Banana Protoplasts: Culture and its Applications

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ABSTRACT

Protoplast technology is an important adjunct to conventional plant breeding. The culture of banana protoplasts has advanced considerably in recent years through the use of embryogenic cell suspensions as a source of protoplasts, combined with nurse cultures. Protoplast transformation and somatic hybridization have also become feasible techniques to support the genetic improvement of banana. The present discussion reviews the advantages and disadvantages of the use of protoplasts compared with other methods of genetic transformation and conventional sexual hybridization. General protocols for protoplast culture, genetic transformation and somatic hybridization are presented.

Keywords: Musa, nurse culture, somatic hybridization, transformation

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INTRODUCTION

Bananas (Musa spp.), including plantain bananas, are one of the most important horticultural crops in tropical and subtropical countries. They also constitute the staple food of some 400 million persons living in developing countries. Banana is a perennial crop that provides more stability to daily living than the annual staple crops such as rice, wheat and corn (maize). However, as banana plants are more vulnerable to attack by pests and diseases, the best option to control these problems is by genetic manipulation through the generation of resistant varieties. The development of pest and disease resistant plants in Musa remains difficult by conventional breeding approaches because of the long generation time, sterility, and triploidy of most cultivated varieties. Non-conventional methods, such as genetic transformation, induced mutation and the selection of somaclonal variants, have been developed by several workers (Tripathi 2003; Arinaite et al. 2004; Chai et al. 2004; Roux 2004; Xu et al. 2006; Tripathi et al. 2008a; Roux et al. 2009), based on tissue culture approaches (Côté et al. 1996; Kosky et al. 2002; Matsumoto and Silva Neto 2003; Strosse et al. 2003, 2004). Indeed, since in vitro techniques have been established for banana, different source tissues such as corn slices, shoot tips, buds, callus, cultured cells and isolated protoplasts, have been exploited for genetic transformation (Hernández et al. 1999; Sági et al. 2000; Tripathi 2003; Pei et al. 2005; Tripathi et al. 2005, 2008b; Sree-ramanan et al. 2005, 2006; Arvanitoyannis et al. 2008; Ghosh et al. 2009).

Protoplasts are naked plant cells whose walls have been removed either mechanically or by enzymatic digestion. Isolated protoplasts are capable of incorporating foreign materials, such as DNA, or can be induced to fuse to transfer genetic material. This phenomenon has attracted scientists involved in gene transfer by transformation and somatic hybridization, and those plant physiologists investigating cell physiology and membrane behavior. During the 1980s and the first half of the 1990s, many scientific papers were published describing the use of isolated protoplasts to generate transgenic plants and somatic hybrids (Davey et al. 2005a, 2005b, 2005c). However, the focus on gene transfer by protoplast-based approaches declined since plant regeneration from isolated protoplasts was, and remains, extremely difficult in many species. Only relatively few species in which culture techniques were well established, such as tobacco, potato, citrus, Brassica and rice, were subjected to protoplast-based techniques (Davey et al. 2005a). In other species, protoplast technologies were superseded by other transformation technologies, and banana was no ex-
exceptions. After demonstration of the possibility of transgenic plant production by biolistic and Agrobacterium-mediated transformation (May et al. 1995; Sagi et al. 1995a), most procedures involving protoplast culture were abandoned. However, recent advances in protoplast culture, combined with marker gene technology, have stimulated a revival in protoplast-based techniques (Davey et al. 2005a, 2005b, 2005c). Haićourt et al. (2004) reviewed the isolation and culture of banana protoplasts. The present paper provides a brief history of the culture of banana protoplasts, and discusses its possibility in contributing to the genetic improvement of banana with focus on genetic transformation and somatic hybridization.

**ISOLATION AND CULTURE OF BANANA PROTOPLASTS**

Biotechnological approaches have been exploited for banana since the early 1970s through in vitro propagation and embryo rescue. The initial attempt at banana protoplast isolation was reported by Bakry (1984), but almost 10 years elapsed before the establishment of a protoplast-to-plant system (Table 1). The culture of banana protoplasts was successful only when suspension cells were used for protoplast isolation. Some of the authors listed in Table 1 had already attempted to culture protoplasts from leaves or other tissues, but were unsuccessful in producing calli or regenerating shoots. Another important observation was the use of nurse (feeder) cells for protoplast culture. Panis et al. (1993) and Megia et al. (1993) reported simultaneously nurse culture techniques for protoplasts of banana with an ABB genome. They also showed that without nurse cells, protoplast densities exceeding 1.0 × 10^6 and 5.0 × 10^5/mL, respectively, were essential to sustain cell division. These critical densities might, however, be underestimated due to the excellent conditions of their embryogenic cells and the response to culture of the banana variety. Based on experiments by the present authors on cultivars of banana with the AAB and AAA genomes, a protoplast density of 2.0 × 10^5/mL was required in the absence of nurse cells, while a density of 1.0 × 10^5/mL was sufficient to culture protoplasts and to regenerate shoots from protoplast-derived tissues when the nurse culture technique was exploited (Fig. 1).

Protoplast isolation and culture in banana is now feasible. Using actively-dividing and fine embryogenic cell suspensions, protoplasts can be isolated in a enzyme solution consisting of 1.5–2% Cellulase RS and 0.15–0.2% Pectolyase Y23. When culture is associated with nurse cells it is possible to maintain isolated protoplasts at a low density of 1 to 5 × 10^5/mL and to regenerate intact plants. Any treatment associated with genetic transformation and somatic hybridization generally causes death of some protoplasts which reduces the number of viable protoplasts. Consequently, the use of the nurse culture technique, combined with high quality banana suspension cells, is essential to generate genetically transformed plants and/or somatic hybrids (Matsumoto et al. 2002; Assani et al. 2005).

**DIRECT GENE TRANSFER FOR PROTOPLAST TRANSFORMATION**

Protoplast transformation mediated by treatment with polyethylene glycol (PEG), electroporation, or a combination of these two treatments, has been used to induce DNA uptake and to generate transiently and stably transformed cell lines. Protoplast transformation has an advantage over both Agrobacterium- and biolistic-mediated gene transfer to tissue explants in that protoplast regeneration is a single cell event. Thus, plants regenerated from transformed protoplasts are not chimeric. The major disadvantage of using protoplasts for genetic transformation is that shoot regeneration from protoplasts of many plants remains extremely difficult and

### Table 1 Some reports of banana protoplast isolation and culture

<table>
<thead>
<tr>
<th>Type of explants</th>
<th>Variety of Musa</th>
<th>Enzyme composition for isolation</th>
<th>Culture medium</th>
<th>Observation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf and callus</td>
<td>Various (AA; BB; AAA; AAB; ABB)</td>
<td>2.5% Cellulase R10, 0.2% Hemilcellulase (Sigma), 0.3% Pectolyase Y23, 0.6% Macerozyme</td>
<td>Not cultured</td>
<td>Protoplasts isolated only from callus</td>
<td>Bakry 1984</td>
</tr>
<tr>
<td>Youngest leaf</td>
<td>Cavendish (AAA); acuminata (AA)</td>
<td>0.5% Cellulysin, 0.5% Rhozyme HR-150, 0.125% Pectolyase Y23</td>
<td>½MS, 0.1 or 2 mg/L 2,4-D, 0.2 M glucose, 0.2 M mannitol, 0.17 M sucrose</td>
<td>Survived 6-15 days, no cell division</td>
<td>Chen and Ku 1985</td>
</tr>
<tr>
<td>Bracts</td>
<td>Maçã (AAB); Nanica (AAA); balbisiana (BB)</td>
<td>0.2% Cellulase R10, 0.2% Macerozyme R10, 0.2% Driselase</td>
<td>8p medium (Kao and Michayluk 1975)</td>
<td>Cell clusters in 20 days</td>
<td>Matsumoto et al. 1988</td>
</tr>
<tr>
<td>Suspension cells</td>
<td>Long Tavoy (AA)</td>
<td>5% Cellulase RS, 2% Pectolyase Y23</td>
<td>N6+8p organic acids, sugar alcohols, 0.35 M glucose, 0.12 M sucrose, 1.9 mM KH2PO4, 0.5 mM MES, with nurse cells</td>
<td>Cellus</td>
<td>Megia et al. 1992</td>
</tr>
<tr>
<td>Suspension cells</td>
<td>Bluggoe (ABB)</td>
<td>1% Cellulase R10, 1% Macerozyme R10, 1% Pectinase SS</td>
<td>½MS, 5 μM 2,4-D, 0.55M mannitol with nurse cells on reservoir medium of ½MS, 5 μM 2,4-D, 0.27 M mannitol</td>
<td>Plants</td>
<td>Panis et al. 1993</td>
</tr>
<tr>
<td>Suspension cells</td>
<td>Bluggoe (ABB)</td>
<td>1.5% Cellulase RS, 0.15% Pectolyase Y23, 0.2% Hemilcellulase (Sigma)</td>
<td>N6+8p organic acids, sugar alcohols, 0.35 M glucose, 0.12M sucrose, 1.9 mM KH2PO4, 0.5 mM MES, with nurse cells</td>
<td>Plants</td>
<td>Megia et al. 1993</td>
</tr>
<tr>
<td>Suspension cells</td>
<td>Maçã (AAB)</td>
<td>1.5% Cellulase RS, 0.2% Pectolyase Y23</td>
<td>½MS, 5 μM 2,4-D, 0.55 M mannitol, 0.06 M sucrose with nurse cells on reservoir medium of ½MS, 5 μM 2,4-D, 0.27 M mannitol</td>
<td>Plants</td>
<td>Matsumoto and Oka 1998</td>
</tr>
<tr>
<td>Suspension cells</td>
<td>Grande Naine (AAA)</td>
<td>2% Cellulase RS, 0.5% Macerozyme (Sigma), 0.2% Hemilcellulase (Sigma), 0.25% Pectolyase Y23</td>
<td>N6+8p organic acids, sugar alcohols, 0.9 μM 2,4-D, 5 μM NAA, 2.3 μM zeatin, 0.4 M glucose, 0.12 sucrose, 1.9 mM KH2PO4, 0.5 mM MES, with nurse cells</td>
<td>Plants</td>
<td>Assani et al. 2001</td>
</tr>
<tr>
<td>Suspension cells</td>
<td>Various (AAA); (AAB); (AA)</td>
<td>1.5% Cellulase RS, 0.15% Pectolyase Y23</td>
<td>N6+8p organic acids, sugar alcohols, 0.9 μM 2,4-D, 4.4 μM NAA, 2.3 μM zeatin, 0.4 M glucose, 0.12 sucrose, 1.9 mM KH2PO4, 0.5 mM MES, with nurse cells</td>
<td>Plants</td>
<td>Assani et al. 2002</td>
</tr>
<tr>
<td>Suspension cells</td>
<td>Mas (AA)</td>
<td>3.5% Cellulase R10, 1% Macerozyme R10, 0.15% Pectolyase Y23</td>
<td>N6 + Morel vitamins, 9 μM 2,4-D, 2.8 mM glucose, 0.12 M sucrose, 0.28 M maltose with nurse cells</td>
<td>Plants</td>
<td>Xiao et al. 2007</td>
</tr>
</tbody>
</table>

**Abbreviations:** ½MS, half-strength Murashige and Skoog (1962) medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; MES, 4-morpholineethanesulfonic acid; Morel, Morel and Wetmore (1951); N6, Chu et al. (1975) medium; NAA, α-naphthaleneacetic acid
generally involves an extended period of culture. For this reason, although protoplast transformation has been exploited for stable transformation, it has been directed primarily to investigations of transient gene expression (Davey et al. 2005a). As summarized in Table 1, a protoplast-to-plant system is now established in several banana cultivars. In these cultivars, protoplast-based transformation could become a feasible technique to generate transgenic plants.

Sági et al. (1994) first reported transient gene expression in electroporated banana protoplasts. Under their experimental conditions, approximately 2% of the protoplast population expressed the β-glucuronidase (gusA or uidA) gene. After investigating various parameters, these authors concluded that optimum efficiency was when using a 960 μF capacitor, an electric field strength of 800 V/cm, ASP electroporation buffer (Tada et al., 1990), a PEG concentration of 5% (w/v), a heat shock of 45°C for 5 min before the addition of PEG, and protoplasts from 1-week-old embryogenic cell suspensions. Optimization of gene constructs was also essential (Sági et al. 1994, 1995b, 1998, 2000).
Unfortunately, these investigators did not report further development of their methods. In laboratory of present authors, transient gene expression assays used a green fluorescent protein (gfp) gene (Fig. 2). More than 25% of the protoplasts that survived the DNA uptake treatment, expressed the gfp gene when diploid AA banana protoplasts were electroporated with 40 μg/mL of plasmid DNA, an electric field strength of 1250 V/cm for 50 μs, with 3 pulses in modified ASP electroporation buffer supplemented with 3% (w/v) PEG. However, this procedure was not considered to be 10-fold more efficient than the former work, because the gene expression frequency was assessed in protoplasts that survived the treatment rather than the total number of protoplasts inoculated, as in the former work. Additionally, the frequency of transformation was often greater when monitored by gfp expression than by expression of the gus gene (Sreeramanan et al. 2005, 2006). However, it is like that the transformation efficiency can be increased by further intensive studies. For example, in tobacco, gus gene expression frequency in nearly 90% of the protoplasts was obtained routinely by protoplast electroporation (Fisk and Dandekar 2005).

**SOMATIC HYBRIDIZATION BY ELECTROFUSION AND PEG-INDUCED FUSION OF PROTOPLASTS**

Until the present time, banana hybrid cultivars have not been generated by somatic hybridization. Hybrid cultivars have been obtained only by conventional cross-breeding, supported by embryo rescue and micropropagation (Tompek et al. 2004; Pedraza et al. 2005; Morán 2006). However, somatic hybridization involving protoplast fusion is currently the only way to obtain hybrids between highly sterile banana varieties, particularly in the triploid Cavendish group. Additionally, it is possible to manipulate some polygenic characters without DNA-level knowledge, by affecting chromosome and/or organelle transfer through asymmetric protoplast fusion. The first tentative attempts at protoplast fusion were reported by Chen and Ku (1985) and, subsequently, by Matsumoto et al. (1992). The former investigators used leaf protoplasts, while the latter employed bract protoplasts. Both research groups demonstrated the fusion of isolated protoplasts, but were unable to culture fusion-treated material. Somatic hybrids were obtained only after the protoplast culture protocol was established, using embryogenic suspension cells as source material (Table 1). Protoplast electrofusion was achieved using an AC electric field of 1 MHz at 200-230 V/cm for a period of 10-30 s, followed by a DC electric field of 1250-2300 V/cm for 30-100 μs with 3-20 pulses (Fig. 3; Matsumoto et al. 2002; Assani et al. 2005). Instead of electrofusion, a treatment with 50% (w/v) PEG solution could also induce reproducible protoplast fusion, although subsequent plant regeneration was not as efficient (Assani et al. 2005).

**PROSPECTS FOR GENETIC IMPROVEMENT OF BANANA**

Although protoplast culture is routine for some cultivars of banana, fine embryogenic cell suspensions are not available in many cultivars. Once embryogenic cell suspensions are established in a range of cultivated varieties, protoplast technology will be widely applicable to the genetic improvement of banana through induced mutation, symmetric and asymmetric somatic hybridization. The latter may include chromosome and organelle transfer which will be relevant to the breeding of inheritable polygenic characteristics.

For the last 10 years, consumer acceptance of genetic transformation has become a key issue. Reduction of the possible environmental impact of transgenes has been discussed, with the eventual requirement to eliminate marker genes from transgenic plants (Day et al. 2005; Ebinuma et al. 2005). Cisgenesis, involving genetic modification of plants by inserting genes of the plant species itself or from sexually compatible relatives, has been suggested as a replacement for transgenesis (Joshi et al. 2008; Schouten 2008). The marker genes for selection, such as the neomycin phosphotransferase (nptII) gene that confers kanamycin resistance on transformed plant cells, is indispensable in the transformation of explants such as those of leaves, embryos or shoot tips, since foreign DNA becomes integrated and expressed in relatively few of the target cells. Consequently, millions of non-transformed cells must be eliminated during culture prior to shoot regeneration from transformed cells. This can be effected by expression of a selectable marker.
gene in transformed cells. The situation is similar with embryogenic cell suspensions, since the cells mostly grow as multi-cell clusters. As all protoplasts have direct contact with the foreign DNA during electroporation or PEG transformation procedures, it should be feasible to generate transgenic bananas without the need for selection, provided the efficiency of protoplast transformation is increased to be comparable to that of tobacco. In this way, protoplasts will have true value in the genetic improvement of banana.

**PROTOCOL FOR ISOLATION, ELECTROPRORATION, ELECTROFUSION AND CULTURE OF BANANA PROTOPLASTS**

**Solutions and culture media**

a) MCP solution for protoplast isolation and electrofusion: 0.6 M mannitol, 0.1 M CaCl2, 0.5% (w/v) polyvinylpyrrolidone (PVP-40), 3.5 mM MES and 8 mg/L bromoresol purple, pH 5.7 (Stabilize by autoclaving at 121°C for 20 min and store at 4°C).

b) Enzyme solution for protoplast isolation: 1.5% (w/v) Cellulase Onozuka RS and 0.2% (w/v) Pectolyase Y23 in MCP solution (Filter-sterilize, 0.2 μm pore size and store at -20°C).

c) KA-MCP solution for electroporation: MCP solution plus 70 mM potassium aspartate, pH 5.6 (Stabilize by autoclaving and store at 4°C).

d) PA3 liquid medium for protoplast culture: ½ strength MS major nutrients, MS minor nutrients and vitamins, 10 mg/L ascorbic acid, 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.06 M sucrose, 0.55 M mannitol, pH 5.8 (Sterilize by autoclaving and store at room temperature).

e) PA3 semi-solid medium for immobilization of nurse cells: PA3 liquid medium plus 1.6% (w/v) agarose of low gelling temperature (Agarose Type VII, Sigma) (Sterilize by autoclaving and store at 4°C).

f) PA5 semi-solid medium for protoplast culture: ½-strength MS major nutrients, MS minor nutrients and vitamins, 10 mg/L ascorbic acid, 5 μM 2,4-D, 0.06 M sucrose, 0.27 M mannitol, 0.2% (w/v) Phytagel, pH 5.8 (Sterilize by autoclaving and distribute 25-mL aliquots into 9-cm diameter plastic Petri dishes).

g) Cell multiplication medium: ½-strength MS major nutrients, MS minor nutrients and vitamins, 10 mg/L ascorbic acid, 5 μM 2,4-D, 1 μM zeatin, 0.09 M sucrose, 0.2% (w/v) Phytagel, pH 5.8 (Sterilize by autoclaving and distribute 25-mL aliquots into 9-cm diameter plastic Petri dishes).

h) Germination medium: MS salts and vitamins, 2 μM indole-3-acetic acid (IAA), 2 μM benzylaminopurine (BA), 0.06 M sucrose, 0.2% (w/v) Phytagel, pH 5.8 (Sterilize by autoclaving and distribute 25-mL aliquots into 9-cm diameter plastic Petri dishes).

**Protoplast isolation**

1) Prepare 5 mL of the enzyme solution and decant into a 5-cm Petri dish.

2) Harvest embryogenic suspension cells (Fig. 1A) at 3-5 days after the last subculture and transfer approximately 0.2 mL settled-cell-volume (SCV) to the enzyme solution in the Petri dish.

3) Incubate at 28 ± 2°C in the dark at 50 rpm on a horizontal platform shaker for 15 hours (overnight).

4) Pass the suspension through a nylon mesh of 25 μm pore size.

5) Centrifuge the suspension at 900 rpm for 4 min.

6) Remove the supernatant and re-suspend the pellet protoplasts in MCP solution.

7) Repeat twice steps (5) and (6).

8) For electroporation, add 1 mL of KA-MCP solution to gently resuspend the protoplasts. For electrofusion, add 1 mL of MCP solution. When only protoplast culture is carried out, add 1 mL of PA3 liquid medium, count the protoplasts using a hemocytometer, adjust the density to 3.0 × 10^5 protoplasts/mL with PA3 liquid medium and proceed to step 24 (Fig. 1B; See Note 1).

**Electroproportion**

9) Count the protoplasts using a hemocytometer and adjust the protoplast density to 1.0 × 10^6 protoplasts/mL by the addition of KA-MCP solution. Prepare protoplasts of the 2 banana varieties to be fused, using the same protocol.

10) Add 40-80 μg/mL of plasmid DNA and mix thoroughly but gently.

11) Add PEG to a final concentration of 3% (w/v) [75 μL/mL of 40% (w/v) PEG stock solution].

12) Transfer the protoplast/DNA/PEG suspension to the electrode chamber of the electroporation apparatus and maintain the chamber on ice for 10 min.

13) Apply an electric field of DC 1250 V/cm, with 3 pulses, each of 50 μs duration (Fig. 2A, B).

14) Keep the chamber with the electroporated protoplasts on ice for 10 min, followed by room temperature for 1 h in the dark.

15) Proceed to the protocol for protoplast culture.

**Electrofusion**

16) From step (8), count the protoplasts using a hemocytometer and adjust the protoplast density to 1.0 × 10^6 protoplasts/mL by addition of MCP solution. Prepare protoplasts of the 2 banana varieties to be fused, using the same protocol.

17) Mix, in equal numbers, the protoplasts of the 2 varieties.

18) Transfer the protoplast mixture to the electrode chamber of the electrofusion apparatus (Fig. 3A).

19) Apply an AC electric field of 1 MHz, 200 V/cm for 10 s to align the protoplasts (formation of “pearl chains”; Fig. 3B).

20) Apply a DC electric field of 1250 V/cm with 3 pulses each of 100 μs.

21) Maintain the chamber containing the protoplast suspension at room temperature, in the dark for 1 h.

22) Proceed to the protocol for protoplast culture.

**Protoplast culture**

23) During the 1-hour incubation of electroporated or fusion-treated protoplasts in the electrode chamber, prepare the nurse cells as detailed below.

24) Liquify the semi-solid PA3 medium using a microwave oven and maintain the molten medium at 35-40°C.

25) Take 1.5 mL settled cell volume (SCV) of nurse cells (rapidly growing banana, rice or Loliurn perenne suspension cells) and suspend in 13.5 mL of PA3 liquid medium.

26) Mix with an equal volume of molten PA3 medium.

27) Wait approximately 2 min. until the medium becomes slightly viscous.

28) Distribute 2-mL of the cell-containing medium over 25-mL of PA5 semi-solid medium in a 9-cm Petri dish and allow the medium containing the nurse cells to become semi-solid.

29) Place an Isopore membrane (25-mm diameter, 5-μm pore size) over the surface of the medium containing the nurse cells.

30) Harvest the protoplasts from the electroporation/electrofusion chamber adding more 2 volumes of PA3 liquid medium. (If the chamber volume is 0.8 mL, add 1.6 mL of the PA3 liquid medium, giving a final protoplast density of 3.0 × 10^5 per mL.)

31) Disperse 0.2 mL of the protoplast suspension on the surface of the Isopore membrane (Fig. 1C); seal the Petri dish (e.g. with PVC film or Parafilm); incubate the cultures at 28 ± 2°C in the dark. Set up all of the treated protoplasts in this way (See Note 2). If the plasmid DNA contains the gfp gene and transient expression assay is intended, dispense approximately 4 mL of the...
suspension of electroporated protoplasts into a 5-cm diameter Petri dish and culture at 28 ± 2°C in the dark at 50 rpm on a horizontal platform shaker for 3 days. Observe transient gene expression with a microscope and UV-blue light at 395 nm (Fig. 2C, 2D).

32) After 20-30 days of culture, transfer the protoplast-derived cells attached to the Isopore membrane to the cell multiplication medium (Fig. 1D). In the case of electrofusion, plasmid DNA containing the hygromycin phosphotransferase (hpt) gene may be used, and transgenic protoplast-derived tissues may be recovered on multiplication medium supplemented with 20-30 mg/L hygromycin B. In the case of electrofusion for somatic hybridization, a selection agent is not applied and somatic hybridity is evaluated by PCR/RAPD analyses (Fig. 3E), and/or ploidy analysis by flow cytometry (see Note 3).

33) Transfer somatic embryos when they are each approximately 1 mm in size (Fig. 3C) to germination medium for shoot development (Figs. 1E, 1F).

NOTES

1. In our laboratory, 1.0 × 10^7 protoplasts are obtained routinely from 0.2 mL SCV of embryogenic suspensions of AAA, AAB and AA group bananas. The enzyme solution of 1.5% Cellulase RS + 0.2% Pectolyase Y23 may be suitable for different several banana cultivars, since enzyme mixtures of similar composition have been used by other workers (Table 1). The quality of cell suspensions should be assessed if protoplast isolation is not successful. Cell suspensions should be rich in embryonic cells, small cell clusters (each < 200 μm in size) being generally preferable as source material for protoplast isolation.

2. In the case of the banana var. Embrapa/CNPMF 2803-01 (AA group), 200 – 500 proembryos are regenerated on a membrane when 0.6 to 10^7 protoplasts (0.2 mL/membrane of a suspension of 3.0 × 10^7 protoplasts / mL) are cultured without electroporation or fusion treatments. When electroporation or fusion procedures are employed, the regeneration efficiency is reduced to less than 50 proembryos/membrane. Consequently, the density of protoplast suspension should be adjusted following preliminary experiments, to facilitate the selection of target cells or proembryos.

3. The protoplast fusion efficiency is 30–40% when observed by light microscopy. Following 3 fusion experiments between AAB and AA genome cultivars, more than 100 nuclei per cell were regenerated from electroporated protoplasts, and 16 of the 24 plants evaluated showed hybrid characteristics as assessed by PCR/RAPD and flow cytometry analyses.

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