Enhancement of banana plant regeneration by incorporating a liquid-based embryo development medium for embryogenic cell suspension

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SUMMARY
Recovering plants from embryogenic cell suspensions is a complex process starting with somatic embryo development which is synchronised on medium lacking plant growth regulators, followed by differentiation and regeneration of plants. An improved method for high frequency recovery of banana plants was investigated by incorporating a liquid-based, embryo-development medium. The highest regeneration rate (approx. 32,000 plants m⁻¹ settled cell volume) was obtained using this liquid protocol. This is one of the highest scores recorded among published data on plant recovery in *Musa* spp. A detailed, comparative histological study has been carried out on somatic embryos developed in semi-solid or liquid-based media. Most mature embryos developed from the liquid protocol enabled differentiation and regeneration within 4 months. Over 3,000 plants are currently under field observation to establish the rate of somaclonal variation.

Over the last few years, several embryogenic banana (*Musa* spp.) suspension culture protocols have been developed. This biotechnique is potentially useful to overcome the difficulties faced in conventional breeding, which is limited due to the high sterility and polyploidy of edible banana varieties. It also provides a high performance micropropagation technique, as well as a single-cell regeneration system that is useful for genetic transformation and somatic hybridisation. However, current protocols for somatic embryogenesis in banana are limited by low embryo development and plant recovery frequencies (Novak et al., 1989; Dheida et al., 1991; Cote et al., 1996; Becker et al., 2000; Ganapathi et al., 2001; Jalil et al., 2003). Most studies on banana somatic embryogenesis systems require high concentrations of auxin, especially 2,4-dichlorophenoxyacetic acid (2,4-D), to induce embryogenic calli, followed by transfer to a liquid proliferation medium (Ganapathi et al., 2001; Jalil et al., 2002; Jalil et al., 2003). The continued presence of 2,4-D in the culture medium causes the disruption of formerly adhering cells, when the differentiated embryogenic calli form pro-embryogenic masses (Georget et al., 2000; Arnold et al., 2002). After several subculturings in liquid proliferation medium, the pro-embryogenic masses will multiply continuously without the appearance of somatic embryos. When embryogenic cells are transferred to auxin-free medium, differentiation of the protoderm occurs, followed by a period of embryo development (Arnold et al., 2002; Feher et al., 2003). However, transferring embryogenic cells from liquid proliferation medium to embryo development medium will incorporate some intracellular 2,4-D into this auxin-free medium. This then prolongs the period of embryo development or requires frequent sub-culturing to reduce intracellular auxin levels as well as those of some inhibitory factors. A simple procedure to synchronise embryo development could be achieved by rinsing the embryogenic cell fraction before transfer to auxin-free medium. The aim of the present work was to improve plant regeneration of embryogenic cell suspensions of cultivar ‘Mas’ (AA) by incorporating a liquid-based embryo-development protocol. The method described in this work is simple and efficient, compared to published data.

MATERIALS AND METHODS
Plant source and culture conditions
Cell suspensions of *Musa acuminate* cv. ‘Mas’ (AA) were established from embryogenic calli derived from immature male flower clusters cultured for 6 months according to the method developed by Jalil et al. (2003). Embryogenic cell suspension cultures were sub-cultured every 2 weeks in M2 medium containing Murashige and Skoog (MS; Murashige and Skoog, 1962) macronutrients and micronutrients supplemented with 0.4 mg l⁻¹ thiamine, 0.5 mg l⁻¹ nicotinic acid, 0.5 mg l⁻¹ pyridoxine, 100 mg l⁻¹ myo-inositol, 10 mg l⁻¹ ascorbic acid, 1.1 mg l⁻¹ 2,4-D (Sigma Chemical Co., MO, USA), 250 µg l⁻¹ transferrin (Sigma) and 20 g l⁻¹ sucrose. The pH was adjusted to 5.7 prior to autoclaving. Throughout the experiment, approx. 2 ml settled cell volume (SCV) of cell aggregates was inoculated into 50 ml M2 medium in a 250 ml Erlenmeyer flask. The cultures were maintained on an
orbital shaker at 70 rpm, at 25° ± 1°C, with a 16 h photoperiod at a light intensity of 31.4 μmol s⁻¹ m⁻².

Comparison of embryo development media

In this work, the use of solidified embryo development media M3 (Côte et al., 1996) and MSO (Murashige and Skoog, 1962), and liquid-based M3 and MSO were compared. The liquid-based medium was formulated by removing the solidifying agent from M3 and MSO (Table I). Suspension cells in M2 medium were sieved through a 450 μm mesh and the filtrate was left to settle in a 50 ml Falcon tube followed by adjusting the SCV to approx. 1.5 (SCV/liquid M2 medium). Cell aggregates were then resuspended and dispersed as 200 μl aliquots into each 20 ml of liquid culture medium. The cultures were again maintained on an orbital shaker at 70 rpm, at 25° ± 1°C, with a 16 h photoperiod at a light intensity of 31.4 μmol s⁻¹ m⁻². For the solid M3 and MSO media, suspension cells were dispersed onto 70 mm-diameter Whatman No.1 filter papers placed on the surface of the medium. Cultures were placed in the dark until formation of somatic embryos was observed.

Differentiation and regeneration of embryos

Various concentrations of 6-benzyladenine (6-BA) were added to the differentiation medium containing half-strength MS macronutrients and micronutrients, supplemented with 1.0 mg l⁻¹ thiamine, 1.0 mg l⁻¹ nicotinic acid, 10 mg l⁻¹ pyridoxine, 100 mg l⁻¹ myo-inositol, 30 g l⁻¹ sucrose and 2.6 g l⁻¹ Phytagel™, pH 5.8. The differentiated embryos were transferred to regeneration media containing full-strength MS basal medium supplemented with similar concentrations of 6-BA for plantlet development. The concentrations of 6-BA used were 0, 1.0, 3.0, 5.0 or 7.0 mg l⁻¹.

Histological studies

For histological studies, samples were fixed for 24 h in formaldehyde-absolute ethanol-acetic acid (FAA) solution [5% (v/v) formaldehyde; 45% (v/v) absolute ethanol; 5% (v/v) glacial acetic acid] and dehydrated in a series of ethanol solutions: 30% for 30 min; 50% for 30 min; 70% for 45 min; 80% for 60 min; 90% for 60 min; 95% for 60 min, and twice in absolute ethanol for 60 min each. After infiltration, specimens were embedded in basic resin (Leica Historesin Embedding Kit; Leica Instruments Nussloch GmbH, Heidelberg, Germany) and cut into 3.5 μm-thick sections. Cell walls and storage proteins were stained with periodic acid-Schiff reagent and naphthol blue black, respectively. Sections were mounted with Cytoseal™ 60 mounting medium (Richard-Allan Scientific, Kalamazoo, MI, USA) on a coverslip and kept in the dark. Photographs were taken under a contrast phase light microscope (Zeiss, Göttingen, Germany) fitted with a Nikon digital camera (Model COOLPIX995; Nikon, Tokyo, Japan).

Acclimatisation of plantlets

Regenerated plantlets were rooted in MSO medium supplemented with 1% (w/v) activated charcoal, 30 g l⁻¹ sucrose and 1.8 g l⁻¹ Phytagel™ at pH 5.8. Rooted plantlets (6-8 cm-long) were obtained after 4–5 weeks and transferred to a small tray containing a peat-soil mix (3:7). The plantlets were acclimatised for 1 month in a netted greenhouse under natural light, without temperature control.

<table>
<thead>
<tr>
<th>Embryo development medium</th>
<th>Conc. of 6-BA in differentiation medium (mg l⁻¹)</th>
<th>Average No. of mature embryos m⁻¹ SCV</th>
<th>No. of recovered plantlets m⁻¹ SCV</th>
<th>Culture duration (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3</td>
<td>0.0</td>
<td>35</td>
<td>0</td>
<td>6 - 8</td>
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<tr>
<td></td>
<td>1.0</td>
<td>45</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>375</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>940</td>
<td>615</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>2,535</td>
<td>1,735</td>
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</tr>
<tr>
<td>M3L</td>
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</tr>
<tr>
<td></td>
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<td>5</td>
<td></td>
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<td></td>
<td>3.0</td>
<td>375</td>
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<td></td>
<td>7.0</td>
<td>2,535</td>
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<tr>
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<td>0</td>
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<tr>
<td></td>
<td>7.0</td>
<td>1,275</td>
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<tr>
<td>MSOL</td>
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<td>3 - 4</td>
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<tr>
<td></td>
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<td>42,835</td>
<td>31,835</td>
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</table>

Cells were necrotic after 1 week of culture.
RESULTS AND DISCUSSION

The effects of liquid and solid-based M3 and MSO media on embryo development and regeneration of plantlets were compared in this study (Table II; Figures 1–4). The highest rate of embryo development was achieved using liquid MS medium (MSOL). Cultures on MSOL produced the highest number of mature embryos on differentiation medium supplemented with 7 mg l⁻¹ 6-BA (Figures 5–8). There were approx. 17-fold and 34-fold increases in the number of embryos obtained on MSOL compared to solidified M3 and MSO media, respectively, when supplemented with 7 mg l⁻¹ 6-BA.

No somatic embryos developed in liquid-based M3 medium (M3L). Most cell aggregates became necrotic after 1 week of culture in M3L, which might have been due to the combined effects of shaking and various plant growth regulators [1-naphthalene acetic acid (NAA), zeatin, kinetin and N⁶-2-isopentenyladenine (2iP)] added to the M3L medium. Dhed’a et al. (1991) suggested that shaking liquid cultures without plant growth regulators could promote the formation of somatic embryos. However, prolonged shaking of suspension cultures could also lead to the breakdown of pro-embryos in the liquid culture medium. Inhibition of embryo

![Image](https://example.com/figure1-8.png)

**Fig. 1–8**

Plant regeneration from embryogenic cell suspensions of banana cv. ‘Mas’ (Musa spp. AA group). Fig. 1: Compact callus with translucent embryo (scale bar = 1 mm). Fig. 2: Proliferating embryogenic cell aggregates (scale bar = 200 µm). Fig. 3: Globular-shaped somatic embryo obtained from MSOL medium after 6 weeks of culture (scale bar = 1 mm). Fig. 4: Globular-shaped somatic embryo obtained from M3 medium after 2 months of culture (scale bar = 500 µm). Fig. 5: Mature embryo. LE, leaf primordia; RI, root initial (scale bar = 5 mm). Fig. 6: Germinated mature embryo on differentiation medium supplemented with 7.0 mg l⁻¹ 6-BA. SD, shoot development; RD, root development (scale bar = 3 mm). Fig. 7: Banana plantlets regenerated from somatic embryos on regeneration medium supplemented with 7.0 mg l⁻¹ 6-BA (scale bar = 1 cm). Fig. 8: Banana plantlets developed from embryogenic cell suspensions were successfully acclimatised (scale bar = 15 cm).
Comparative histological studies of somatic embryos of banana 'Musa' developed on semi-solid (M3) and liquid-based (MSOL) media. Fig. 9: Aggregates from suspension cell were observed in M2 maintaining medium (Dheda et al., 1991). Cell walls and storage proteins were stained with periodic acid Schiff (red colour) and napthol blue-black (blue-black colour), respectively (scale bar = 60 µm). W, wall; NU, nucelolus; N, nucleus. Fig. 10: Oblong embryos developed in MSOL medium (scale bar = 100 µm). Fig. 11: Elongated embryos, with pro-cambial strands (PS) present in the central core, were developed in MSOL medium (scale bar = 100 µm). Fig. 12: The protocorm (P) of a somatic embryo in M3 medium was not well-developed and consisted of highly vacuolated cells (scale bar = 100 µm). Fig. 13: Highly vacuolated cells containing visible medas were observed in an oblong embryo on M3 medium (scale bar = 75 µm). Fig. 14: Most of the highly vacuolated cells (VC) present in somatic embryos developed from M3 medium (scale bar = 100 µm); GE, globular embryo.
development occurred when the breakdown of the protoderm allowed direct contact between cortical cells and plant growth regulators added to the culture medium (Georget et al., 2000). Differences in the results between solid and liquid media could be due to the higher cell density in the former, which might inhibit the formation of somatic embryos.

Work on banana cell suspensions showed that shaking liquid medium leads to a disorganisation of cell structure which could hinder the regeneration of cells (Georget et al., 2000). However, our study showed that cell suspensions in MSOL medium without plant growth regulators and subjected to continuous low shaking (70 rpm) appeared to enhance cell growth. Therefore, we suggest that a low shaking speed (70 rpm) could facilitate synchronisation of banana embryo development processes (Figure 3). In addition, the use of liquid culture medium could overcome problems associated with high cell-density cultures, which usually inhibit the formation of somatic embryos (Figure 4).

When comparing media composition between M3 and MSOL, the use of various types of cytokinins in M3 appeared to affect the conversion of pro-embryonic masses into somatic embryos. Our results showed that incorporation of cytokinins during earlier embryo development stages suppressed the formation of embryos. However, Dheda et al. (1991) showed a contrasting effect of cytokinin on embryo formation. The exact role of cytokinins during the different stages of somatic embryogenesis was not demonstrated in this study.

The effect of 6-BA concentration on the differentiation and regeneration of embryos was shown in this study. The highest percentages of mature embryos (Figures 5–6), and regenerable somatic embryos, were obtained in differentiation (half-strength MS) and regeneration (full-strength MS) media supplemented with 7 mg l⁻¹ 6-BA (Figure 7). The high concentration of 6-BA used in this study produced approx. 32,000 regenerable somatic embryos ml⁻¹ SCV. However, development of plantlets on higher 6-BA concentrations (≥ 7 mg l⁻¹) could lead to the induction of somacidal variation. To assess this possibility, over 3,000 suspension-derived banana plantlets are currently under field observation for somaclonal variation.

Histological analyses of somatic embryo development (Figures 9–14) showed various differences between embryos developed on MSOL medium and those established on M3 medium (Coët et al., 1996). The initial cell aggregates, maintained in M2 medium were characterised by cells with a dense cytoplasm, small vacuoles and rich in protein (Figure 9). Histological observations indicated an earlier accumulation of storage starch, and well-defined pro-cambial strands in somatic embryos obtained from MSOL medium (Figures 10 and 11). Most elongated somatic embryos (Figures 5–6; 10, 11) were successfully regenerated into complete plants.

On the other hand, somatic embryos developed on solid M3 medium were composed of highly vacuolated cells with rarely visible nuclei (Figures 12–14). Less embryo development on solid medium (Table II), compared to liquid medium, was expected due to these highly vacuolated peripheral cells.

To conclude, this study has shown that liquid-based MSOL medium was superior to the solid M3 medium commonly used to develop somatic embryos of Musa in terms of growth rate. Greater dispersion of phenolics and other tissue by-products may have overcome the inhibition of growth on solid media. The histological features of our somatic embryos have been described in detail, but the molecular mechanisms that control embryo differentiation are not yet understood and need to be studied further to gain a better understanding of somatic embryo induction and development in banana.

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