Cell suspension culture and plant regeneration of a Brazilian plantain, cultivar Terra

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Abstract – The objective of this study was to establish cell suspension culture and plant regeneration via somatic embryogenesis of a Brazilian plantain, cultivar Terra Maranhão, AAB. Immature male flowers were used as explant source for generating highly embryogenic cultures 45 days after inoculation, which were used for establishment of cell suspension culture and multiplication of secondary somatic embryos. Five semisolid culture media were tested for differentiation, maturation, somatic embryos germination and for plant regeneration. An average of 558 plants per one milliliter of 5% SCV (settled cell volume) were regenerated in the MS medium, with 11.4 µM indolacetic acid and 2.2 µM 6-benzylaminopurine. Regenerated plants showed a normal development, and no visible somaclonal variation was observed in vitro. It is possible to regenerate plants from cell suspensions of plantain banana cultivar Terra using MS medium supplemented with 11.4 µM of IAA and 2.2 µM of BAP.

Index terms: *Musa*, culture media, propagation, somatic embryogenesis.

Cultura de células em suspensão e regeneração de plantas de bananeira cultivar Terra

Resumo – O objetivo deste trabalho foi estabelecer a cultura de células em suspensão e a regeneração de plantas via embriogênese somática de bananeira cultivar Terra Maranhão, AAB. Flores imaturas masculinas foram utilizadas como fonte de explante para obtenção de culturas altamente embriogênicas 45 dias após a inoculação, as quais foram utilizadas para o estabelecimento de suspensões celulares e multiplicação de embriões somáticos secundários. Cinco meios de cultura semi-sólidos foram testados para a diferenciação, maturação, germinação dos embriões somáticos e para a regeneração de plantas. A média de 558 plantas por mililitro de 5% SCV (volume de células sedimentadas) foi regenerada, em meio MS com 11,4 µM de ácido indolacético e 2,2 µM de 6-benzilaminopurina. As plantas regeneradas apresentaram desenvolvimento normal, e não foi observada a ocorrência de variação somaclonal in vitro. É possível a regeneração de plantas a partir de células em suspensão de bananeira 'Terra' utilizando meio MS suplementado com 11,4 µM de AIA e 2,2 µM de BAP.

Termos para indexação: *Musa*, meio de cultura, micropropagação, embriogênese somática.

Introduction

Bananas and plantains (*Musa* spp.) are very important fruits for both social and economical reasons. They are worldwide cultivated in the tropical countries and constitute a staple food for nearly half a billion people. The Brazilian cultivar Terra is a French plantain consumed in stews or fried, mainly in the North and Northeast of the country. This cultivar is resistant to yellow sigatoka (*Mycosphaerella musicola* Leach) and fusarium wilt [*Fusarium oxysporum* f. sp. *cubense* (E.F. Smith)], but it is susceptible to black sigatoka (*Mycosphaerella fijiensis* Morelet), nematodes and weevil borers [*Cosmopolites sordidus* (Germar)] (Silva et al., 2001).

The materials used for conventional propagation of banana and plantain include corms, large and small suckers, and sword suckers (Cronauer & Krikorian, 1984). However, conventional planting materials are

not the ideal propagule, because of the low number of suckers (Vuylsteke, 1989) and the fact that it could be a potential source of dissemination of weevils, fungal pathogens, nematodes, and viruses (Sagi et al., 1998). The rapid production of healthy planting material of desired clones, within a short time period, is facilitated by large-scale micropropagation. Different in vitro micropropagation protocols have been used in several *Musa* spp. of divergent genomic constitution and ploidy (Cronauer & Krikorian, 1984; Vuylsteke, 1989; Scott et al., 1999; Arinaitwe et al., 2000; Roels et al., 2005). However, in vitro propagation through shoot-tip culture has shown low level of success in some varieties, mainly due to oxidation caused by phenolic compounds that form a barrier around the tissue, preventing nutrient uptake and hindering growth (Strosse et al., 2004). Micropropagation of plantain using meristem tip culture has shown low level of success, mainly due to oxidation and low proliferation caused by a high degree of apical dominance. The presence of a B genome can also affect multiplication (Ariwnaitwe et al., 2000; Roels et al., 2005).

Somatic embryogenesis and cell suspension have been obtained for different genotypes of banana (Navarro et al., 1997; Strosse et al., 2003, 2006). Cell suspensions with high regeneration capacity have application for mass clonal propagation, germplasm handling and cryopreservation (Panis et al., 1990; Navarro et al., 1997; Kosky et al., 2002) and for improving *Musa* spp. through nonconventional strategies, i.e. protoplast culture and fusion, mutagenesis, somaclonal variation and genetic transformation (Ganapathi et al., 2001; Matsumoto et al., 2002; Assani et al., 2005; Houllou-Kido et al., 2005; Kumar et al., 2005; Pei et al., 2005). In addition, the incorporation of biotechnological procedures in breeding programs depends upon efficient and reliable plant regeneration protocols.

The objective of this work was to establish a protocol of cell suspension culture and plant regeneration via somatic embryogenesis of the Brazilian plantain cultivar Terra Maranhão, AAB, applicable for large-scale propagation, genetic transformation and somatic hybridization studies.

Materials and Methods

Explant donor plants of plantain cultivar Terra (cooking banana, AAB) are maintained at Embrapa's Banana Germplasm Bank, in Cruz das Almas, BA, Brazil. The experiment was carried out during 2006. Male buds were harvested and peeled off in the laboratory to approximately 10 cm size. The size-reduced male buds were washed in tap water and transferred to a clean bench for further steps of surface sterilization. They were sprayed twice with 70% ethanol, and flamed. Immature flowers from the position 4 to 14, counting down from the top, were removed under stereomicroscope and placed on Petri dishes containing 30 mL of culture medium constituted of MS salts and vitamins (Murashige & Skoog, 1962), supplemented with 4.1 µM biotin, 18 µM 2,4-D (dichlorophenoxyacetic acid), 5.7 µM IAA (indoleacetic acid), 5.4 µM NAA (naphtaleneacetic acid), 87 mM sucrose, and solidified with 0.7% type III agarose. The pH was adjusted to 5.7 before autoclaving at 121°C for 20 min. Eight male buds, containing five explants per Petri dish, were used. The cultures were kept in the dark, at 27±1°C, for up to six months. Cultures were observed weekly for the presence of embryo formation.

Immature male flowers of the Brazilian plantain cultivar Terra, AAB, was used to induce embryogenic callus (Figure 1 A). Friable embryogenic calli with many embryos were transferred to 125 mL Erlenmeyer flasks containing 15 mL of liquid medium constituted of MS salts and vitamins supplemented with 4.5 µM 2,4-D, 680 µM glutamine, 4.1 µM biotin, 10 mg L^{-1} ascorbic acid and 130 mM sucrose. The pH of the medium was adjusted to 5.3, before autoclaving. The cultures were maintained in the dark, at 27±2°C, in orbital shaker at 120 rpm. After one month, the cell suspension was homogenized by passing through a 0.6-mm-mesh sieve selecting the single cells and small cell aggregates. Subcultures were done at every ten days period. Cell samples were observed through an inverted microscope (Zeiss, Germany).

The density of cell suspensions was adjusted to 5% of settled cell volume (SCV) by dilution of 1mL of settled cells into 19 mL of fresh medium. One milliliter of the diluted culture was placed on a filter paper in a Petri dish containing 35 mL of culture medium. Five different culture media were used (Table 1). Four treatments were tested, with different use sequences of culture medium for each phase of the regeneration process (differentiation, embryo maturation, germination and

whole plant regeneration) (Table 2). The culture media A, B, C and D (Assani et al., 2001; Matsumoto et al., 2002; Strosse et al., 2003) were used for cellular differentiation. The cultures were maintained in these media until plant regeneration, except for the cultures in medium A that, after embryo formation, were transferred to media C, D and E, for embryo maturation, germination and plant regeneration, respectively (Table 2). Five replications were used for each stage of regeneration, and the number of regenerated plants with roots and leaves was evaluated, at the end of the last stage of regeneration.

For plant regeneration, the cultures were kept in the dark for one month, and then transferred to a growth chamber at 27±2°C, under white fluorescent light with 16-hour-photoperiod at 50 µmol m^{-2} s^{-1}. The cultures were kept for 30 days in the culture medium, for differentiation and maturation, 15 days in the medium for germination, and 45 days in the regeneration medium. Regenerated plants were rescued and transferred to Magenta box containing 50 mL of solid growth regulator-free MS medium, at ratio of 16 plants per box. One hundred plants were selected at random, from each treatment, for acclimatization under greenhouse conditions.

Analysis of variance was carried out using the Generalized Linear Model procedure. Means

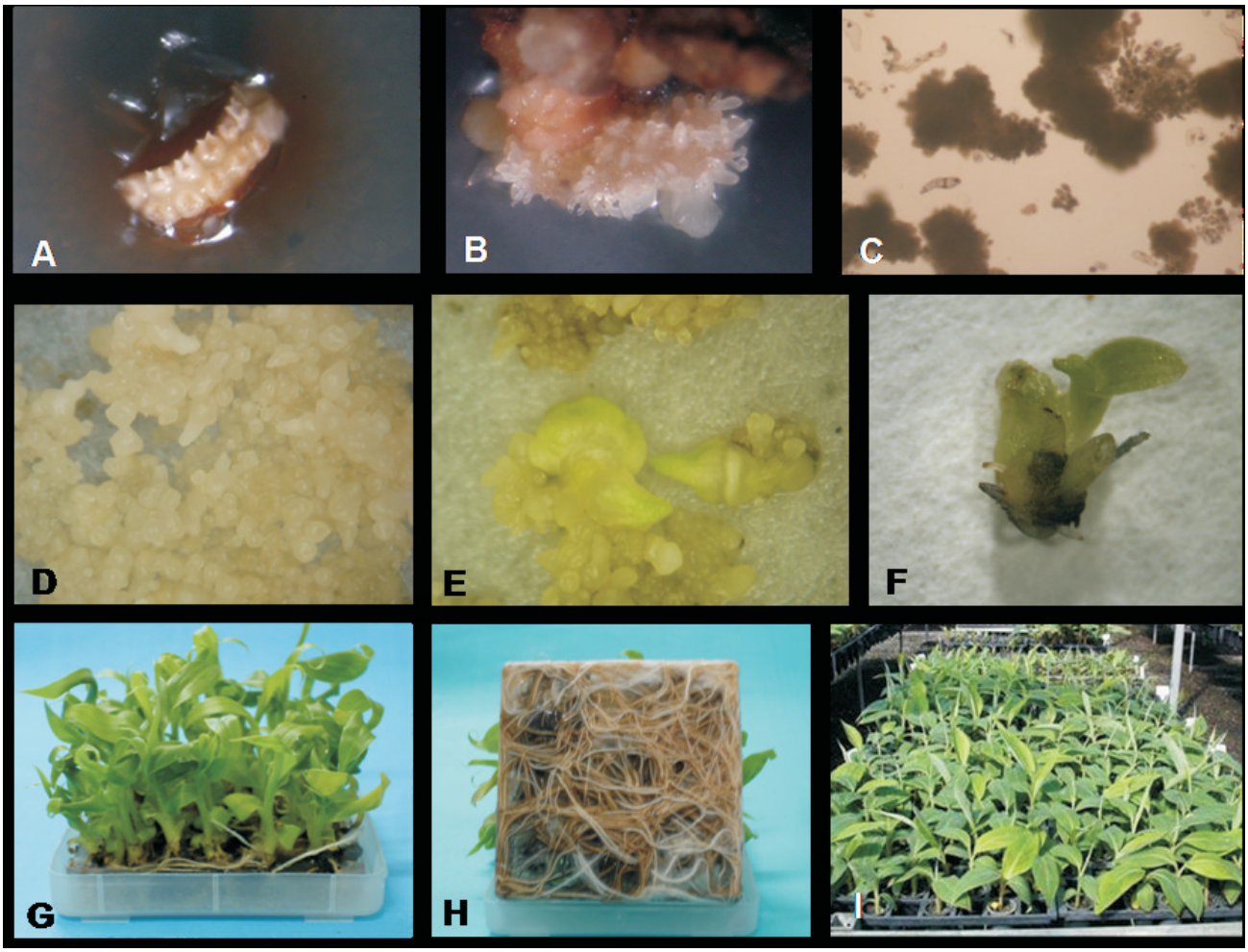

Figure 1. Plant regeneration from somatic embryos of a Brazilian plantain cv. Terra (AAB). A, initial explant, cluster of immature male flower; B, embryogenic mass bearing a group of somatic embryos; C, morphological aspects of the embryogenic suspension type II aggregates; D, somatic embryo formed after 30 days in semisolid medium; E, fully developed somatic embryos after 15 days, in medium of germination; F, germination of embryos; G and H, normal rooted plants germinated from somatic embryos, side view and bottom view, respectively; I, acclimatization of plants regenerated in greenhouse.

separation was done by the Tukey test using SAS (SAS Institute, 2000).

Results and Discussion

Forty-five days after explant inoculation, embryogenic calli appeared with numerous whitish translucent somatic embryos (Figure 1 B). Somatic embryos with similar features have been previously reported (Côte et al., 1996; Navarro et al., 1997; Khalil et al., 2002; Jalil et al., 2003). At 5–6 months of culture, 20% of the explants produced embryogenic calli (Figure 1 B). Different authors have reported that somatic embryogenesis is genotype dependent (Assani et al., 2002; Gahan & George, 2008). However, the embryogenic frequency in banana was not only dependent on the genome group, but it also varied with the variety within genome groups and even from one experiment to another (Strosse et al., 2006).

Suspension cells could be observed after two weeks of culture. Several cell types were observed: large and highly vacuolated cells, dense cytoplasm cells, single cells and cell aggregates (Figure 1 C). After homogenization, single cells and small cell aggregates were selected. After two months of cultivation, the suspension was set up by dense cytoplasm cell aggregates. Domergue et al. (2000) have described such cells as type II ones, highly embryogenic with the producing capacity of 130,000 to 150,000 embryos m^{-1} of PCV. When the cell suspensions were 4–5 months old, fine embryogenic cells were observed as yellowish white in color. The yellow pigmentation of cytoplasm indicated the embryogenic character of the cell suspensions (Ganapathi et al., 2001; Jalil et al., 2003). The number of cells had doubled at every subcultivation period.

Early stages of embryo development could be observed two weeks after transferring to differentiation medium (Table 2). After 30 days, the embryos were completely formed (Figure 1 D). The embryos were transferred to maturation medium and maintained there for another 30 days (Figure 1 E). Mature embryos were transferred to germination medium. After 15 days, the embryos started germinating, displaying roots and leaves, and after 45 days, they were transferred to whole plant regeneration medium (Figure 1 F). All treatments generated normal and vigorous plants with good root system development (Figure 1 G and H), with few losses during the greenhouse acclimatization step (Figure 1 I), and without occurrence of morphological variation in plants. The treatment using C medium achieved the best results in all stages of the regeneration process, with an average number of 558 plants mL^{-1} of 5% SCV (Table 3). Domergue et al. (2000) got an estimated number of 154,000 embryos from 1 mL PCV for 'Grand Naine'; Strosse et al. (2006) reported estimates of 48,000 and 20,000 regenerated plants per 1 mL PCV, using cultivars from the subgroup Cavendish and plantains, respectively.

Lemos et al. (2001) used micropropagation of banana 'Terra' with traditional cultivation in semisolid media compared with a temporary immersion bioreactor, and reported that the temporary immersion system presented 2.20 times more viable shoots than the

Table 1. Composition of culture media used to obtain somatic embryogenesis of Brazilian plantain cv. Terra (ABB).

Component	Culture media[1]				
	A	B	C	D	E
IAA (µM)		2.2	11.4	2	1
BAP (µM)		2.2	2.2	2	1
NAA (µM)	1.1				
Zeatin (µM)	0.2				
2-ip (µM)	0.7				
Kinetin (µM)	0.5				
Glutamine (µM)	680				
Proline (mM)	2				
Sucrose (mM)	130	87	87	87	87

[1]All media used in this study contained MS salts (Murashige & Skoog, 1962) and vitamins, solidified with Phytagel (2.0 g L^{-1}) and adjusted to pH 5.8, before autoclaving at 121°C for 20 min.

Table 2. Culture media sequence used for differentiation, maturation, germination and plant regeneration from embryogenic cell suspension.

Step	Sequence of culture media[1]			
	T1	T2	T3	T4
Differentiation	A	B	C	D
Maturation	C	B	C	D
Germination	D	B	C	D
Plant regeneration	E	B	C	D

[1]Treatments.

Table 3. Number of plants regenerated from cell suspension of Brazilian plantain cv. Terra in different culture media[1]

Treatment	Average number of regenerated plants from 1 mL of 5% PCV suspensions
T1	168.20±33.95c
T2	323.00±135.80bc
T3	558.60±108.31a
T4	375.40±65.44b

[1]Means followed by the same letters do not differ significantly, according to Tukey's test, at 5% of probability.

traditional semisolid system, in 60 days of subculture. However, the number of regenerated plants in the present work, for each milliliter of 5% SCV (around 558 plants), was similar to that obtained for another plantain (AAB) via temporary immersion bioreactor using shoot tips (Roels et al., 2005). This number was also proportionally higher than the obtained by Kosky et al. (2002), for the hybrid cultivar FHIA-18 (AAAB), in a temporary immersion system (RITA) using 2 g of somatic embryos in 200 mL of the same medium used in the present study (medium C): 748 plants.

The protocol developed in this work is suitable for large-scale propagation of type French plantains, as well as for the use in nonconventional genetic improvement strategies, after studying the genetic stability, including the somatic hybridization and the genetic transformation.

Conclusions

1. It is possible to regenerate plants from cell suspensions of plantain banana cultivar Terra.

2. The protocol using MS medium supplemented with 11.4 µM of IAA and 2.2 µM of BAP is efficient in plant regeneration from 'Terra' plantain cell suspension.

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