RESEARCH

Plant regeneration of plantain 'Barraganete' from somatic embryos using a temporary immersion system

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ABSTRACT

Banana and plantain (*Musa* spp.) are highly-demanded fruits at the international level. *In vitro* propagation of plantain in solid medium by using apical meristems, as initial explants, is a very slow method that is always accompanied by high phenolization of culture medium, which leads to poor shooting or death of cultured tissue. This paper reports the successful regeneration (84.5 %) of 'Barraganete' plantain (AAB) plants from somatic embryos, obtained from embryogenic cell suspensions and using a temporary immersion system. The low percentage (1.55 %) of somaclonal variation in plants grown under greenhouse conditions indicates that this method could be used for commercial propagation. However, regenerated plants should be monitored for a longer period to rule out the possibility of somaclonal variation in adult plants.

Keywords: plantain, *in vitro* propagation, cell suspensions, somatic embryos, temporary immersion system

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RESUMEN

Regeneración de plantas de plátano 'Barraganate' a partir de embriones somáticos mediante el uso de un sistema de inmersión temporal. Los bananos (*Musa* spp.) producen frutos de gran demanda en el mercado internacional. La propagación *in vitro* de plátano, utilizando el medio de cultivo sólido y los meristemos apicales como los explantes iniciales, es un método lento y siempre está acompañado por una alta fenolización, tanto de los explantes como de los medios de cultivo o la muerte de los brotes. En esta investigación se obtuvieron los embriones somáticos de plátano de la variedad 'Barraganete' (AAB) en las suspensiones celulares. El 84.5 % de estos se regeneraron en plantas mediante el uso de biorreactores de inmersión temporal, utilizando el medio de cultivo líquido. El bajo porcentaje de variación somaclonal (1.55 %) en las plantas evaluadas permitió pensar que el método empleado puede ser utilizado para la propagación comercial de plátano, aunque es necesario seguir el estudio de la variación somaclonal en las plantas adultas.

Palabras clave: plátanos, propagación *in vitro*, suspensiones celulares, embriones somáticos, sistema de inmersión temporal

Introduction

Banana and plantain (*Musa* spp.) are highly demanded fruits of immense economic importance for countries that produce them. The estimated area planted with banana and plantain worldwide reaches 10 million ha for an approximate production of 84 million tons of fruits, which only over 10 % is exported worldwide. The main banana producing countries are: India, Brasil, Colombia, Indonesia, Filipinas and China. In Ecuador, over 12 % of the population works in the production and marketing of this fruits and contributes to about 28 % of total exports producing (1.9 billions of US dollars in 2010 and 2.2 billions in 2011 that is 32 % of world trade in bananas) [1-3].

Currently, Ecuador reports a total of 144 981 ha planted with plantain. The main cultivated varieties are 'Dominico', which is used for local consumption and 'Barraganete' which is mostly exported. It is estimated that about 90 000 tons of this cultivar are exported annually [4, 5]. The fast *in vitro* multiplication of plants of different species has contributed enormously to the development of agriculture [6]. But there have been limitations, since multiplication of different varieties of plantains by tissue culture, using apical meristems of selected plants, has always been accompanied by high phenolization of initial explants and culture medium. This has led to a poor and slow shooting or death of tissue-cultured plants [7].

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To overcome these, high rates of shooting have been observed in propagation of *Musa* spp. in liquid medium using the meristems of vitroplantlets as initial explants and a temporary immersion system (TIS) [8, 9]. The use of somatic embryos obtained from cell suspensions of *Musa* spp. and their regeneration into plants in liquid medium, using permanent agitation or the bioreactors of TIS have been reported in the international literature [10-12]. However, neoformation of somatic embryos in plants of the Ecuadorean plantain 'Barraganete' using the mentioned system has not yet been reported.

In Ecuador, this is the first report of the development of embryogenic cell suspensions of *Musa* spp. capable to produce somatic embryos and regenerate them into plants. This system and the ability to raise large-scale plant production could help Ecuador to restore rapidly the plantain or banana plantations lost due to intensive rain or crop age.

The objective of this study was to obtain somatic embryos of 'Barraganete' plantain from embryogenic cell suspensions and regenerate them into plants using TIS.

Materials and methods

Obtainment of embryogenic callus

The vitroplantlets of 'Barraganete' plantain were used as initial biological material to obtain embryogenic

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calli from multimeristems in proliferation or scalps [7, 13, 14].

For the generation of a higher number of embryogenic calli, necessary for the establishment of cell suspensions, obtained calli were multiplied by dividing them into small fragments and growing in the modified Murashige and Scoog medium (MS) in the presence of auxins 2,4 D-diclorophenoxyacetic acid (2,4 D) and naphthalene acetic acid (NAA) [15].

Establishment of cell suspensions

In order to obtain cell suspensions, 100 mg of embryogenic calli were placed into an Erlenmeyer flask with 10 mL of MS liquid medium in the presence of 1 mg/L of 2, 4 D [16]. This culture was maintained under a regime of continuous agitation in a rotating orbital shaker at 90 rpm, and the old medium replaced every 10-14 days with fresh liquid medium used for calli disaggregation [17-19].

Obtainment and germination of somatic embryos

For the induction of embryos from plantain cell suspensions, 1 mL of cells (0.15 mL settled cells) was placed on the surface of solid modified MS medium [13]. Two months later, to induce rapid germination, the subculture of obtained embryos was performed into MS modified culture medium in the presence of 6-furfurylaminopurine (1 mg/L) and absence of 6-benzylaminopurine (BAP), to induce rapid germination [20].

Conversion of 'Barraganete' somatic embryos into plants

One month later, the content of one Petri dish was placed into a TIS bioreactor. Embryos were immersed in 200 mL of liquid MS modified culture medium [16] during 1 min every 12 h until shooting of the embryos [21-24]. Obtained shoots, at 1 to 6 cm in height, were separated from the total biological material present in the bioreactor and recultured in solid medium for size homogenization before transplanting to the greenhouse. Shoots shorter than 1 cm and embryos at different developmental stages were returned to the bioreactor for further regeneration of plants. Later on, at greenhouse level, somaclonal variation in plants was done by visual assessments.

Results

Obtainment of embryogenic calli from scalps

The first indication of the presence of multimeristem structures (scalps) of plantain 'Barraganete' was observed 3-4 months after the vitroplantlets meristematic domes were planted in the TDZ and P4 induction culture medium [7, 13]. Two months later, after reculturing the scalps in the medium with 2,4 D, the presence of calli with different colors and consistency was observed: friable white-bone, some white with strong consistency and others. The phenolized calli were observed after placing fragments of scalps with 3-4 proliferating multimeristems in the culture medium in the presence of 2,4 D. The friable part of calli cultured for 2 months, for further multiplication in the presence of the NAA, allowed to increase the volume by 4-5 times and were later used for development of embryogenic cell suspensions (Figure 1).

Establishment of embryogenic cell suspensions of 'Barraganete' and embryos formation

After placing the friable callus in liquid medium, different types of cells were observed under the microscope (40×), including the individual embryogenic ones and clustered cells. Eight to ten weeks later, a established cell suspension was obtained showing the presence of more than 80 % of the embryogenic cells, small and round (Figure 2A and B), as described by Côté [25]. After subculturing the cells, for 2-3 months on solid medium in the absence of phytohormones, the embryonic structures of whitish color were observed and in different developing stages (globular, heart among others). Subculturing of these structures in the culture medium lacking BAP led to germination of embryos (Figure 2C and D).

Regeneration of 'Barraganete' somatic embryos into plants using TIS

After 2-3 days of placing the formed embryos into the bioreactor, containing the liquid MS modified culture medium, the phenolization of some structures was observed. However, the culture was not affected seriously although the transparency of the medium changed to purple, apparently due to the stress of the biological material caused by the hormonal content of the fresh culture medium used. The plantain shoots (578) developed in the TIS bioreactors (Figure 3A) that were larger than 1 cm in height and subcultured to solid medium for homogenization after four weeks were transplanted to the greenhouse. The rest of the

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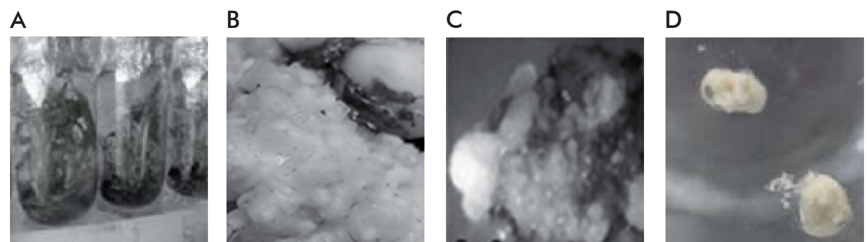

Figure 1. Different production stages of embryogenic calli of 'Barraganete' plantain from *in vitro* plants and its multiplication. A) *In vitro* propagated plants. B) Multimeristematic structures (scalps) grown in modified MS medium, 3-4 months after starting the process. C) Embryogenic 'Barraganete' calli obtained 2 months after planting scalps on solid medium containing 2,4 D (1 mg/L), D) Friable embryogenic calli ready to use for establishing cell suspensions.

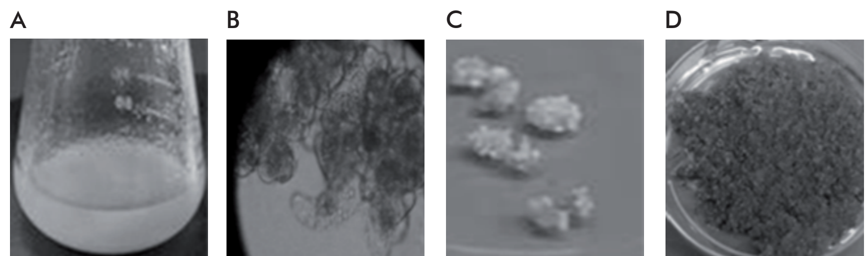

Figure 2. Different production stages of plantain 'Barraganete' embryos from cell suspensions: A) Established embryogenic cell suspension. B) Microscopic view of established embryogenic cell suspensions (40 ×). C) Embryos formed from cells grown in culture medium without 6-benzylaminopurine (BAP). D) Embryos grown for 1-2 months in culture medium with 6-furfurylaminopurine (1 mg/L).

evaluated embryos at different developmental stages (globular, heart, torpedo) and shoots shorter than 1 cm in height were returned to the bioreactor for further development and 4-5 weeks later, the second harvest of the vitroplantlets (280) was performed for further experiments.

Culture of 'Barraganete' plants in the greenhouse and evaluation of somaclonal variation

In total, the 858 vitroplantlets were obtained from 1015 somatic embryos (84.5 %) using TIS. Only the first harvest of 578 vitroplantlets was planted in the greenhouse and successfully developed into plants with a low mortality rate (10.1 %) (Figure 3B and C). In the phases 1 and 2, the detected somaclonal variation by visual assessment (off type) in plants grown in the greenhouse for 3 months was also low (1.55 %).

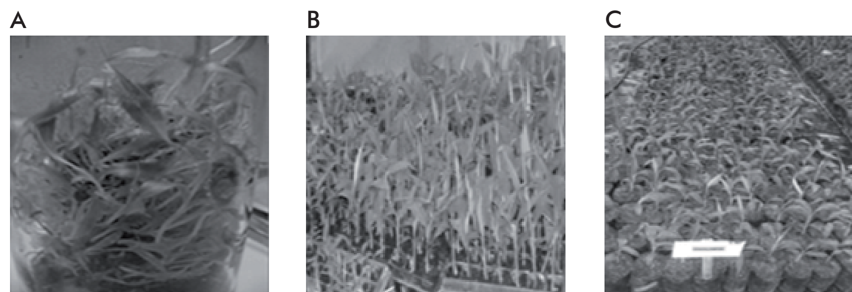

Figure 3. Plantlets of 'Barraganete' plantain obtained from somatic embryos at different developmental stages. A) Plants obtained after two months in the temporary immersion system bioreactor. B) Greenhouse phase 1; plants 6 weeks after transplantation from the bioreactor. C) Greenhouse phase 2; plants 10 days after transplantation from phase 1 for visual assessment of morphological aspects (off type plants).

Discussion

From 1 mL of embryogenic cell suspension (with 0.15 mL of pelleted cells) of plantain 'Barraganete' a total of 1015 embryos were obtained. After 4 months of culture, 858 plants were regenerated using the TIS. The conversion capacity of embryos into plants was 84.5 %. It was much higher than the one obtained by Gómez *et al*. [11], of only 295 embryos of Pelipita variety (genotype ABB) with a low percentage of regeneration into plants and obtained from 50 mL of cell suspensions applying continuous agitation of embryos in liquid medium. However, comparable results were obtained by other authors using TIS (RITA®) [12, 24].

According to the results obtained in our study, from 20 mL of established embryogenic cell suspensions of plantain (2-3 mL of precipitated cells), we could obtain 17 000-20 000 vitroplantlets by using a TIS system.

In all the processes described above, the most time consuming stages were devoted to obtain the proliferating multimeristems (scalps), the development and the multiplication of calli required for embryogenic cell suspensions.

Although the process for obtaining plantain embryogenic cell suspensions requires expertise and adequate conditions, once established the embryogenic callus and cell suspensions, a large amount of plantain plants could be obtained, even more than if they were obtained by applying conventional *in vitro* propagation methods using apical meristems explanted from corms, where culture medium phenolization and low proliferation rate of shoots occur (0.85-0.90 for each donor plant after 6-8 weeks of culture) [7].

However, a drastic decrease in time (from 10-14 months to 4-6 months) of the mentioned process could be obtained if apical meristems of *in vitro* plantlets were used to obtain embryogenic cell suspensions by planting those directly in liquid medium, without passing through the obtention of calli on the solid culture medium [20].

Although somatic embryogenesis in banana and plantain is well established and standard techniques are available, obtainment of somatic embryos is complex and not yet a common procedure. This is mainly due to a low embryogenic response of banana tissues and plantain, the long periods required to obtain embryogenic cell suspensions, the risk of somaclonal variation and the occurrence of contamination of cell suspensions [25, 26].

For these reasons, the development of embryogenic cell suspensions of the 'Barraganete' plantain following conversion into plants of somatic embryos through the use of the TIS is very important for mass production of crops in countries like Ecuador, which has an important participation in fruit exports and domestic consumption. The semi-automated process of *in vitro* propagation is needed to reduce costs in the plant propagation industry [22].

In summary, the embryogenic cell suspensions of the plantain 'Barraganete' were established and somatic embryos were obtained. Using TIS it was possible to convert somatic embryos into plants at a high percentage of regeneration (84.5 %). This method is more efficient than the conventional one using fast propagation *in vitro* through organogenesis. Finally, the low percentage of somaclonal variation in plants grown in the greenhouse (1.55 %) suggests that this method could be used for commercial propagation.

Further improvement of the methodology may involve the use of apical meristems of plantlets for faster development of embryogenic cell suspensions. Somaclonal variation in plants obtained by the method described must be followed in the field.

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