for protoplast culture. In this modified M2 medium, named medium-B, cell colonies were obtained from both liquid and feeder-layer culture systems. Similar results have been reported in various plant species, e.g. *Vitis vinifera* L. (Zhu et al. 1997) and *Triticum aestivum* (Xia et al. 1995). This could be explained by an easier adaptation of protoplasts to the original medium used for the donor tissues. In addition, in our studies, compared to medium-A, medium-B was cheaper and easier to manipulate.

Effects of BAP, zeatin and TDZ on somatic embryo formation of PDCC

It have indicated that embryo formation of many plant species required a high cytokinin supplemented medium, e.g. *Coffea canephora* (Hatanaka et al. 1991), *Coronilla varia* (Moyer and Gustine 1984), *Medicago truncatula* (Nolan et al. 1989), *Thevetia peruviana* (Kumar 1992) and *Eleusina caracana* (Eapen and George 1989). It also has been shown that cytokinin was essential for embryo formation of banana ECS (Nova et al. 1989; Dhedà et al. 1991; Ma 1991; Côte et al. 1996; Grapin et al. 1996). In protoplast culture of banana, BAP was also commonly used for somatic embryogenesis (Megia et al. 1993; Assani et al. 2001), but the conversion rate of microcalli or PDCC into somatic embryos was very low (about 2%). In this study, we found for the first time that TDZ at 0.4 μM was most effective and gave the highest frequency of embryo formation. Various reports have indicated that TDZ may act through a modulation of endogenous plant growth regulators (Visser et al. 1992; Hutchinson and Saxena 1996), either directly or as a result of induced stress (Murch and Saxena 1997). But the exact mechanism of TDZ to promote embryo formation of the PDCC needs to be investigated further.

Effects of AC on root development

Addition AC to the medium for root development is a common practice (Gould et al. 1991; Rasai et al. 1994; Ouma et al. 2004), but the exact mechanism for the promoting effects of AC on root development is still unknown. In this study, when the regenerated plantlets were cultured on MS medium with AC, the roots were more vigorous and more lateral roots were developed (Table 4, Fig. 2-j-1). Sanchez and San-Jose (1996) suggested that the stimulatory effects of AC on root development may involve: (1) the reduction of light intensity at the base of shoots, providing an environment conducive to the accumulation of auxin or cofactors, or both; and (2) the absorption of substances such as inhibitory phenolics and any excess auxin or cytokinin carried over from previous media. These effects may be true of our results.

The next stage of our investigation will be the extension of this protocol to produce somatic hybrids to generate novel genotypes between two *Musa* species.

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