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AN EFFICIENT METHOD OF PROTOPLAST ISOLATION IN BANANA (*MUSA* SPP.)

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Abstract

Protoplast cultures are essential commodity for transformation and this can be achieved through electroporation and somatic hybridization. Like other monocot systems, embryogenic cell suspension is the material of choice for the isolation of protoplasts. In banana protoplasts have been successfully isolated but so far sufficient quantities of protoplasts for practical application are not routinely met. Enzyme mixture comprises of 3% cellulose R-10, 1% macerozyme R-10 and 1% pectinase was used for protoplast isolation. Leaf, leaf bases and corm tissue were used as an explant source and corm tissue gave the highest yields of protoplasts. Average yield of protoplasts from corm tissue ranged from 2.5 x 10^5 to 8.7 x 10^5 protoplasts. Freshly isolated protoplasts were colorless and 80% protoplasts stained with FDA shows high level of viability. Protoplast were cultured for callus induction on SH medium and cell wall formation was observed after 3 days of culture and cell division was achieved within 6-8 days.

Introduction

Banana (*Musa* spp.) is a monocotyledonous, poor man fruit crop in tropical and subtropical countries. Most of the edible bananas are highly sterile, triploid and seedless. These features are barriers for the implementation of breeding strategies. Biotechnology including induced mutations, gene technology and somatic hybridization together with conventional methods could assist in overcoming these problems in developing new banana cultivars. Somatic hybridization technology is commonly used now in some crops like potato (Wenzel *et al.*, 1979; Mollers & Wenzel, 1992). Protoplasts of monocotyledonous plants have generally been much more difficult to isolate and regenerate than of dicot plants. A good regeneration system for banana protoplast is prerequisite for somatic hybridization. Protoplast from plant tissue provides a model system for physiological, biochemical and virological studies.

Protoplasts are naked cells that lack cell walls. They are spherical with a plasmolysed cell content and are contained within a plasmalemma. In principle, each individual protoplast can reform a cell wall, and later initiate either a callus through sustained divisions, or an embryo, defined as a somatic embryo. In banana protoplasts can be obtained from *In vivo* tissues or *In vitro* cultures (Cronauer & Krikorian, 1986). Banana is now easily amenable to *In vitro* culture, and plants are regenerated from various explants through organogenesis (Khatri *et al*., 1997), embryogenesis (Novak *et al*., 1989; Cote *et al*., 1996), anther culture (Kerbelec, 1996) and even from cultured protoplasts (Panis *et al*., 1993; Megia *et al*., 1993). This has created opportunities for other biotechnological applications.

Bakry (1984 b) reported the first successful isolation of viable banana protoplast from inflorescence of Cavendish banana (AAA) and was confirmed by Cronauer & Krikorian (1986) and by Da Silva Conceicao (1989). Later on regular progress was made by several scientists. Initially banana calli from protoplasts were developed by Megia (1992), followed by plant regeneration directly from protoplasts (Panis *et al*., 1993;

Megia *et al*., 1993;), protoplast transformation (Sagi *et al*., 1994) and somatic hybridisation (Haicour *et al*., 1993; Matsumoto *et al*., 2001, 2002,). Matsumoto *et al*., (2002) obtained protoplasts from *In vivo* bracts, leaf explants (Bakry, 1984 a; Da Silva, 1989; Chen & Ku, 1985; Assani *et al*., 2002; Matsumoto & Oka, 1998), slices of shoot tissue, roots (Cronauer, 1986), Immature female flowers (Grapin *et al*., 2000), Immature male flowers (Ma, 1991; Cote *et al*., 1996) or callus (Bakry, 1984 a; Da Silva, 1989; Assani *et al*., 2002; Matsumoto & Oka, 1998) were the first choice as starting material for protoplast isolation because of their convenience. In fact, protoplasts can be obtained in banana from almost any tissue, including young leaves, sheaths, bracts, roots, and callus, but yields depend on the explant source. Yields are quite low with most tissues and range from 0.1 to 2.8 × 10^6 protoplasts per gram, but the protoplasts are unable to divide. However, cell suspension-derived protoplasts gave high yields which ranged from 4 to 5 × 10^5 per gram with 'Long Tavoy'; from 1.1 to 3 × 10^5 per gram with *malaccensis* (Haicour *et al*., 1994); 2 to 20 × 10^6 per gram with 'Maça' (Matsumoto and Oka, 1998)] to 6.6 × 10^7 per millilitre of packed cell volume (PCV) with 'Bluggoe' (Panis *et al*., 1993). Megia *et al*., (1992), reported first sustained cell divisions in protoplast culture leading to calluses formation. Megia *et al*., (1993) and Panis *et al*., (1993) improved the protocol. Regeneration of plantlets from embryogenic protoplasts were reported by many researchers (Matsumoto & Oka, 1998; Assani *et al*., 2001 and 2002).

In the present research paper we have describe an efficient method for protoplast isolation in large quantity from *in vitro* rhizome tissue (corm) used as explant.

Material and Methods

Rhizome tissue, leaf and leaf base excised from 3-4week old micro propagated *In vitro* plantlets of banana (*Musa* spp.) cv. Basrai were used as a source of protoplast isolation. Explants were cut into small pieces and digested with 3 different enzyme solution i) 3% cellulose R-10, 1% macerozyme R-10, 1% pectinase; ii) 2% Cellulase R10, 2% Gencor cellulase 150 L, 0.03% Macerozyme R10 and iii) 1% Cellulase RS, 0.1% Pectolyase Y23 containing 0.05% $CaCl_2.2H_2O$, 6 mM MES and 13% mannitol at pH 5.8. Digestion was conducted at 35 rpm at 25^{o}C in dark for 16 hours. The digestion mixture was filtered through 80 µm nylon mesh, centrifuge at 100 g for 10 minutes. The pallet was suspended in washing solution same as with enzyme solution without enzymes and centrifuge twice at 100 g for 10 minutes. The flotation purification was carried out with 21% sucrose at 100 g for 5 minutes. The viability of protoplast was determined by fluroescein diacetate (FDA) and counting was carried out by haemocytometer. The protoplast were cultured at a density of 2-3 x10^5 protoplast/ml in a 3cm Petri dishes in culture media containing SH salts, Staba vitamins and growth hormone with 13% mannitol. The cultures were maintained at 25^{o}C in the dark.

Table1. Effect of enzyme combination on isolation of protoplast from three different explants of banana (*Musa* spp.).

Enzyme solution	Leaf		Basal part of leaf		Corm	
	0.4 M	0.6 M	0.4 M	0.6 M	0.4 M	0.6 M
Enzyme mixture 1	+	+	++	++	+++	+++
Enzyme mixture 2	-	-	+	+	+	+
Enzyme mixture 3	-	-	-	-	-	-

Enzyme mixture 1= Cellulase R10 3%, Macerozyme R10 1 %, Pectinase 1%,
Enzyme mixture 2 = Cellulase R10 2%, Gencor cellulase 150 L 2%, Macerozyme R10 0.03 %,
Enzyme mixture 3 = Cellulase RS 1%, Pectolyase Y23 0.1%
M = Mannitol morality
+ = Fair to poor, ++ = Good, +++ = Excellent, - = None

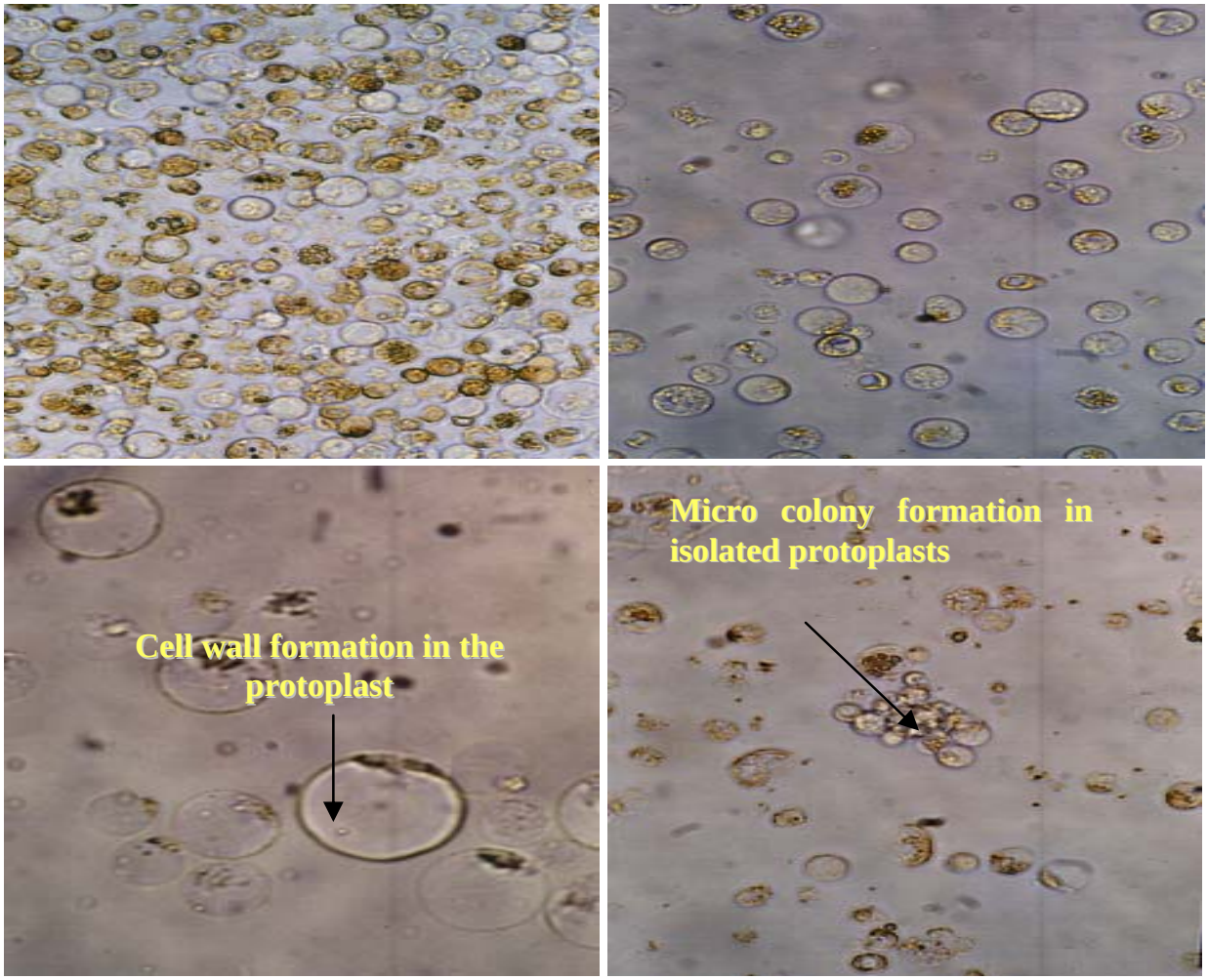

Fig 1. a. Protoplast with enzyme solution, b. Freshly isolated protoplast, c. Protoplast with newly synthesized cell wall, d. Initiation of micro colony formation.

Results

Freshly isolated protoplasts were generally colorless and population was heterogeneous with respect to the size (Fig. 1a & b). The yield of protoplast was better from rhizome tissue. An average yield of protoplast ranged from 2.5 x 10^5 to 8.7 x 10^5 protoplasts/ml was achieved from corm tissue with 3% cellulose, 1% macerozyme and 1% pectinase. Also viable protoplasts were isolated from basrai leaves, but the yield was very low and protoplast could not divide. These results confirmed that rhizome base is the best source of protoplast for isolation in banana. The isolated protoplasts comprised of two broad types, one is large, highly vacuolated and usually includes starch grains, and the second type is smaller, denser and has much fewer starch grains. No clumps of undigested cells detected in protoplast purification. Purification of protoplasts on sucrose gradient yielded very clean protoplasts that are free of raphide crystals (Kancharopoova *et al*., 2001). However, very low levels of contamination by intact single cells on average 0.05 % were revealed. These cells were elongated, thick and generally plasmolysed. They are readily distinguished microscopically from protoplast. More than 80% of the protoplast stained with FDA was highly fluorescent under UV light indicating a high level of viability immediately after isolation. In general the larger, more vacuolated cells remained as single unit while smaller, more densely cytoplasmic cells aggregated together. Protoplast was cultured by suspending the protoplast in liquid medium. Cell wall regeneration was obtained after 2 days of culture

while first cell division was achieved within 6-8 days of culture (Fig. 1c). Micro colony formation was observed in the protoplast culture (Fig. 1d) and remained viable upto 3 weeks in protoplast culture medium.

Efficiency of protoplast isolation, culture and regeneration depends on several factors such as duration of subculture of micropropagules, explant source and growth regulators. While the other important factor for protoplast development was culture system. It was clearly seen that all combination of enzyme affects protoplast yield. Addition of cellulose 3% and macerozyme 1% promoted a higher yield of protoplast in three different enzyme combination used in this study. Concentration of cellulose less than 3% did provide high yield of protoplasts.

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