Journal of Horticultural Research 2017, vol. 25(2): 37-47
DOI: 10.1515/johr-2017-0020

DE GRUYTER
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IN VITRO PROLIFERATION AND CRYOCONSERVATION OF BANANA AND PLANTAIN ELITE CLONES

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Received: May 2017; Accepted: October 2017

Abstract

Agriculture and modern biotechnology are increasingly becoming interdependent, and many new techniques have brought new opportunities for enhancing production and marketing. Germplasm storage is an alternative for the conservation of plant genetic diversity, contributing to the improvement and maintenance of propagation programs for species of interest. In this work, banana corms were collected as plant material from relatively young commercial plantations of three different cultivars: 'Williams', Valery (AAA genome; Cavendish subgroup), and 'Barraganete' (AAB genome; Plantain subgroup). Their shoot tips were introduced into *in vitro* conditions, and subcultured monthly to obtain the required number of shoots. The shoots were subsequently rooted and stimulated to invigoration in order to extract apical meristems (0.8–1.0 mm), which were prepared for cryopreservion in liquid nitrogen (−196 °C) following preconditioning in PVS2 vitrification solution. Thereafter, the explants were rapidly thawed and then recovered and regenerated using two different methods – by Panis (2009) and Korneva et al. (2009) – consisting of two different sets of recovery and subsequent regeneration media. Statistical analysis of the results showed that the banana cultivar 'Williams' demonstrated higher survival and regeneration rates after cryopreservation using the Korneva method, whereas in cultivars 'Valery' and 'Barraganete', there were no significant differences between the tested methods. The 'Barraganete' cultivar had the lowest survival and regeneration rates, regardless of the applied method.

Key words: vitrification, cryopreservation, *in vitro* propagation, *Musa* spp, meristems

INTRODUCTION

Bananas and plantains belong to the genus *Musa* of the family Musaceae (Swennen 2000). They include cultivars of paramount importance due to their commercial, agronomic and nutritional value, and have played a significant role in the development of various communities in Latin America (Albarrán et al. 2011). Most commercial bananas and plantains belong to the *Musa* genomic groups AAA and AAB, respectively. Edible plantains and bananas are grown in more than 150 countries in the tropics and subtropics, representing a total planted area exceeding 10 million ha, with an estimated production of 100 million tons. Of this, more than 10% is exported around the world (Singh et al.

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2011). In Ecuador, plantains are grown mainly for domestic consumption, while bananas are intended for export, directly or indirectly benefiting more than 12% of the local population through each of the links in the production chain (Diario El Universo 2014). Bananas are the second-best-selling fruit in Ecuador and account for approximately 28% of total exports, generating more than 2000 million dollars of income per year (AEBE 2005).

Plant technology holds promise for enhancing agricultural production, and its development has enabled tremendous advances ranging from genetic improvement to the protection of certain plant species from extinction (Villalobos & Engelmann 1995; Rao 2004; González-Arnao et al. 2009). The development of *in vitro* cell and plant tissue culture over the last 40 years has enabled the storage and multiplication of germplasm collections of countless orthodox and recalcitrant species (Bajaj 1987; Benson et al. 2006; García-Águila et al. 2007; Cousins & Adelberg 2008; González-Arnao et al. 2008; Coronel & Henríquez, 2010; Marco-Medina & Serrano-Martínez 2012; Imarhiagbe et al. 2016; Höfer 2016). The storage of *Musa* germplasm has been carried out through *in vitro* conservation, which involves modifications of chemical and physical conditions that will reduce growth rate and enable long-term storage without frequent explant transplantations. These include supplementing culture media with growth retardants, osmotic inhibitors or tissue dehydrators, and using lower temperatures or lower light conditions (Engelmann 1997; Daniells et al. 2001; Buitink et al. 2002; Engelmann 2004; Benson 2008; Reed 2008). However, this conservation strategy has been questioned, as it can lead to long-term genetic instability (Roca et al. 1991; Patiño Torres 2010) and morphogenetic abnormalities (Adams et al. 1999; Dumet & Benson 2000). In *Musa*, these problems have been overcome by cryopreserving apical meristems, a process that is generally performed by incorporating the tissue in the cryogenic process under conditions of dehydrating and cooling (Esterbauer et al. 1988; Panis & Swennen 1995). The main purpose of the cryopreservation of selected genotypes of bananas and plantains is the long-term storage of plant genetic resources (PGRs), because some species of banana urgently require protection due to habitat loss (Agrawal et at. 2014). This storage requires minimal space and a low technical level of maintenance. Different techniques for banana storage have been developed based on pre-growth desiccation, vitrification and droplet-vitrification (Fahy et al. 1984; Gámez-Pastrana et al. 2004; Heverlee et al. 2011; Yamamoto et al. 2011, 2012). It is important to devise specific protocols for *Musa* species because they are somewhat recalcitrant with regard to cryopreservation (Panis 2009). Vitrification enables hydrated living cells to be cooled to cryogenic temperatures in the absence of ice. This technique has achieved the safe storage in liquid nitrogen of 926 accessions belonging to different genomic groups from the World Collection of *Musa* germplasm (MusaNet 2016), thus becoming an essential technique to store *Musa* cultivars.

In this work, two different methods for the recovery of cryopreserved apical meristems from the banana cultivars ‘Williams’ and ‘Valery’ (AAA *Musa* genomic group) and the plantain cultivar ‘Barraganete’ (AAB) were tested, with the aim of improving the rate of regeneration.

MATERIALS AND METHODS

Plant material collection and sterilization

Corms from three *Musa* cultivars – the ‘Williams’ and ‘Valery’ bananas (AAA genomic group) and the ‘Barraganete’ plantain (AAB) – were collected from commercial plantations (four to five years old) from the Vinces and Baba regions (Los Rios province, Ecuador). Thirty to forty healthy and vigorous corms of 30–45 cm height, each weighing approximately 500 g, were selected for each cultivar.

Plant material was established *in vitro* according to a modified protocol from Korneva et al. (2010). External bracts from the corms were removed to obtain sections 5 cm long and 3 cm in diameter containing shoot tip. The sections were washed with a flow of running water and then sterilized with a 2% commercial chlorine solution for 20 minutes, followed by three washes with sterile distilled water. After sterilization, shoot tips were reduced to 1 × 1 cm.

Organogenesis initiation and multiplication

The explants obtained thereby were placed on MS (Murashige & Skoog 1962) semi-solid medium (2 g·dm^{-3} Gelrite) and supplied with 4.43 μM 6-Benzylaminopurine (BAP). The explants were maintained for 30 days under light conditions (fluorescent tubes, 2500–3000 lux).

Following the scale of oxidation according to Cajacuri (2007), the explants were classified after 30 days as slightly oxidized (++), moderately oxidized (+++), oxidized (++++) and necrotic (+++++).

Thereafter, the explants were transferred to a multiplication medium MS supplied with 10.65 μM BAP and 1 μM indole-3-acetic acid (IAA) – for 60 to 90 days, with subcultures every 30 days.

Rooting stage

The resulting *in vitro* shoots were placed on a modified MS semisolid medium – 0.17 M sucrose without growth regulators – for 30–45 days to induce rooting and shoot thickening with the objective of extracting apical meristems capable of withstanding low temperatures. During this phase, the explants were incubated at 28 ± 2 °C, 70% humidity and with a 16-hour photoperiod.

Cryoconservation

Healthy-looking *in vitro* rooted shoots with a good radicular system were selected. Leaves, roots and parts of the pods of the apical dome were removed using a stereomicroscope under aseptic conditions of laminar flow in order to extract meristems (1 × 1 × 1 mm). Fifteen explants, with four replicates per cultivar (60 meristems), were obtained for use in the subsequent experiment. Due to the variable extraction time of meristems (six to eight minutes) it was difficult to achieve a uniform treatment by the cryoprotectants. Thus, the meristems were placed in an MS liquid medium with 0.17 M sucrose until the required number of explants had been obtained, in order to ensure that the material had undergone homogeneous treatment prior to the application of the loading solution.

Subsequently, the meristems were placed in a loading solution (LS) consisting of MS liquid medium with 2 M glycerol and 0.4 M sucrose (10 ml per explant). After 20 minutes in LS, the meristems were transferred to an in-ice, pre-cooled (0 °C) plant vitrification solution 2 (PVS2), consisting of MS liquid medium plus 3.26 M glycerol, 2.42 M ethylene glycerol, 1.90 M dimethyl sulfoxide (DMSO) and 0.40 sucrose as cryoprotectants (Sakai et al. 1990) for 15 minutes. Then, eight to ten PVS2 cool solution drops (15 μl each) containing single meristems were placed on a pre-cooled piece of aluminum foil (4 × 15 mm). Finally, each closed piece of foil containing meristems was placed in a pre-cooled cryovial and immersed in liquid nitrogen for 30 minutes.

Thawing of plant material

The cryovials were removed from the liquid nitrogen and heated in water bath at 40 ºC for 1 minute. Then, the meristems were placed on aluminum foils within a Petri dish (15 ml) with a thawing solution (TS) consisting of MS liquid medium with 1.2 M sucrose. This was constantly shaken for 15 minutes at room temperature, in a sterile air stream of laminar flow, in order to quickly defrost it and to remove the toxic PSV2 solution residues. A thermostat was not used due to the good thermic conductance of aluminum foil (Sakai & Engelmann 2007).

Recovery and regeneration

The thawed meristems were recovered by placing them on Whatman filter papers on a semi-solid medium in the dark for 24 hours. They were then transferred to a regeneration medium (without filter paper), incubated in the dark for the first week, and subsequently transferred to the light conditions under photoperiod 2500–3000 Lux for 16 hours at 26 ± 2 ºC. For this two-step process (survival followed by regeneration), two methods were tested using distinct media. 1. Recovery on MS semi-solid medium with 3 g·dm^{-3} Phytagel and 0.30 M sucrose, and regeneration on MS medium enriched with 0.08 M sucrose, 2.22 μM BAP, according to Panis (2009). 2. Recovery on half-strength MS semi-solid medium with 2 g·dm^{-3} Phytagel and 0.08 M sucrose and regeneration on MS medium enriched with 0.08 M sucrose and 100 ml·dm^{-3} coconut water, without synthetic growth regulators, according to Korneva et al. (2009). Fifteen meristems were cultured, with four repetitions per cultivar and method.

After four to six weeks on the regeneration medium, the number of meristems considered alive (green and turgid) was evaluated to determine the percentage of survival (number of living meristems/ number of tested meristems × 100). Three months

later, the number of meristems that had regenerated into plantlets was evaluated to determine the percentage of regeneration (number of sprouting meristems/number of tested meristems × 100).

The pH of all culture media was adjusted to 5.9 before sterilization by autoclaving (121 °C at 1.05 kg·cm^{-2} for 20 minutes).

Experimental design and statistics

A completely randomized block design was used, with a bifactorial (cultivar, regeneration method) arrangement. The effect of cryoconservation on meristem survival and regeneration was assessed using descriptive statistics by estimating the proper parameters of central tendency and dispersion. An F test was applied to check the homogeneity of variances, and Tukey's post-hoc test was applied after the analysis of variance for the formation of homogeneous subgroups. All the statistical inferences were realized with a 5% significance level using InfoStat and the SPSS 12.0 program.

RESULTS

In vitro initiation of cultures

Explant development under *in vitro* conditions was successful. Only 3-15% (depending on cultivar) of initial explants were visibly contaminated during the 30 days of culture on the initiation medium (Table 1). It is noteworthy that the method used for the disinfection of the explants had satisfactory results despite the fact that the samples were collected during the rainy season.

After 30 days in this medium, visually healthy explants were transferred to a multiplication medium. A thickening in the meristematic tissue with moderate oxidation in the reserve area was observed in some explants, while the rest maintained green and increased in size (Fig. 1).

The degree of oxidation was slight for the 'Williams' and 'Valery' cultivars (AAA) and moderate for the 'Barraganete' (AAB). No necrosis was observed in the 'Valery', while the 'William's and the 'Barraganete' exhibited necrosis rates of 3% and 15%, respectively (Table 1).

Organogenesis initiation and multiplication

Organogenesis was carried out by all the explants, but there were different responses to propagation conditions between cultivars. In the third subculture, the multiplication rates of 'Williams' and 'Valery' were 2.20 and 2.12, respectively, while the 'Barraganete' needed a fourth subculture to obtain a 1.95 multiplication rate (Table 2).

Rooting stage

After rooting, plantlets of 'Williams' and 'Valery' cultivars presented appropriate morphological characteristics, while those of the 'Barraganete' had a smaller diameter and roots, and were not suitable for meristem extraction until further one or two rooting weeks.

Cryoconservation and thawing of plant material

After thawing, for the first 24 hours after in-darkness culture, phenolization was observed around some explants, due to the enzymatic reaction. However, majority of the meristems retained a clear color for a couple of days and began phenolization on the third day. After the application of the method 1, the percentage of phenolization was as follows: 'Williams' 48%, 'Valery' 55% and 'Barraganete' 90%. After the application of the method 2, the percentages were: 67% 'Williams', 63% 'Valery' and 72% 'Barraganete' (Fig. 2).

Table 1. Oxidation and percentage of initial explants contamination and necrosis for each tested cultivar after the establishment in *in vitro* conditions, after 30 days

Cultivar	Number of explants	Oxidation scale	Rates (%)	
			Contamination	Necrosis
Williams	30	++	6	3
Valery	30	++	3	0
Barraganete	40	+++	15	15

Table 2. Number of shoots per cultivar and subculture obtained from meristems. Each subculture lasted 30 days

Cultivar	Number of sprouts per subculture				Multiplication rate
	1	2	3	4	
Williams	54	108	280	-	2.20
Valery	58	118	264	-	2.12
Barraganete	56	106	197	345	1.95

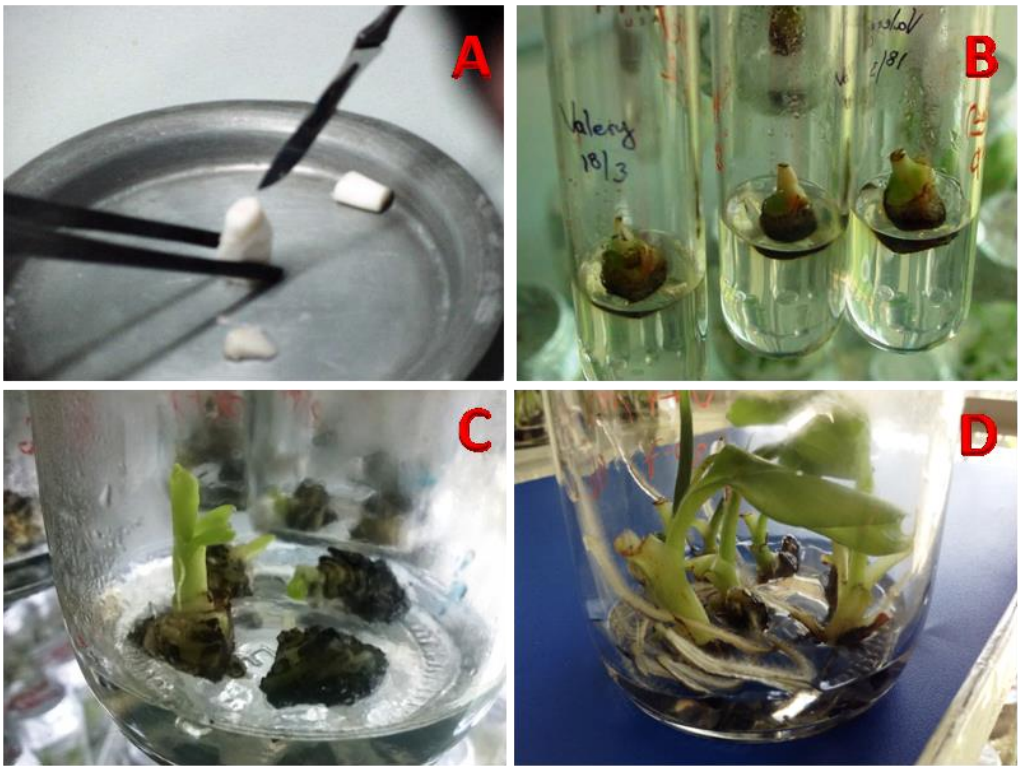

Fig. 1. Steps followed in the *in vitro* culture of banana meristems. A. Extraction of shoot tips from corms collected from 4 or 5 years old commercial plantations; B. Initial growth on MS medium supplemented with 4.43 µM BAP; C. Proliferation on MS medium supplemented with 10.65 µM BAP and 1 µM IAA; D. Rooting on MS medium without growth regulators

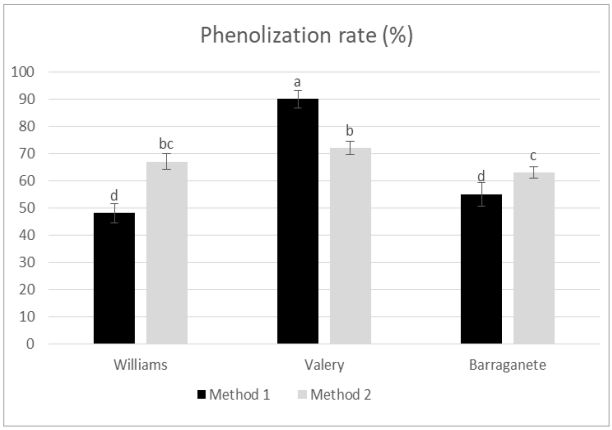

Fig. 2. Percentage of phenolization of thawed apical meristems after cryopreservation. Method 1. Recovery on MS medium with 3 g·dm^{-3} Phytagel and 0.30 M sucrose, and regeneration on MS medium with 0.08 M sucrose and 2.22 µM BAP. Method 2. Recovery on ½ MS medium with 2 g· dm^{-3} Phytagel and 0.08 M sucrose and regeneration on MS medium with 0.08 M sucrose and 100 ml· dm^{-3} coconut water. Different letters indicate significant differences (p = 0.05) ± SD.

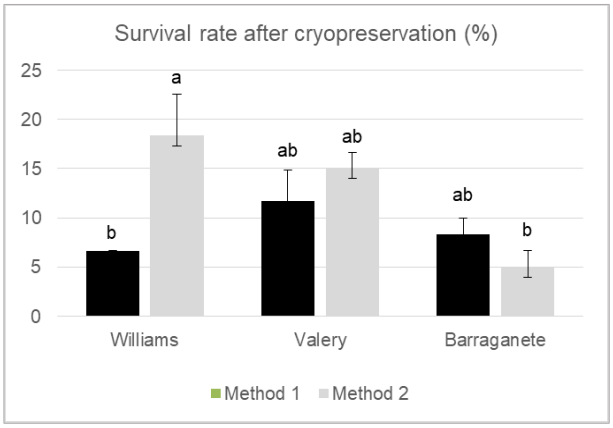

Fig. 3. Percentage of survival of meristems after cryopreservation using two different methods as in Fig. 2. Different letters indicate significant differences (p = 0.05) ± SD.

Cryoconservation and thawing of plant material

After thawing, for the first 24 hours after in-darkness culture, phenolization was observed around some explants, due to the enzymatic reaction. However, majority of the meristems retained a clear color for a couple of days and began phenolization on the third day. After the application of the method 1, the percentage of phenolization was as follows: 'Williams' 48%, 'Valery' 55% and 'Barraganete' 90%. After the application of the method 2, the percentages were: 67% 'Williams', 63% 'Valery' and 72% 'Barraganete' (Fig. 2).

Survival rate

After four to six weeks on the regeneration medium, the explants that had undergone method 2 had the highest survival rate for the 'Williams' cultivar, but no significant differences were noticed in the other cultivars. The survival results were as follows: 'Williams' 7%, 'Valery' 12% and 'Barraganete' 8% for the method 1, and 'Williams' 18%, 'Valery' 15% and 'Barraganete' 5% for method 2. For both methods, the 'Williams' and 'Valery' cultivars achieved better survival rate results than the 'Barraganete' using method 2, but these differences were significant only for the 'Williams' cv. (Fig. 3).

Some meristems that survived thawing were initially at a latent stage before they turned green after six to seven weeks incubation on the regeneration medium. For the 'Barraganete' cultivar, this occurred when method 2 was used. In addition, some meristems of 'Williams' and 'Valery' looking initially necrotic turned green in the method 2 (data not shown). In any of the cases, no presence of calli was observed that developed during the recovery and regeneration step.

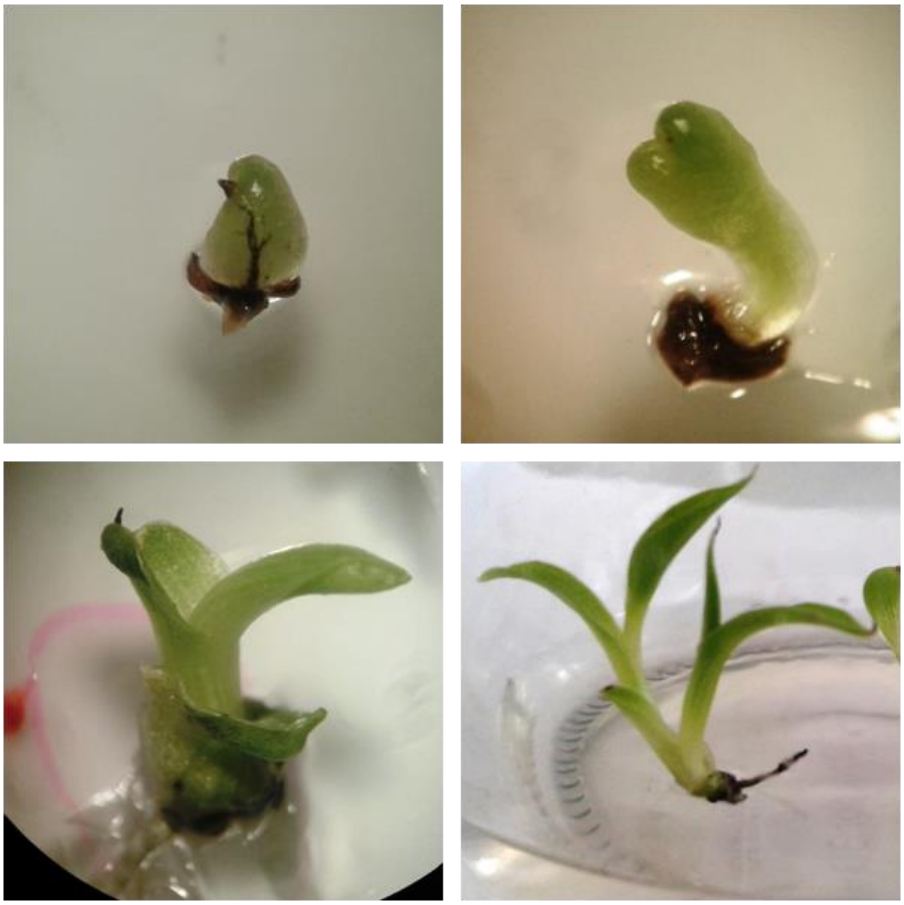

Fig. 4. Shoot development from thawed meristems

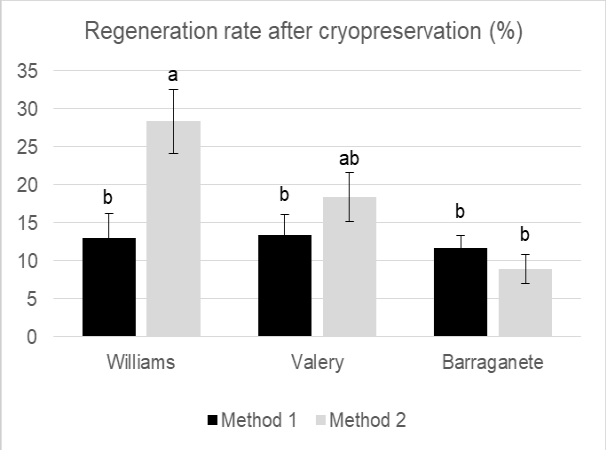

Fig. 5. Percentages of meristem regeneration after cryopreservation (apex elongation and development of green shoots meristems). Methods as in Fig. 2. Different letters indicate significant differences (p = 0.05) ± SD

Regeneration

After eight to ten weeks, apex elongation and the development of green shoots (approximately 4–6 mm in height) were observed (Fig. 4). A part of the meristems considered initially as non-living, was able to regenerate after 3 months. Only the meristems that were dark or translucent were presumed dead. The regeneration rate was calculated with respect to the initial number of meristems. In the method 1., the 'Williams', 'Valery' and 'Barraganete' cultivars showed 13%, 13% and 12% regeneration, respectively. In the method 2., the respective percentages were 28%, 18% and 9% (Fig. 5). Again, the 'Williams' cultivar yielded the best result using method 2., but these differences were not significant in the case of the 'Valery' or 'Barraganete'.

During the recovery and regeneration stages, no contaminant agents were observed, which indicates that the sterilization process was optimal.

DISCUSSION

The average degree of oxidation was higher for plantain (AAB) than for bananas (AAA) after 30 days of culture in the initiation medium. These results are in agreement with those obtained by Perea and Angarita (1984), who claimed that the AAB cultivars have higher tendency for oxidation due to higher activity of the phenol oxidases. The results were satisfactory with regard to necrosis (0.0% in 'Valery', and 3% and 15% in 'Williams' and 'Barraganete', respectively) whereas in Martin et al. (2007) study, necrosis was developed in 28% in AAA cultivars and 39% in AAB explants.

The organogenesis initiation and multiplication (1.95–2.20 multiplication rate) was effective in comparison with those obtained by Korneva et al. (2013), where 1.86 and 0.8 multiplication rates for AAA banana and AAB plantain genotypes, respectively was reported. Our results confirmed the opinion that AAA bananas are easier to multiplicate in vitro than AAB plantains.

Rooting of shoots was slower in plantains than in bananas. Root induction, and shoot thickness are important for the vigor and wellness of meristems that can resist the subsequent cryoconservation procedure. This assumption is fully in line with Henshaw et al. (1985), who indicated that the physiological condition of the donor plantlets is a particularly important and significant factor in the morphogenesis of the explants after immersion in liquid nitrogen.

After thawing, the phenolization of the cryopreserved meristems was higher for plantains than for bananas. Bananas had better survival rates than plantains regardless of the method used. Korneva et al. (2009) obtained higher survival rate for 'Williams' cv. (34%). Nobody reported cryopreservation of 'Valery' and 'Barraganete' cultivars, but their survival rates can be assumed to be similar to those of other genetically related cryopreserved cultivars: 14–27% for AAA cultivars and 5–35% for AAB cultivars (Panis et al. 2002).

The concentration of sucrose in the method 2. was lower than the counterparty, and BAP was partially replaced with coconut water. Sucrose can be a very efficient membrane protector, inducing physiological and metabolic changes that lead to cryoprotection (Crowe et al. 1987). This compound can maintain the crystalline state of the membrane and stabilize proteins under freezing conditions (Kendall et al. 1993). Nevertheless, it has been shown that a low sucrose concentration could have a beneficial effect on rehydrating the cryopreserved tissues. The lower concentration reduces the osmotic potential, thus enabling the cell

to incorporate water gradually. This reduces stress response of the tissue (Digilio 2015). On the other hand, the addition of coconut water to the culture media yields good results and, if it is used with the addition of auxins, it leads to strong cellular division in the meristems (Ayerbe Mateo-Sagasta 1990). The mechanism by which coconut water promotes cell division is unclear, but it is believed that it could be due to the presence of zeatin (Steward & Krikorian 1971).

Percentage of regeneration was higher for 'Williams' cultivar using method 2., but in the 'Valery' and 'Barraganete' results did not differ significantly. It should be noted that in the other study Korneva et al. (2009) found that regeneration rates depend on individual characteristics of the cultivar in response to thermal or cold stress. This effect could not be related to a different behavior between genomic groups; in other words, the accession response is different within the same genotype. According to Thinh et al. (1999), many different *Musa* genotypes have been cryopreserved using drop-vitrification, which up to then had been considered the most appropriate method for meristemal apex culture (Panis & Lambardi 2005). However, it was reported that the quality of meristematic tissue of AAB genome cultivars is too low to be used for such method of cryopreservation. An alternative would be the use of proliferating clumps obtained on a medium containing high concentrations of cytokinins (10 $mg \cdot dm^{-3}$ TDZ) (Strosse et al. 2008). Better results of shoot regeneration were obtained from cryoconserved explants of the AAA and ABB genomes (69% and 43%, respectively) than from the AAB genome (20%) (Korneva et al. 2009). However, this method was not used because a higher risk of somaclonal variation that has been reported from it (Pocasangre 1992). Cryopreservation may have a positive effect on the elimination of bacteria, viruses, and other pathogens (Wang et al. 2008). Relatedly, the success of cryotherapy in the elimination of the mosaic virus (CMV) in the 'Williams' banana cultivar has been reported (Helliot et al. 2003; Torres et al. 2011).

In summary, this work clearly demonstrates that the protocol described by Korneva et al. (2010) achieves satisfactory disinfestation and multiplication rates for the two cultivars used here. The two methods tested for the survival and regeneration of cryopreserved tissue, by Panis (2009) and. Korneva et al. (2009) led to different results between genotypes, with the best survival and regeneration rates for the 'Williams' cultivar using method 2, although there were no significant differences in the 'Valery' and 'Barraganete' cultivars. Furthermore, the 'Barraganete' cultivar showed the worst results regardless of the method. The use of BAP could be a problem when regenerating the 'Williams' cultivar, but its effect on the 'Valery' and 'Barraganete' cultivars remains unclear. The plants obtained from cryopreservation will be used to study the performance with regard to somaclonal changes under greenhouse conditions. There are many banana and plantain cultivars in Ecuador whose genetic characteristics have not yet been studied; consequently, it is advisable to evaluate a possibility of their conservation in a safe way.

Acknowledgements

This work was funded by the Centro de Investigaciones Biotecnológicas de Ecuador (CIBE). It was also supported by C. Aguaguiña and O. Ruiz, Graduate and PhD in Statistics from ESPOL, respectively. This work fulfils part of the requirements for G. Reyes's Master's Degree.

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