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Cryopreservation of banana's cv Grand Naine *in vitro* rhizomes

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ABSTRACT

The preservation of banana genetic material is usually performed through seedlings. However, most banana cultivars do not produce seed and are propagated vegetatively. Therefore, cryopreservation is a feasible technique that allows the preservation of banana genotypes indefinitely. For the success of cryopreservation protocols, the selection of cryoprotectants and pre-freezing techniques are important factor. Therefore, the objective of this study was to verify the effects of different cryoprotectants with and without 1% phloroglucinol and pre-cooling periods on the development of a protocol for cryopreservation of *in vitro* rhizomes of *Musa accuminata* (AAA) cv Grand Naine banana. The addition of 1% phloroglucinol to the cryoprotective solutions, such as PVS2 enhanced recovery of cryopreserved banana rhizomes. In addition, pre-cooling of explants in ice for 3 hours in PVS2 + 1% of phloroglucinol allowed efficient cryopreservation of banana rhizomes, followed by successful recovery and regeneration of *in vitro* shoots of banana cv Grand Naine.

Key words: *Musa acumminata*, *in vitro* conservation, cryoprotectants, pre-cooling.

INTRODUCTION

Currently, seeds are used for the preservation of banana plant material. However, not all banana species and/or cultivars produce seeds, and mostly are propagated vegetatively. Therefore, alternatives to banana germplasm storage need to be addressed. The preservation of genetic material under *in vitro* conditions is a promising technique and ensures the conservation of plant material for short time periods. However, genetic variation and contamination are potential risks (Carvalho and Vidal 2003).

Cryopreservation is a feasible technique that reduces the risk of contamination and genetic variation, while allowing successful long-term storage of plant material, as demonstrated in several plant species, including banana (Panis et al. 1990). The classical cryopreservation technique involves controlled cooling of plant material to a pre-determined temperature, followed by rapid immersion in liquid nitrogen. However, protocols for cryopreservation need to be adjusted to prevent intracellular ice formation, which causes disruption

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of the cell membrane system, resulting in loss of semi-permeability and cell compartmentalization; and consequently cell collapse and death (Nogueira 2010).

New cryopreservation protocols have been developed, which involve the use of cryoprotectants to allow cooling of plant cell contents into a vitrification state without the formation of ice crystals (Panis et al. 2007). Vitrification allows water to transition from the liquid phase to an amorphous solid and metastable phase, thus avoiding the formation of ice crystals within the cell (Santos 2001, Engelmann 2011), and preserving cell integrity (Wowk 2010). There are different classes of cryoprotectants according to their ability to penetrate plants cells and induce the vitrification state.

Sugars such as sucrose, trehalose and glucose have been used as cryoprotectants and are regarded as vitrification agents without causing toxicity to plant cells, even when accumulated in the cytoplasm. They are highly effective in stabilizing cell membranes during freezing over traditional cryoprotectants (Panis et al. 2002). Phloroglucinol (1,3,5–trihydroxybenzene) or phloroglucin (PG tautomer), a benzenetriol that has growth regulating properties (Sarkar and Naik 2000) and promotes plant growth (Teixeira da Silva et al. 2013, Perez et al. 2016) has been used as a cryoprotectant. Phloroglucinol has shown to enhance growth and rate of axillary shoots on *in vitro* cultures in several woody plants, to initiate adventitious roots in *in vitro* shoots of different woody species, to enhance survival of meristems and/or shoot tips *in vitro* (Jones and Hatfield 1976, James and Thurbon 1979, 1981), to enhance shoot multiplication and elongation (Gururaj et al. 2004, Giridhar et al. 2005, Siwach and Gill 2011, Wang et al. 2011, Bairwa et al. 2012), and root proliferation (Romais et al. 2000, Buthuc-Keul and Deliu 2001, Sujatha and Kumar 2007, Bopana and Saxena 2009, Kumar et al. 2010, Tallon et al. 2012), embryogenesis induction (Find et al. 2002, Reis et al. 2008) and improved recovery of cryopreserved protocorms (Vendrame and Faria 2011).

In banana, cryopreservation protocols have been developed for *in vitro* apical banana meristems (Thinh et al. 1999, Panis et al. 2005a) and banana meristem clusters (cauliflower-like structures) (Dhed'a et al. 1991, Schoofs 1997, Strosse et al. 2006). However, the process involves a series of steps, which increase the time for recovery of cryopreserved material and can also increase the risk of contamination (Panis 2009). Therefore, the development of new cryopreservation protocols that could use alternative tissues, such as rhizomes, could reduce the number of steps necessary for recovery of cryopreserved banana tissues, thus accelerating regrowth and also reducing the risk of contamination.

The objective of this study was to develop a protocol for cryopreservation of *in vitro* rhizomes of banana *Musa accuminata* (AAA) cv Grand Naine. The effects of different pre-cooling periods and cryoprotectants with and without 1% phloroglucinol for the cryopreservation protocol development were also evaluated.

MATERIALS AND METHODS

Banana plants were selected for this study from plants growing in a shade house at the Tropical Research and Education Center (TREC), University of Florida, in Homestead, Florida, USA. Well-developed plants were transferred to a greenhouse at TREC for continued growth and development. Fully developed plants with healthy rhizomes were used as source of explants (Fig. 1a).

Plantlets with healthy rhizomes were sprayed with systemic fungicide Daconil a day prior to rhizome extraction for *in vitro* culture establishment. The upper part of plants were removed prior to removal of rhizomes (Fig. 1b). Removed rhizomes were transferred to the Ornamental Horticulture's

Laboratory at TREC were cryopreservation experiments were subsequently performed.

In vitro RHIZOME CULTURE ESTABLISHMENT

In the laboratory, the outer sheaths of the rhizomes were removed and reduced in size to approximately 6-cm long x 3-cm in diameter. Disinfection of tissues was performed with 0.5 mg L^{-1} streptomycin for 20 minutes. The explants were then transferred to a laminar flow hood, where they were immersed in ethanol (75%) for 5 minutes, followed by sodium hypochlorite (2%) for 30 minutes, and then rinsed 3 times in autoclaved distilled water (Fig. 1c).

The culture medium was comprised of MS (Murashige and Skoog 1962) salts and vitamins supplemented with 7.0 mg L^{-1} 6-benzylaminopurine (BAP), 30 g L^{-1} sucrose, 2.5 g L^{-1} activated charcoal, and pH adjusted to 5.8 ± 0.1, before adding 7 g L^{-1} agar. The medium was autoclaved at 121 °C and 20 psi for 20 minutes. Explants were further reduced in size to 3-cm long segments and placed into 200 ml flasks containing 30 mL of semi-solid MS culture medium. Cultures were maintained in the dark at 27 ± 2 °C for 2 weeks.

Two experiments were performed to evaluate different cryoprotectants and different pre-cooling periods, respectively. A total of 200 rhizomes were selected for the first experiment on cryoprotectants, and another 200 rhizomes for the experiment on pre-cooling periods.

EXPERIMENTS

Experiment I: Cryopreservation of banana rhizomes using different cryoprotectants

A total of 200 healthy rhizomes were selected and again reduced in size to 0.5 cm prior to exposure to different treatments (Fig. 1d). For each treatment, 1 ml of wash solution (WS) composed of 2 M glycerol and 0.4 M sucrose (Nishizawa et al. 1993) was added to 15-ml Falcon tubes. A pre-vitrification solution (PVS2) was also added to the treatments (Fig. 1e) and tubes were pre-cooled in ice (Fig. 1f). The PVS2 solution consisted of 30% (w/v) 3.26 M glycerol, 15% (w/v) 2.42 M ethylene glycol, 15% (w/v) 1.9 M DMSO and 0.4 M sucrose (Sakai 1997). Treatments consisted of:

T1 – No cryoprotectants (control);

T2 – 2 M Glycerol (20 minutes) + PVS2 (10 minutes);

T3 – 2 M Glycerol (20 minutes) + PVS2 with 1% phloroglucinol (10 minutes);

T4 – 0.4 M sucrose (20 minutes) + PVS2 (10 minutes);

T5 – 0.4 M sucrose (20 minutes) + PVS2 with 1% phloroglucinol (10 minutes);

T6 – 2 M Glycerol (20 minutes) + 0.4 M sucrose (20 minutes) + PVS2 (10 minutes); and

T7 – 2 M Glycerol (20 minutes) + 0.4 M sucrose (20 minutes) + PVS2 with 1% phloroglucinol (10 minutes).

For all treatments, 15-ml Falcon tubes with samples were wrapped in aluminum foil and placed in Liquid Nitrogen (LN) for 48 hours (Fig. 1g and h). After 48 hours in LN, Falcon tubes were removed and rapidly heated to 40 °C in a water bath for 2.5-3.0 min, and the cryoprotectant solutions removed from the tubes. The explants were washed in ½ MS + 1.2 M sucrose (pH 5.7) for 15 min and introduced into ½ MS + 0.3 M sucrose (pH 5.7), solidified with 7.0 g L^{-1} agar and maintained in the dark at 27 ± 2 °C for 24 hours (Panis 2009).

After 24 hours, the rhizomes were subdivided and transferred to MS medium containing 4.0 mg L^{-1} BAP (Fig. 1i). Cultures were maintained for 30 days in a growth chamber under controlled conditions at 27 ± 2 °C, 60 µmol m^{-2} s^{-1} PAR; and 18/6 light/dark photoperiod under 4 9A fluorescent lamps (Philips®).

After 30 days of culture establishment, any oxidized parts of banana's explants were removed and the explants transferred to fresh MS medium containing 4.0 mg L^{-1} BA and maintained in

Figure 1 - Cryopreservation procedures for Grand Naine banana. Plants selected for the study were located in a shade house at the Tropical Research and Education Center, University of Florida (**a**). Upper portion of plants were removed prior to removal of rhizomes (**b**). Disinfection of rhizomes under laminar flow hood (**c**). Detail of rhizome after disinfection and size reduction for cryopreservation (**d**). Rhizomes placed in Falcon tubes containing pre-vitrification solution (PVS2) (**e**). Pre-cooling of samples prior to cryopreservation (**f**). Falcon tubes are wrapped in aluminum foil (**g**) and placed in liquid nitrogen (**h**). After cryopreservation, rhizomes are subdivided and placed in MS medium for growth and development (**i**).

a growth chamber under the same controlled conditions as described above.

At 10 and 30 days after transfers to fresh medium, culture oxidation was evaluated and ranked using scores from 1 to 3, where 1 = low oxidation, 2 = medium oxidation, and 3 = high oxidation of explants. The color of the meristem was also evaluated, whereby green meristems are an indication of organogenic potential. In addition, percentage of explant survival (%), and the development of plantlets (shoot multiplication, length of shoots, root multiplication, and length of roots) were evaluated. All variables were submitted to analysis of variance and means compared by Tukey's test at a = 5%.

Experiment II: Cryopreservation of banana rhizomes under different pre-cooling periods

A total of 200 healthy rhizomes were selected and again reduced in size to 0.5 mm prior to exposure to different treatments. For each treatment, 1 ml of wash solution (WS) composed of 2 M glycerol and 0.4 M sucrose (Nishizawa et al. 1993) was added to 15-ml Falcon tubes. The PVS2 solution was also added to the treatments. The pre-cooling treatments were in 0 °C. The treatments consisted of:

T1 – PVS2, no pre-cooling (control 1);

T2 – PVS2 +1% phloroglucinol, no pre-cooling (control 2);

T3 – PVS2, pre-cooling for 1h;

T4 – PVS2 + 1% phloroglucinol, pre-cooling for 1h;

T5 – PVS2, pre-cooling for 2h;

T6 – PVS2 + 1% phloroglucinol, pre-cooling for 2h;

T7 – PVS2, pre-cooling for 3h;

T8 – PVS2 + 1% phloroglucinol, pre-cooling for 3h.

For all treatments, 15-ml Falcon tubes with samples were used and placed in Liquid Nitrogen (LN) for 48 hours. After 48 hours in LN, Falcon tubes were removed and rapidly heated to 40 °C in a water bath for 2.5-3.0 min, and the cryoprotectant solutions removed from the tubes. The explants were washed in ½ MS + 1.2 M sucrose (pH 5.7) for 15 min and introduced into MS + 7.0 g L^{-1} BA + 2.5 g L^{-1} activated charcoal, solidified with 7.0 g L^{-1} agar and maintained at 27 ± 2 °C in the dark for 1 week.

After 1 week, rhizomes were subdivided and transferred to MS medium containing 4.0 mg L^{-1} BAP and maintained for 30 days in a growth chamber under controlled conditions at 27 ± 2 °C, 60 µmol m^{-2} s^{-1} PAR; and 18/6 light/dark photoperiod under 4 9A fluorescent lamps (Philips®).

Explants were removed from dark at 7 and 30 days and evaluated for oxidation using the same scores as used above (1-3). The percentage of explant survival (%), and the development of plantlets (shoot multiplication, length of shoots, root multiplication, and length of roots) were also evaluated. All variables were submitted to analysis of variance and means compared by Tukey's test at a = 5%.

EXPERIMENTAL DESIGN

Both experiments were conducted using a completely randomized design with 5 replications and 4 explants per replication. Of the total 200 rhizomes selected for each experiment, 160 rhizomes were used in the first experiment and 140 rhizomes in the second experiment. This is because of contamination in some of the rhizomes initially selected, which were discarded.

Data were normalized by the formula √x + 0.5 and subjected to analysis of variance. Means were separated by the Tukey's test at 5% probability using the statistical software Sisvar (Ferreira 2011).

RESULTS AND DISCUSSION

EXPERIMENT I

For any cryopreservation protocol, success is determined by the recovery of viable propagules

(Reed 2008) and oxidation can be a problem for the survival of the explant.

After removal from liquid nitrogen and thawing, it is a common procedure to wash the plant tissues with a solution of sucrose (Panis 2009). Therefore all explants were submitted to a 1.2 M sucrose treatment for 15 minutes and 0.4 M sucrose in the media for 1 week. Sugars, such as sucrose are regarded as vitrification agents and therefore used as cryoprotectants because they have no toxic effects on plant cells even when accumulated in the cytoplasm (Teixeira da Silva et al. 2013). They are highly effective to induce osmotic and colligative effects, i.e. reducing osmotic potential, leading to a reduction in the water content of scalps so that sugars may function directly to confer dehydration and/or freezing tolerance (Agrawal et al. 2004, Halmagyi and Deliu 2007, Panis et al. 2002, Panis 2009). In addition, the concentrations of sugars may influence proteins, membrane fatty acids and amino acids within cells, imparting tolerance to dehydration and cryopreservation (Panis et al. 2002, Sipen et al. 2011). However, in our study, this procedure was not satisfactory and most of the explants showed oxidation 5 hours after introduction into culture. It is possible that in our study the concentration of sucrose was not sufficient to avoid oxidative damage to the cells at the membrane level as well as to their proteins. It is also possible that the reactive oxygen species (ROS) formation increased due to dehydration, resulting in lipid peroxidation, denaturation of proteins, and nucleic acid damage (França et al. 2007). However, additional studies would be necessary to evaluate the specific causes of oxidation.

Similar results with banana (*Musa*) cvs. Pisang Mas, Pisang Nangka, Pisang Berangan and Pisang Awak demonstrated that sucrose at 0.4 or 0.5 M was not effective for cryopreservation of scalps of all banana cvs. tested, with no or low survival following preculture (Sipen et al. 2011). However, a pre-culture with 0.4 or 0.5 M sucrose for 2 weeks was effective for cryopreservation of scalps of banana cvs. Bluggoe (ABB), Monthan (ABB), Kamaramasenge (AB), Guyod (AA) and Grand Naine (AAA), with survival between 12 - 72% (Panis et al. 1996a, 2002).

Although banana Grand Naine scalps had good results according Panis et al. (2002), in our study rhizomes did not promote the same response to sucrose treatment, showing external oxidation in most of the explants. These results are similar to those reported by Panis et al. (1996b), who observed that media containing higher sucrose levels retard the growth of proliferating meristems considerably. By increasing the sucrose level, lower survival rate and a concomitant blackening of tissue was observed in banana's cultivar Bluggoe (*Musa* spp., ABB group) (Panis et al. 1996b).

Ten days after rhizomes were subdivided and placed in fresh MS medium, the percentage of survival ranged from 22% to 89%. Despite the survival percentage of T3, T5 and T7 were not statistic different, the higher survival percentage (89%) was observed for explants submitted to treatment 7 (T7), which contained 2 M Glycerol + 0.4 M sucrose + PVS2 with 1% phloroglucinol (Table I). For all treatments containing 1% of phloroglucinol, the percentage of explant survival was higher (61% for treatment 3 – T3 and 79% for treatment 5 – T5) (Table I).

Cryoprotectans are important substances that can prevent the formation of ice crystals within the cells. Tao and Li (1986) classified the cryoprotectants used in cryopreservation of plants according to their ability to penetrate cells, that is, unable to penetrate the cell wall (high molecular weight polymers - PEG6000, PVP - polysaccharides and proteins); only able to penetrate cellular wall (oligosaccharides, mannitol, amino acids and low molecular weight polymers); and able to penetrate the cell wall and plasma membrane (DMSO and glycerol). Most vitrification solutions include a mixture of penetrating and non-penetrating

TABLE I

Experiment I. Cryopreservation of banana rhizomes using different cryoprotectants. Data shows survival (%), oxidation score (1-3), shoot length (cm), number of roots, and root length (cm) for *in vitro* shoots 10 and 30 days after removal from cryopreservation. Two cryoprotectants were evaluated: 1) PVS2: 30% (w/v) 3.26 M glycerol, 15% (w/v) 2.42 M ethylene glycol, 15% (w/v) 1.9 M DMSO and 0.4 M sucrose; and 2) PG: 1% phloroglucinol.

Treatments	Survival (%)		Oxidation score	Shoot length (cm)		Number of roots		Root length (cm)	
Days	10	30	10	10	30	10	30	10	30
T1 – No cryoprotectants	0.00b	0.00c	2.83c	0.00c	0.00c	0.00a	0.00c	0.00a	0.00b
T2 – 2M glycerol + PVS2	0.00b	0.00c	1.91b	0.00c	0.00c	0.00a	0.00c	0.00a	0.00b
T3 – 2M glycerol + PVS2 + 1% PG	61ab	2.68b	0.69a	1.58b	4.88ab	0.00a	2.20b	0.00a	4.53a
T4 – 0.4 M sucrose + PVS2	22b	0.40bc	1.04b	1.11 b	1.33b	0.00a	0.00c	0.00a	0.00b
T5 – 0.4 M sucrose + PVS2 + 1% PG	79a	4.80a	0.40a	3.52a	4.99ab	0.00a	2.56 b	0.00a	4.75a
T6 – 2M glycerol + 0.4M sucrose + PVS2	27b	2.48b	1.11b	2.22b	1.63b	0.00a	0.00c	0.00a	0.00b
T7 – 2M glycerol + 0.4M sucrose + PVS2 + 1% PG	89a	4.88a	0.28a	3.60a	5.71a	1.20a	3.65a	2.14a	4.86a

cryoprotectants in order to reduce the toxicity and help stabilize the vitrification state (Reed 2008).

Phloroglucinol is a precursor in the lignin biosynthesis pathway and effectively controls hyperhydricity through the process of lignification, thus maximizing the multiplication rate of woody species and other species that are difficult to propagate (Teixeira da Silva et al. 2013). A number of studies report the use of phloroglucinol to improve *in vitro* shoot and root multiplication in ornamentals and herbaceous plants, with positive responses on *in vitro* morphogenesis. These include *in vitro* enhancement in growth and rate of axillary shoots and adventitious roots in woody plants, survival of meristems and shoot tips (Jones and Hatfield 1976, James and Thurbon 1979, 1981), shoot multiplication and elongation (Gururaj et al. 2004, Giridhar et al. 2005, Siwach and Gill 2011, Wang et al. 2011, Bairwa et al. 2012), root proliferation (Romais et al. 2000, Buthuc-Keul and Deliu 2001, Sujatha and Kumar 2007, Bopana and Saxena 2009, Kumar et al. 2010, Tallon et al. 2012), embryogenesis induction (Find et al. 2002, Reis et al. 2008) and improved recovery of cryopreserved tissues (Vendrame and Faria 2011).

Despite removal of oxidized parts of the rhizomes before transfer to fresh medium, oxidation was still observed afterwards. However, treatment 7 (T7) had the lowest oxidation in addition to high survival, which promoted better *in vitro* plant shoot and root development (Table I, Figure 2a). This is due to a possible synergistic effect among glycerol, sucrose and phloroglucinol in T7 in reducing the oxidative damages caused by dehydration.

For all developed rhizomes, only one shoot formed initially, varying in length from 1.11 cm (T5) to 3.60 cm (T7). Both treatments T5 and T7 contained 1% phloroglucinol (Table I), showing a positive effect on the development of *in vitro* shoots of banana Gran Naine. This is the first study using phloroglucinol in rhizome banana's cryopreservation showing that it can increase the survival of *in vitro* explants. Similarly, Vendrame and Faria (2011) showed that the addition of 1% of phloroglucinol to the medium increased recovery and survival of *Dendrobium nobile* protocorms to 68%. Phloroglucinol has antioxidant properties

(Athanasas et al. 2004, Benson and Bremner 2004, Kang et al. 2006), which may have contributed to the survival of banana cv Grand Naine by reducing oxidative stress in the cells.

Rooting of explants was initially observed only in treatment 7 (T7), with average root length of 2.14 cm (Table I). Subsequently, treatment T7 had the best root development and therefore the largest number of roots as compared to other treatments (Table I, Figure 2a). Treatments T3 and T5 were not significantly different, with root length of 2.20 cm and 2.56 cm, respectively. However, the combination of cryoprotectants with phloroglucinol shows a positive response in root formation, as depicted in Table I and this might have enhanced the cryoprotective effect by reducing potential oxidative stress damage caused to cells and membranes. Therefore, additional studies are warranted to evaluate the effects of cryoprotectants combined with phloroglucinol in reducing oxidative stress and increasing plant antioxidant defenses and tolerance to dehydration. After 30 days of evaluation of rhizomes, an increment in the number of shoots was observed (Table I) for several treatments, such as 4.80 shoots for T5 and 4.88 shoots for T7 (Figure 2a). The greatest length of shoots, 5.71cm and 4.99 cm was also observed for T5 and T7, respectively (Table I). Although the length of shoots was not significantly different from other treatments, treatments with 1% phloroglucinol added to the cryoprotectant solution demonstrates its positive action on plant development after cryopreservation.

Possibly the combination of sucrose, glycerol and PVS2 associated with phloroglucinol promoted greater protection of banana Grand Naine rhizomes, providing better tissue differentiation of *in vitro* plants. Cryoprotectants may have acted to replace water in the cells, while the PVS2 changed the non-penetrating freezing behavior of the solution by osmotically dehydrating the intracellular solution. Subsequently, the lower temperature restricted the mobility of the water molecules inside the cell, thus preventing the nucleation of ice crystals (Reed 2008), and consequently promoting better organogenic response of *in vitro* tissues.

EXPERIMENT II

Rhizomes that were not submitted to pre-cooling in either PVS2 or PVS2 with 1% phloroglucinol did not survive after cryopreservation.

Cryopreservation provides stable, long-term, low-cost storage of plants safe from diseases or environmental damage. However, storing plants at extremely low temperatures does have some deleterious effects (Uchendu et al. 2010). Chilling and freezing injury promote many sub-lethal changes, such as metabolic uncoupling, which can then lead to increased production of free radicals (Day et al. 2000). Reactive oxygen species (ROS) include superoxide radicals (O_2), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2) (Asada 2006), which can lead to a marked oxidation in the plants. In our first experiment, we expected sucrose to act positively in enhancing recovery of cryopreserved rhizomes and consequently reducing oxidation. However, because the treatment with sucrose did not return positive results for the first experiment, this treatment was not used in the second experiment. In our study we added 2.5 $g.L^{-1}$ activated charcoal to the MS medium to mitigate oxidation.

Oxidation was observed in all treatments (Table II). Treatments not submitted to pre-cooling had the highest scores (2.73) for oxidation, and no plant regeneration was observed. For treatments submitted to pre-cooling for 1 or 2 hours, oxidation was reduced (0.52 - 1.97), but some rhizomes showed the capacity for regeneration. Despite the addition of activated charcoal in MS medium for 7 days, oxidation was still present.

For treatments with pre-cooling (T3-T8), survival percentages 7 days after cryopreservation

Figure 2 - Grand Naine banana's plantlets developed after cryopreservation. Experiment I evaluated the effect of different cryprotectants in plantlet development after cryopreservation (**a**). Experiment II evaluated the effects of different pre-cooling periods after cryopreservation (**b**).

ranged from 40 to 100% (Table II). Treatments submitted to 3 hours of pre-cooling with PVS2 or PVS2 + 1% phoroglucinol had 100% survival (Table II).

The key to successful cryopreservation by vitrification is the careful control of the procedures for dehydration and cryoprotectant permeability to prevent injury by chemical toxicity or excess osmotic stress during dehydration (Niino et al. 2007). Therefore, optimizing the exposure time of tissues to PVS2 is the most important parameter for ensuring successful plant regeneration from cryopreserved seeds following vitrification. The exposure duration of plant cells to the vitrification solution is critical for their survival because dehydration may result in cell injury by chemical toxicity and excess osmotic stress from cryoprotectants. The suitable dehydration duration can be related to several factors, including the nature and the size of the sample, and the composition and loading procedure of cryoprotectants (Chen and Wang 2002).

The length of the shoots formed 7 days after removal from cryopreservation was highest (4.52 cm) for the treatment submitted to 3 hours of pre-cooling in PVS2 + 1% phloroglucinol. This result indicates that the slow cooling of explants for 3 hours in cryoprotective agent (PVS2) + 1% phloroglucinol influences rhizome regeneration after the cryopreservation process.

The incubation time and temperature of the PVS2 solution affect the germination rate of cryopreserved seeds because overexposure to PVS2 can cause chemical toxicity. By determining the best dehydration time of explants at 0 °C, the toxicity of the vitrification solution can be reduced and, consequently, the exposure time to PVS2 solution can be increased for a successful cryopreservation protocol (Johari et al 2009, Vendrame et al. 2008). Vendrame et al. (2008) showed that pre-cooling treatment (ice) combined with PVS2 treatment for a period of time between 1 and 3 h was essential to allow proper germination of cryopreserved seeds of four commercial *Dendrobium* hybrids genotypes (Galdiano et al. 2014). Likewise, in this study, the dehydration period was similar and 1 h in PVS2 can be recommended. A moderate germination rate for the 6-h treatment in PVS2 was observed, leading to the hypothesis that this *Dendrobium* hybrid could have an intrinsic trait of high dehydration tolerance. Although they are different species, the ice pre-cooling treatment is efficient on the organogenic regeneration of plants, varying with the species and/or genotype studied.

Panis et al. (2005b) showed that regrowth of banana meristems is not influenced by loading solution exposure time (0 - 5 hours). Although the precise mechanism of loading is not yet fully understood, it has been proven for different plant species that loading can dramatically enhance the tolerance of isolated meristems to dehydration by the vitrification solution (Matsumoto et al. 1994, Takagi et al. 1997, Panis 2009).

Treatments submitted to 3 hours of pre-cooling with PVS2 or PVS2 + 1% phoroglucinol had the highest number of shoots per explant 30 days after cryopreservation, with an average of 3.04 shoots per explant for T7 and 3.67 shoots per explant for

TABLE II

Experiment II. Cryopreservation of banana rhizomes under different pre-cooling periods. Data show survival (%) oxidation score (1-3), and shoot length (cm) for *in vitro* shoots 7 days after removal from cryopreservation and number of shoots, shoot length (cm), number of roots and roots length (cm) for *in vitro* shoots 30 days after removal from cryopreservation . Two cryoprotectants were evaluated: 1) PVS2: 30% (w/v) 3.26 M glycerol, 15% (w/v) 2.42 M ethylene glycol, 15% (w/v) 1.9 M DMSO and 0.4 M sucrose; and 2) PG: 1% phloroglucinol.

Treatments	Survival (%)	Oxidation score	Shoot length (cm)	Number of shoots	Shoot length (cm)	Number of roots	Root length (cm)
T1 – PVS2, no pre-cooling	0c	2.73d	0.00b	0.00b	0.00b	0.00a	0.00a
T2 – PVS2 + 1%PG, no pre-cooling	0c	2.73d	0.00b	0.00b	0.00b	0.00a	0.00a
T3 – PVS2 + 1 hour pre-cooling	40ab	1.97c	1.76ab	1.58ab	3.76ab	0.00a	0.00a
T4 – PVS2 + 1%PG +1 hour pre-cooling	50ab	1.04c	1.60ab	1.87ab	3.60ab	0.00a	0.00a
T5 – PVS2 + 2 hours pre-cooling	55ab	1.48c	1.92ab	1.6ab	3.92ab	0.00a	0.00a
T6 – PVS2 + 1%PG + 2 hours pre-cooling	60ab	0.52b	1.67ab	2.08b	3.67ab	0.00a	0.00a
T7 – PVS2 + 3 hours pre-cooling	100a	0.00a	2.96a	3.04a	4.96a	0.00a	0.00a
T8 – PVS2 + 1%PG + 3 hours pre-cooling	100a	0.00a	4.52a	3.67a	5.52a	2.48a	1.80a

T8 (Table II). For other treatments submitted to pre-cooling, the number of shoots ranged from 1.58 to 2.08 shoots per explant (Table II). The number of shoots per explant was higher for all treatments that included 1% phloroglucinol (Table II).

Differences in *in vitro* shoot elongation and multiplication for banana cv Grand Naine were evidenced in concentrations of 200 to 1000 µM phloroglucinol in MS medium. Results of this preliminary study showed that 200 µM phloroglucinol promoted increased *in vitro* shoot multiplication (unpublished data).

Treatments submitted to 3 hours of pre-cooling also had greater shoot length. Treatment 7 (PVS2) had shoot length average of 4.96 cm, while treatment 8 (PVS2 + 1% phloroglucinol) had average shoot length of 5.52 cm (Table II, Figure 1b). For treatments 3 through 6, the average length of the shoots ranged from 3.6 cm to 3.92 cm.

Root formation was only observed for treatment 8 (PVS2 + 1% phloroglucinol), which was submitted to 3 hours of pre-cooling. The average root number was 2.48 roots per explant and the average root length was 1.80 cm (Table II, Figure 2b).

It has been reported that phloroglucinol acts synergistically with auxin during the most sensitive phase of root initiation (Hammatt 1994, Dobránszki and Teixeira da Silva 2010, Daud et al. 2013). According to De Klerk et al. (2011), phenolic compounds such as phloroglucinol protect the auxin indole-3-acetic acid (IAA) from decarboxylation in tissue culture shoots of apple. The results of our study show a synergistic effect of phloroglucinol with IBA, an analog of auxin, which can be degraded by decarboxylation, a reported by De Klerk et al. (2011) and Daud et al. (2013). Phloroglucinol at 79 µM also stimulated the growth of *in vitro* papaya plants with significant differences between treatments and control (Perez et al. 2016). In banana cv Grand Naine root induction was

improved with 200 μM phloroglucinol added to MS medium (unpublished data).

This study shows that the use of cryoprotective agents (sucrose, glycerol, PVS2 and 1% phloroglucinol) in combination with a 3-hour ice pre-cooling treatment provides enhanced *in vitro* plant development of banana rhizomes cv Grand Naine after cryopreservation. This is the first report of cryopreservation of banana rhizomes using phloroglucinol. Additional studies should be performed using antioxidants added to the PVS2 solution, aiming at the reduction of oxidation of plant tissues in banana.

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