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New explant for somatic embryogenesis induction and plant regeneration from diploid banana ('Calcutta 4´, *Musa* AA)

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ABSTRACT

A method has been developed for plant regeneration via embryogenic cell suspensions from diploid cultivar 'Calcutta 4´. For callus induction with embryogenic structures, different plant tissues such as scalps from cauliflower-like meristems and meristematic domes of axillary sprouted buds in combination with several culture media were evaluated as explants. The best embryogenic response (8%) was noticed with meristematic domes of axillary sprouted buds in culture medium Murashige and Skoog salts at 50%, MS vitamins, 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 1 mg l^{-1} 2,4-D, 0.22 mg l^{-1} Zeatine and supplemented with 100 mg l^{-1} malt extract, 100 mg l^{-1} glutamine, 1 mg l^{-1} biotin, 200 mg l^{-1} casein hydrolysate, 4.0 mg l^{-1} proline and solidified with 2.0 g l^{-1} Gelrite. Embryogenic cell suspensions were established and the highest increase of cellular biomass with 0.50 ml settled cell volume (SCV) was obtained 18 days after culture initiation. In a RD1 culture medium, embryogenic masses from 1362 to 2480 embryos were formed. An average of 54.5% germinated embryos in Temporal Immersion System (TIS) was obtained. Results were significantly higher in comparison with the use of semisolid culture media (28%). Regenerated plants are in field conditions to value their genetic stability.

Keywords: axillary buds, somatic embryogenesis, Temporal Immersion System

Abbreviations: gFW - grams of fresh weight; SCV – Settled Cell Volume; MS - Murashige and Skoog (1962) medium; BA- 6-benzyaldenine; 2,4-D - 2,4-dichlorophenoxgace acid; IAA - indole-3-acetic acid.

INTRODUCTION

Bananas and plantains are important fruits in tropical and subtropical countries and provide employment, nutrition and food security. They are grown in more than 100 countries (Escalant and Jain, 2004), however, due to the appearance of 'Black Sigatoka' disease caused by *Mycosfaerella figiensis*, the yields losses are estimated at 33-76% being necessary the development of biotechnological techniques that can supplement the genetic improvement programs.

The integration of genetic engineering into breeding programs may provide powerful tools to overcome these limitations by introducing specific genetic changes that can be utilized for banana improvement within a short period of time. However, these applications require reliable plant regeneration protocols for banana (Khalil *et al.,* 2002; Pérez and Rosell, 2008). On the other hand, the Global *Musa* Genomics Consortium, uses 'Calcutta 4', *Musa* (AA) as the standard genotype for genomic works and in this respect, it is recommended to develop diploid embryogenic cell suspensions for mutagenesis (INIBA and IPGRI, 2002) but embryogenic cell suspensions from this genotipe are not available (Strosse *et al.,* 2003; Strosse *et al.,* 2004). For that reason is necessary to develop a protocol that allows the regeneration of plants starting from somatic

embryos in this diploid genotype for their use in the search of resistant or tolerant plants to 'Black Sigatoka' disease.

The objective of this investigation was to develop a protocol for plant regeneration through embryogenic cell suspensions from cultivar 'Calcutta 4'.

MATERIALS AND METHODS

Plant Material

Flowering 'Calcutta 4' (*Musa acuminata* AA) plants from the germplasm bank at 'Instituto de Investigaciones de Viandas Tropicales' (INIVIT) were used. Plants were established *in vitro* according to proceedings described by Vuylsteke (1989).

Callus formation with embryogenic structures

Different tissue cultures were taken as explants from scalps and meristematic domes from axillary sprouted buds as follow:

Scalps

Scalps were excised from cauliflower like meristems cultures, subcultured in P4 culture medium (composed by Murashige-Skoog (1962) salts (MS) and vitamins, 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 22.5 mg l^{-1} BA, 0.18 mg l^{-1} IAA and solidified with 2 g l^{-1} Gelrite^{MT}). From 1 to 15 subcultures were carried out until they formed clusters of small, white meristems surrounded by only a few, very small leaves according to a procedure described by Dhed´a *et al.* (1991) and refined by Schoofs (1997).

Plant materials were cultured in 125 mm test tubes with 10 ml ZZ culture medium volume and incubated during four months in dark at 27 ± 2 ºC.

A study was carried out to determine the effect of subculture number in P4 culture medium on the embryogenic response of 125 explants (scalps) which were incubated for four months in ZZ semisolid medium (Schoofs 1997).

Meristematic domes from axillary sprouted buds

In order to obtain meristematic domes from axillary sprouted buds, three subcultures from 15 to 18 days each one were developed in multiplication medium constituted by salts and vitamins (MS), 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 2.25 mg l^{-1} BA, 0.18 mg l^{-1} IAA, 0.4 mg l^{-1} ancimydol and solidified with 2 g l^{-1} Gelrite (López *et al*., 2004).

Later, 2-3 mm meristematic domes were excised from recent axillary sprouted buds under a stereoscopic microscope and incubated in different culture media (Table 1) with monthly transfer to fresh media during the first three months. In each culture medium treatment, 125 explants were incubated for four months in the same conditions described for callus induction with embryogenic structures from scalps.

Establishment and multiplication of embryogenic cell suspensions

Embryos in globular stage coming from calli of axillary sprouted buds (0.08-0.1 gFW) were used to establish embryogenic cell suspensions in 10 ml Erlenmeyer flask, in culture medium III (10 ml MS medium reduced to half strength salts concentration, MS vitamins, 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 0.22 mg l^{-1} zeatin, 100 mg l^{-1} malt extract, 100 mg l^{-1} glutamine, 200 mg l^{-1} hydrolyzed casein, 1 mg l^{-1} biotin and 4 mg l^{-1} L-proline (Sigma) (according to results obtained in previous experiments). Explants were put on an orbital shaker, model INFORS (HT) at 90 r.p.m., at a temperature of 27 ± 2 ºC and a 16 h light regime (62-68 ì mol $m^{-2}s^{-1}$).

After establishing embryogenic cell suspensions for a month, settled cell volume (SCV) was adjusted to 3.0% according to Schoofs (1997) in the same medium in order to determine the growing of embryogenic cell suspensions. Three 100 ml Erlenmeyer flasks were used as replicates. The cell volume growth was measured each third day through SCV, according to Schoofs (1997).

Somatic embryo formation

SCV was adjusted to 12% cell density according to Cabrera *et al*. (2002) for somatic embryo formation. The RD1 culture medium described by Dhed´a *et al.* (1991) was used and it was composed by half-strength MS salts, MS vitamins, 30 mg l^{-1} sucrose, 10 mg l^{-1} ascorbic

Table 1. Modifications to ZZ semisolid culture medium used for callus formation with embryogenic structures from axillary sprouted buds of *Musa acuminata* cv. 'Calcutta 4' (AA).

Culture Media	Concentration 2,4-D (mg l^{-1})		
Malt extract 100 mg l^{-1}, glutamine 100 mg l^{-1}, biotin 1 mg l^{-1} and casein hydrolysate 200 mg l^{-1}.	1^{I}*	2^{V}	3^{VIII}
Malt extract 100 mg l^{-1}, glutamine 100 mg l^{-1}, biotin 1 mg l^{-1}, casein hydrolysate 200 mg l^{-1} and L-proline 2 mg l^{-1}.	1^{II}	2^{VI}	3^{IX}
Malt extract 100 mg l^{-1} glutamine 100 mg l^{-1}, biotin 1 mg l^{-1}, casein hydrolysate 200 mg l^{-1} and L-proline 4 mg l^{-1}.	1^{III}	2^{VII}	3^{X}
ZZ Schoofs (1997) (Control)**, (1962) 50% salts, vitamins MS, sucrose 30 g l^{-1}, ascorbic acid 10 mg l^{-1} and zeatin 0.22 mg l^{-1}.	1^{IV}	-	-

* Roman numbers from I to X, different variants of culture medium.
** ZZ control culture media according to Schoofs (1997) MS 50 percentage of salts and MS vitamins, 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 1.11 mg l^{-1} 2,4-D, 0.22 mg l^{-1}, zeatin and solidified with 2 g l^{-1} Gelrite.

acid without growth regulator, pH 5.7 and solidified with Gelrite (2 g l^{-1}).

Five Petri dishes were used and four 2 cm^2 plastic mesh with 200 ?m holes were placed on each one to pour 200 ì L cells at 25% SCV. Incubation conditions were at 27 ± 20 ºC under a 16 light regime (62-68 ì mol m^{-2} s^{-1}). After 45 days of culture, the number of somatic embryos formed was evaluated.

A 0.1 gFW embryo was weighted and it was added separately to each Petri dish which contained a mixture of Gelrite-water. Counting was done under stereoscopic microscope.

Germination of somatic embryos

The Temporal Immersion System, type RITA® of 500 ml capacity and 200 ml culture medium was used for germination of somatic embryos. The medium consisted of MS salts and vitamins, 100 mg l^{-1} myo-inositol, 10 mg l^{-1} ascorbic acid, 2 mg l^{-1} IAA, 0.5 mg l^{-1} BA and pH 5.7. Incubation took place with a 0.5 gFW somatic embryos.

The immersion frequency was three times daily, during one minute each according to Escalant *et al*. (1994). As control, five 90 mm Petri dishes with 25 ml of culture medium for germination solidified with 2.0 g l^{-1} Gelrite (Sigma Co.) were used. Each Petri dish contained 50 somatic embryos.

Incubation was developed at 27 ± 2 ºC under a 16 light regime (40 ì mol m^{-2} s^{-1}). After 45 days, the number of somatic embryos which resulted in entire plants was evaluated.

The experimental statistical analysis was done by Curve expert 1.3 versions 96-97.

RESULTS AND DISCUSSIONS

Callus formation with embryogenic structures

Scalps

Callus formation with embryogenic structures was characterized in both explant types using scalps from cauliflower-like meristems as starting material in the embryogenic process; a very low formation frequency of calli with embryogenic structures was obtained. 0.8% embryogenic response was noticed with explants from subcultures 14 and 15 on P4 culture medium. Schoofs *et al*. (1999) stated that high quality cauliflower-like meristems may be obtained from a few days up to more than a year, although, some cultivars (*Musa* spp.) show a recalcitrant response for developing somatic embryogenesis. According to INIBA (2003) from January 2001 to December 2002, 1872 scalps from cv. 'Calcutta 4´ were incubated for callus formation without any embryogenic response.

Meristematic domes from axillary sprouted buds

When meristematic domes from axillary sprouted buds were used as explants in combination with different culture media, a better embryogenic performance was obtained in comparison with scalps from cauliflower-like meristems.

After four months of incubation, the best culture medium combination (Figure 1) for callus formation with embryogenic structures were constituted by culture medium III (MS salts at 50% and vitamins, 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 1 mg l^{-1} 2,4-D, 0.22 mg l^{-1} zeatine, 100 mg l^{-1} malt extract, 100 mg l^{-1} glutamine, 1 mg l^{-1} biotine, 200 mg l^{-1} casein hydrolysate, 4 mg l^{-1} L-proline and solidified with 2 g l^{-1} Gelrite).

Due to results obtained in this experiment, 2,4-D concentration was not determinant for callus formation with embryogenic structures in a range from 1.11 to 2 mg l^{-1}, however, embryogenic response was observed when media were enriched by L-proline. Bieberach (1995) used this amino acid to induce callus with embryogenic structures from male inflorescences in banana cv. 'Grande naine' (*Musa* spp. AAA) and these embryogenic structures were increased from 0.25% to 1.75%.

Several studies have shown the influence of some amino acids like L-proline on the development of somatic embryogenesis as rate regulator in the protein synthesis during the morphogenetic process. Torné and Santos (2005) were able to increase the embryogenesis efficiency using L- proline for corn callus culture and they found that callus percentage with embryogenic cluster, in relation to non-embryogenic calli was higher in culture media containg L-proline.

Other authors as Khalil *et al*. (2002) added proline and casein hydrolysate into the callus formation medium and increased callus proliferation and production of primary somatic embryos, and the embryogenic potential in other banana cultivars may also be increased.

On the other hand, explants selection could be a key factor for determining success or failure in an embryogenesis protocol (Krisshnaraj and Vasil 1995). In case of somatic embryogenesis in edible bananas and plantains, three main principles have been described and as explants, rhizome sections, leaf bases (Novak *et al*., 1999), scalps from cauliflor like multibuds (Dhed´a, 1991; Shoofs, 1997) and immature female and male flowers (Escalant *et al*., 1994) are used. However, the use of meristematic domes from sprouted buds in combination with the culture medium permitted to obtain somatic embryos, similar to those obtained by López *et al*. (2005) who used this explant type for somatic embryogenesis in plantain (AAB).

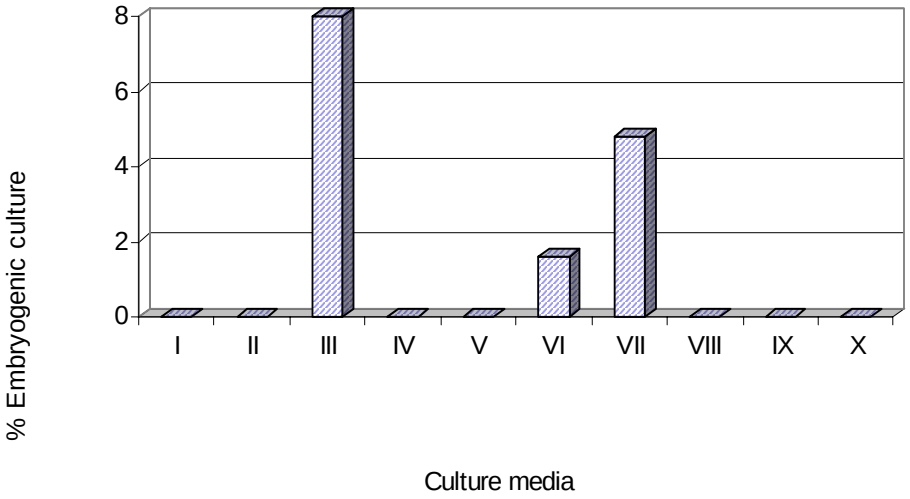


Figura 1. Percentage of callus formation with embryogenic structures coming from axillary sprouted buds in different culture media in *Musa* acauminata cv. 'Calcutta 4' (AA).

Establishment and multiplication of embryogenic cell suspensions

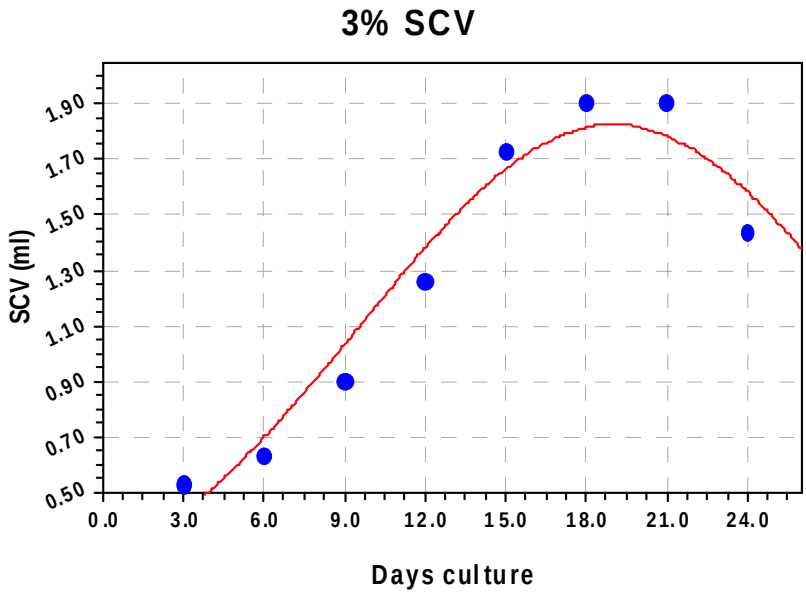
Cell suspensions are characterized by cell aggregates which occupy from 80 to 95% of the suspension and sizes from 80 to 300 ì m.These cells aggregates were constituted by small and spherical embryogenic cells with dense cytoplasmic content, small vacuoles, starch grains and a high nucleus/ cytoplasm rate. Similar results had been obtained by De Vries *et al*. (1988) in the growing of carrot cells and by Dhed'a *et al*. (1991) in cell suspension cultures of cooking banana cv. 'Bluggoe' (*Musa* spp. ABB group). According to Williams and Maheswaran (1986), previous cell characteristics are considered as an indicative of embyogenic conditions for cell suspensions.

The growth curve to the cell density of 3.0% SCV (Figure 2) showed a phase of well-defined and continuous exponential growth, similar to results obtained by Barranco (2001) who studied the cell density effect on the multiplication stage of embryogenic cell suspensions in the hybrid 'FHIA-18' (AAAB). The highest increment of cell biomass with 1.90 ml SVC was noticed 18 days after culture.

Results have shown that the optimum moment for subculturing cell suspensions can not coincide with the maximum expression of cell density in suspension, neither with the moment in which main energy sources are exhausted. Subcultures must be performed in the last exponential growing stage, and it infers that subcultures have to be developed each 15 days at a cell density of 3.0% SCV.

Somatic embryos formation and germination

After 45 days of inoculating 200 ì l cells at 12.0% SCV in RD1 culture medium, 0.42 gFW embryos were formed and a total of embryos from 1362 to 2480 in a globular stage were obtained. Temporal Immersion Systems showed better performance than semisolid culture media when germination of somatic embryos was studied. As can be observed (Table 2) a significantly higher average (54.51%) of germinated embryos in comparison with semisolid culture media (28%) was obtained.



Gaussian Model $y=ae^{-(x-b)^2/2c^2}$

CV	A	B	C
3.0%	1.820788	18.978315	9.3906454

Figure 2. Effect of cell density on growth dynamics of the embryogenic cell suspensions in 'Calcutta 4´, (*Musa* AA)

Table 2. Germination of somatic embryos from 'Calcutta 4', (*Musa* AA) in Temporal Immersion Systems (TIS) and on semisolid culture medium.

TREATMENTS	Germination percentage
TIS	54.49
Semisolid medium	28.00

Similar results on the use of TIS in the germination of somatic embryos in *Musa* spp. have been reported by Escalant *et al*. (1994), Bieberach (1995) and Kosky *et al.* (2002). Later, regenerated embryos were transferred to the acclimatization stage of plants to study the genetic stability of regenerated plants in the field conditions.

The scientific results obtained in this work, permitted to develop somatic embryogenesis in 'Calcutta 4' and plant regeneration from embryogenic cell suspensions, all this open great possibilities for genetic improvement of the crop. This research constitutes the first report on plant regeneration via embryogenesis somatic through embryogenic cell suspensions in *Musa* acuminata 'Calcutta 4'.

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