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Cryopreservation of Naranjilla (*Solanum quitoense* var. *quitoense*) Shoot tips by Vitrification

Pedro Jose González[1*] • Andrea Arias[1] • Venancio Arahana[2] • Maria de Lourdes Torres[2]

[1] Department of Agronomy, Iowa State University, Ames, IA 50011 – 1010, USA
[2] Laboratorio de Biotecnología Vegetal, Universidad San Francisco de Quito, Cumbayá - Ecuador

Corresponding author: * **pedrog@iastate.edu**

ABSTRACT

Shoot tips of *in vitro* germinated naranjilla (*Solanum quitoense* var. *quitoense*) plants were cryopreserved by vitrification. Shoot tips (1-3 mm) of five week-old plants were pre-cultured on Murashige and Skoog (MS) culture medium with increasing sucrose concentrations (0.4, 0.5, 0.6 and 0.7 M) for 7 days under a 16-h photoperiod and maintained at 23 ± 2°C. Then, shoot tips were treated with a loading solution 2 M glycerol + 0.4 M sucrose, at 26°C for 25 min in a 2 mL cryo-tube; and cryoprotected with 1 mL of pre-cooled PVS 2 solution at 4°C for 60 min. Finally, shoot tips were immersed into liquid nitrogen and kept there for 10 days. Post-warming culture of these shoot tips was carried out on a basal MS medium supplemented with 0.1 mgL^{-1} 6-benzylaminopurine (BAP), 0.05 mgL^{-1} indole-3-butyric acid (IBA) and 0.1 mgL^{-1} gibberellic acid (GA_3). The optimized protocol for *S. quitoense* shoot tips preservation consisted of pretreatment with MS + 0.5 M sucrose, followed by a treatment with loading solution for 25 min at room temperature, PVS 2 at 4°C for 60 min and immersion in liquid nitrogen. Cryopreserved shoot tips started growing 15 days after re-warming. Shoot tips recovery was 70 to 95% with the above described protocol. Plantlets with a normal phenotype after 21 days of culture were acclimatized for 4 weeks in controlled conditions before they were finally transferred to the greenhouse. This is the first report of an effective protocol for the cryopreservation of *S. quitoense* shoot tips by vitrification.

Keywords: cryoprotection, gibberelic acid, glycerol, liquid nitrogen, pre-treatment, sucrose
Abbreviations: BAP, 6-benzylaminopurine; **GA_3**, gibberellic acid; **IBA**, indole-3-butyric acid; **Me_2SO**, dimethylsulfoxide; **MS**, Murashige and Skoog

INTRODUCTION

Conservation of plant genetic diversity is critical and one of the most important topics recently focused on during international forums of environmental management. Genetic erosion of economically important cultivars around the world has become a real concern for farmers and scientists, who now realize that once germplasm is lost, it will definitely affect every aspect of future agricultural processes (Takagi *et al.* 1997). Genetic diversity is the key factor for the development of breeding programs, so germplasm conservation plays an essential role for agricultural practices and environmental management.

For decades, gene banks around the world have collected and stored germplasm of economically and environmentally important plants from everywhere. Methods used for maintaining plant diversity have been based on field collections, seed banks, or *in vitro* culture conservation (Thinh *et al.* 1999; Keller *et al.* 2006; Nukari *et al*. 2009). Each of these has disadvantages. Field collections require extended areas for growth, demand high amounts of care, can preserve a limited number of species, and are subjected to environmental factors such as climate changes and diseases (Matsumoto *et al.* 2001; Engelmann 2004; Keller *et al.* 2006). *In vitro* culture techniques have also been applied for germplasm preservation for short-medium term storage. This strategy achieves conservation by reducing the growth rate of plants and increasing the time interval between subcultures (Escobar *et al.* 1997; Engelmann 2004). However, *in vitro* cultured plant germplasm can develop somaclonal variations and genotypes can be lost because of contamination problems (Harding 2004; Scocchi *et al*. 2004). Therefore, none of these methodologies are completely satisfactory for long-term preservation (Engelmann 1997).

Cryopreservation enables plant materials to be theoretically stored for an unlimited period of time without physiological changes (Engelmann 2004); it requires minimal storage space, minimal maintenance and considerably low costs are involved in the process (Sakai 2000; Matsumoto *et al.* 2001; Touchell *et al.* 2002). Formerly, the majority of cryopreservation procedures involving encapsulation-dehydration or desiccation, were utilized for temperate plant species rather than for tropical species (Gonzalez-Arnao and Engelmann 2006; Keller *et al.* 2006). Today, encapsulation-dehydration and vitrification techniques are applied to a wide range of species. Moreover, vitrification has proven to be a cryopreservation method of high efficiency for freezing tissues of several plant species (Wang *et al.* 2005; Keller *et al.* 2006; Sakai and Engelmann 2007; Nukari *et al.* 2009). Vitrification involves rapid cooling (4°C to -196°C in seconds) in cryopreserving solutions composed of dimethylsulfoxide (Me_2SO), glycerol and sucrose that slowly dehydrate and preserve the explants from osmotic stress produced in the cell (Matsumoto *et al.* 1994; Sakai and Engelmann 2007; Gonzalez-Arnao *et al.* 2008). Vitrification is effective because it avoids intracellular ice crystal formation and allows the cells to solidify into a non-crystalline, glassy-state (Fahy *et al.* 1984). Ice crystals are the primary problem with cryopreservation since they can cause the disruption of the cellular membranes (Fahy *et al.* 1984; Hirai and Sakai 1999; Lambardi *et al.* 2000; Sakai 2000; Touchell *et al.* 2002).

Solanum quitoense, is an Andean fruit crop native to Ecuador and Colombia (Velez 1988), where it is cultivated and used for agro-industrial purposes. This species grows as a bush which can attain a height of 2 m; it has big leaves

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that normally present spines. Fruits are round, small and orange in color (Heiser 1999). In Ecuador, *Solanum quitoense* var. *quitoense* is the most frequently cultivated variety, because of its organoleptic qualities (Soria 1997). *S. quitoense* is economically important income for Ecuadorian farmers (Soria 1997), has great acceptance in the local markets and an increasing potential as an exotic fruit for exportation. However, this fruit crop is very susceptible to fungal infections and nematode attacks; which can result in the loss of cultivars and genetic diversity (Velez 1988). Therefore, scientists seek new breeding and preserving measures to improve the genetic variability and pathogen resistance of this species (Fiallos 2000).

The objective of this study was to optimize a standardized vitrification protocol using the PVS2 for cryopreserving shoot-tips of *S. quitoense*.

MATERIALS AND METHODS

Plant material

Solanum quitoense var. *quitoense* fruit were acquired from local markets in Quito. Into a laminar air-flow hood, seeds were extracted and sterilized by immersing them in 70% ethanol for 3 min, then in sodium hypochlorite 2.5% for 15 min and finally washed several times with sterile distilled water. Sterilized seeds were germinated in Murashige and Skoog (MS) solid basal medium (7 g L^{-1} agar). Plantlets, approximately 2 cm high, were incubated for 5 weeks in a culture room at 23 ± 2°C and a 16-h photoperiod (approx. 40 µmol m^{-2} s^{-1} light intensity). The shoot tips (1.5 mm long and with 2 pair of leaf primordia), were aseptically dissected from the plants' apical bud under a laminar air-flow hood. Individual shoot tips were excised with scalpel blade under a binocular microscope.

Pre-treatment

Shoot tips were placed in plastic Petri dishes containing semi-solid MS medium (0.8% agar) with 4 sucrose concentrations (0.4, 0.5, 0.6, 0.7 M), and incubated for one week under the same conditions as mentioned above.

Vitrification

After 7 days of pre-treatment, shoot tips were exposed to a vitrification treatment. Five shoot tips were put into a cryo-tube (Quality Scientific Plastics: 2.0 mL Conical Screw Cap Microtube), which contained 1 mL of loading solution (2 M glycerol + 0.4 M sucrose) (Matsumoto *et al*. 1994); and incubated at 26°C for 25 min. The shoot tips were then incubated with 1 mL of PVS2 solution (30% w/v glycerol + 15% w/v ethylene glycol + 15% w/v Me_2SO, containing 0.4 M sucrose; in liquid MS medium) (Sakai *et al*. 1990); for 60 min at 4°C. Finally, cryo-tubes were rapidly immersed into liquid nitrogen, where they were kept for 10 days (Wang *et al*. 2005).

Re-warming and re-growth

For re-warming, cryo-tubes containing the shoot tips were taken out from the liquid nitrogen container, and placed directly into a 40°C water bath for 90 seconds (Wang *et al*. 2005). Shoot tips were then washed with a series of sucrose solutions of decreasing concentrations (1.2, 0.8 and 0.4 M) in liquid MS medium, for 15 min each. Then, the shoot tips were randomly chosen and cultivated into two different semi-solid MS medium (5 g L^{-1}). One was supplemented with 0.1 mg L^{-1} of 6-benzylaminopurine (BAP), 0.05 mg L^{-1} indole-3-butyric acid (IBA,), 0.1 mg L^{-1} gibberellic acid (GA_3) and 30 g L^{-1} sucrose (modified from Wang *et al*. 2005); and the other with the same concentrations of BAP, IBA and sucrose, but without GA_3 (Wang *et al*. 2005).

Shoot tips were placed at 25 °C and maintained in the dark for 3 days and then incubated in a culture room under controlled conditions (16-h photoperiod; 23 ± 2°C) for 4 weeks. Afterwards, the shoot growth was assessed. Developed plantlets were sub-cultured into basal MS medium (30 g L^{-1} sucrose) without plant growth regulators. Plantlets developed roots within three weeks and were then transferred into soil (Agro Terra Potting Mix; Canterbury Research S.A.) to begin the acclimation process under the same controlled conditions as stated above. Acclimation consisted of transplanting plantlets to containers with controlled relative humidity of 100%. The acclimation took 4 weeks, where relative humidity was progressively dropped to the same level as the greenhouse, where the plantlets were then taken to.

Reagents

MS, agar and all PGRs were all SIGMA Plant cell culture tested, BioReagents.

Data analysis

The cryopreservation experiments were repeated three times to assess results consistency. Every experiment consisted of four pre-treatments; each pre-treatment had four replications (Petri dish with 10 shoot tips). Thus, a total of at least 40 shoot tips per pre-treatment were used. Besides, a basal MS pre-treatment medium, supplemented with the regular amount of sucrose (0.1 M) was utilized as a negative control in the experiments. For the vitrification phase, around eight cryo-tubes were used per treatment, each cryo-tube containing five shoot tips. During the vitrification process, a few shoot tips were lost due to manipulation.

For the GA_3 tests after vitrification, 10 shoot tips from each pre-treatment medium were cultivated with GA_3 and 10 without the plant growth regulator for each replication. These results were basic preliminary data to assess the final survival rate percentages presented below.

Means and their respective standard deviations were calculated for all the results obtained in this research using Microsoft Excel. Data was subjected to an analysis of variance (ANOVA) and the treatment means were compared using Duncan's multiple range test (DMRT) (α=0.05) using SAS program (SAS 2003)

RESULTS

Pre-treatment

The variation in the survival rate after vitrification of shoot tips previously subjected to a range of different sucrose concentrations is shown in **Fig. 1**. The control group, pre-treated with MS basal medium (0.1 M sucrose), showed a low mean survival rate (3.35 ± 1.43%). Shoot tips pre-treated in 0.4 M sucrose had a mean survival rate of 59.2 ± 8%. At the highest sucrose concentration tested (0.7 M), 64.7 ± 4.6% of shoot tips survived. However, greater survival rates were obtained at 0.5 M sucrose (82.2 ± 12.9%) and 0.6 M sucrose (69.7 ± 0.4%); these data demonstrated that the intermediate sucrose concentration tested (0.5 M) was better suited to prepare *S. quitoense* shoot tips for vitrification. DMRT showed no significant difference ($P < 0.05$) between 0.5 and 0.6 M sucrose concentrations, but it showed significant difference between 0.5 M and the three remaining sucrose concentrations as indicated in **Table 1**.

Some callus growth was observed at the base of shoot tips pre-treated with 0.7 M sucrose, in early stages of plantlet development after warming. Nevertheless, when these plantlets were sub-cultured into a new basal MS culture medium, they grew normally showing healthy roots, good development and no physiological abnormalities.

Re-warming and re-growth

The experiments to test the GA_3 effect in the recovery medium showed that shoot tips re-growth significantly better ($P < 0.005$) in the presence of GA_3. Shoot tips extracted from liquid nitrogen and plated on MS culture medium supplemented with 0.1 mg L^{-1} BAP, 0.05 mg L^{-1} IBA and 30 g L^{-1} sucrose (Wang *et al.* 2005); grew and developed into plantlets in a low percentage (40.45% ± 6.7). However, when plated on a modified culture medium, with the addition of 0.1 mg L^{-1} GA_3, shoot tips development was much

Table 1 Survival of shoot tips exposed to different sucrose concentrations in pre-treatment medium.

Sucrose concentration (M)		**0.1**	**0.4**	**0.5**	**0.6**	**0.7**
	Means †	**3.23**	**59.3**	**82.3**	**69.7**	**64.7**
0.1	3.23 c	0	56.1 *	79.1 *	66.5 *	61.5 *
0.4	59.3 b		0	23.0 *	10.4 n.s.	5.4 n.s.
0.5	82.3 a			0	12.6 n.s.	17.6 *
0.6	69.7 ab				0	5.0 n.s.
0.7	64.7 b					0

0.5 M sucrose presents significant differences with 0.1, 0.4 and 0.7 M sucrose. * = significant difference ($P < 0.05$); n.s. = no significant differences between means. † Different letters indicate significant differences according to Duncan's Multiple Range Test ($P < 0.05$).

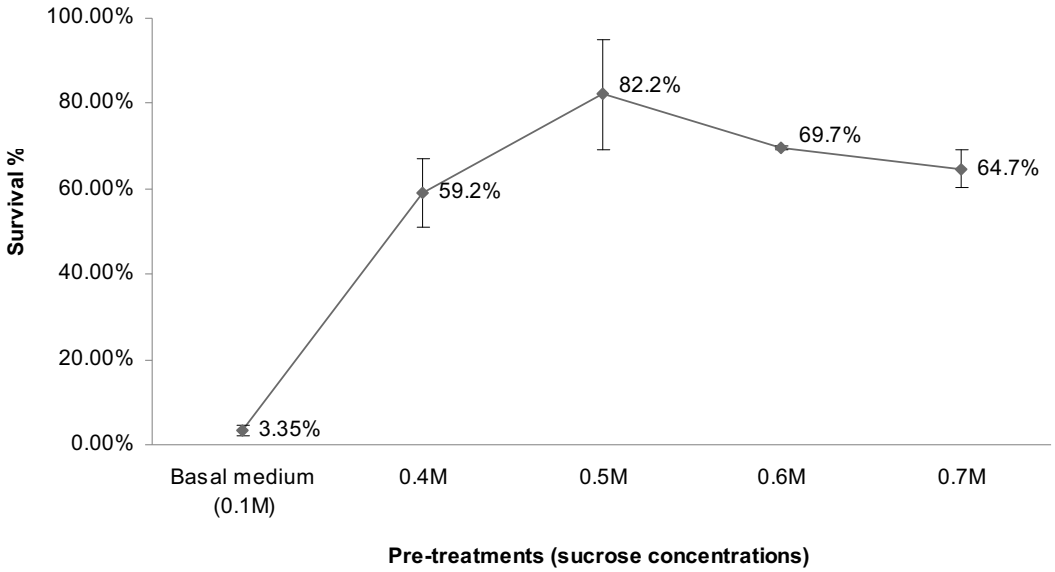

Fig. 1 Effect of the sucrose concentration of the pre-treatment medium on the survival percentages of shoot tips in three experiments. Every concentration presents a survival percentage above the 50%. Treatment with 0.5 M sucrose medium achieved the greatest percentage of survival in all experiments. Data are presented as means ± standard error.

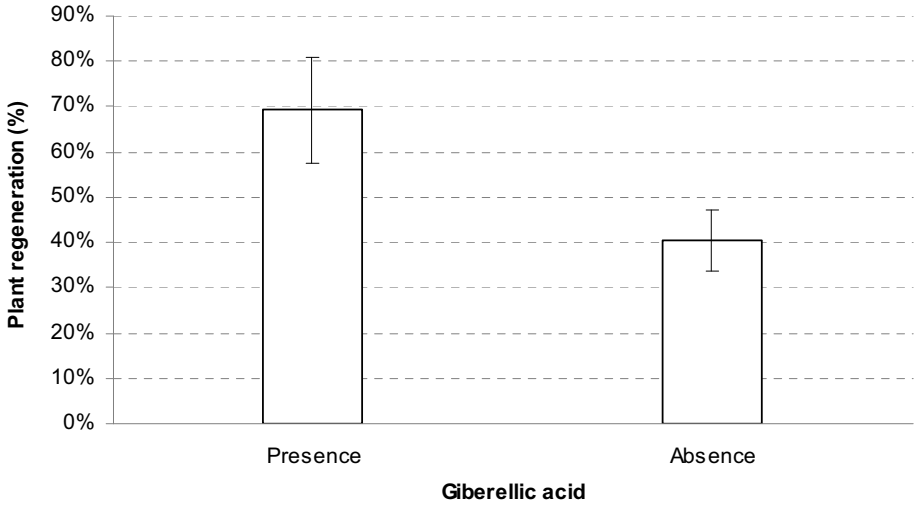

Fig. 2 Effect of GA_3 on the percentage of plant regeneration after liquid nitrogen storage. One week after liquid nitrogen storage, shoot tips were cultured in medium supplemented with GA_3. Data are presented as means ± standard error.

more efficient reaching 69% ± 11.7 (**Fig. 2**). Thus, GA_3 seems to improve *S. quitoense* shoot development. These experiments were conducted as a previous stage of this research. The vitrification results formerly shown, comparing the survival rates of the shoot tips with different pre-treatment media, were achieved using GA_3 as a component of the culture medium for all treatments.

Rooting process on MS medium took three weeks with 100% success. Acclimation was 85% successful and took four weeks. Plantlets were transferred to the greenhouse when they were 10cm in height, and possessed at least five fully expanded leaves (**Fig. 3**).

DISCUSSION

In spite of the important economical characteristics that *Solanum quitoense* represents for Ecuadorian farmers, no germplasm conservation strategy has been developed yet in the country (Soria 1997). There are no reports on the use of the vitrification method for the preservation of *S. quitoense* shoot tips. Thus, results showed above present a successful cryopreservation procedure that will be useful for the germplasm preservation of the species, as done with several other species before (Keller *et al*. 2006; Nukari *et al*. 2009). Based on the vitrification protocol developed by Sakai *et al.* (1990) this research applied the protocol standardized by

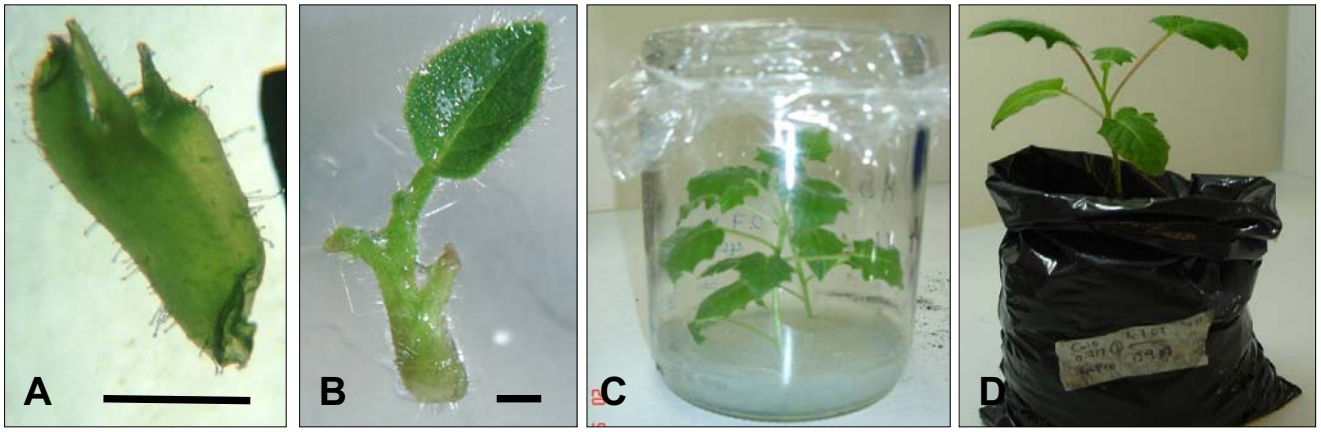

Fig. 3 Shoot-tip development, rooting and acclimation after cryopreservation. (**A**) dissected shoot tips used for vitrification; (**B**) Shoot tip elongated three weeks after thawing; (**C**) Rooting of elongated plants occurred 6 weeks after thawing; (**D**) Acclimatized plant 4 weeks after rooting.

Wang *et al.* (2005) for the cryopreservation of *Carica papaya* and was modified for *S. quitoense*.

The aim of vitrification is to increase the solute concentration inside the cells to avoid ice crystal formation (Matsumoto *et al.* 1994; Harding 2004; Sakai and Engelmann 2007). This is progressively done by first exposing the explants to a constant source of carbon, which helps to stabilize the cell and to exchange inner water with the cryogenic liquids (Uchendu and Reed 2008; Varghese *et al*. 2009). In this study, treatment on medium with 0.5M sucrose worked best for protecting *S. quitoense* shoot tips from PVS2. Several studies have reported that sucrose concentrations between 0.5 and 1M are ideal to achieve plant regeneration after cryopreservation (Scocchi *et al*. 2004; Gonzalez-Arnao *et al*. 2008; Uchendu and Reed 2008; Rey *et al*. 2009; Varghese *et al.* 2009). As shown in **Fig. 1**, when shoot tips were pre-treated with low sucrose concentration (0.1 M), the regeneration was highly reduced. This indicates that pre-treating the cells with sucrose increases cell tolerance to vitrification solutions such as PVS2 (Scocchi *et al.* 2004; Sakai and Engelmann 2007). On the other hand, increasing sugar concentration above 0.5 M did not improve protection in this species. Mandal and Dixit-Sharma (2007), reported a decline in recovery growth of *Dioscorea deltoidea* shoot tips when sucrose was increased to levels of 0.9 M. It is not clear how sucrose exerts its action, but it seems that sucrose exposure effects include solute accumulation and reduction of isotonic water content in the plant cells (Gonzalez-Arnao and Engelmann 2006; Keller *et al.* 2006). Thus, a suitable pre-treatment protocol needs to be developed for cryopreservation as reported for several tropical species (Thinh *et al*. 2000; Mandal and Dixit-Sharma 2007).

Another critical phase in this procedure is the exposure of plant cells to vitrification solutions such as Me_2SO and glycerol, after an adequate pre-treatment procedure. This exposure must be carried out carefully because these chemical compounds are toxic for plant cells (Sakai and Engelmann 2007; Gonzalez-Arnao *et al.* 2008; Susuki *et al.* 2008). Several publications describe different methodologies to expose different plant material to cryopreserving solutions. A gradual increment of the concentration of chemicals in vitrification solutions or the addition of pre-cooled vitrification compounds are two of the most common used strategies to safely expose plant materials to the vitrification solutions (Sakai 2000; Wang *et al.* 2005; Tsai *et al.* 2009). These strategies are aimed mainly to avoid cell damage, such as chemical toxicity or osmotic stress (Matsumoto *et al.* 1994; Takagi *et al.* 1997; Thinh *et al.* 1999; Sakai 2000; Sakai and Engelmann 2007; Tsai *et al.* 2009) during the exposure to vitrification solutions in tropical and temperate plants. In this study, exposure of *S. quitoense* shoot tips to a loading solution (2 M glycerol + 0.4 M sucrose); followed by the immersion of shoot tips into PVS 2 solution for 60 minutes at a controlled temperature (4°C) appeared to be adequate treatment. Both, loading solution and PVS2, allowed an adequate pre-conditioning of *S. quitoense* shoot tips for liquid nitrogen exposure, with no obvious toxicity to the plant material. The loading solution then acts as a cryoprotective agent that provides tolerance to the shoot tips to the dehydration potential of PVS2 solution (Sakai and Engelmann 2007).

Inclusion of growth regulators in the post-vitrification culture medium was essential for elongation of *S. quitoense* shoot tips that survived the cryopreservation process. In this study, gibberellic acid (GA_3) was found to be a suitable growth regulator since it stimulated efficient shoot growth. The response of *S. quitoense* shoot tips to an exogenous source of GA_3 is related to the fact that vitrification process can be highly stressful; and the addition of exogenous plant growth regulators may help explants to overcome such effect (Sarkar and Naik 1998).

Chang and Reed (1999) have reported callus formation on shoot tips during re-growth as an effect of growth regulators; however, in this study that was not the case. Damage to the tissue can be caused at any of the stages of manipulation during the whole cryopreservation process. Thus, it is not rare to observe abnormal growth in some part of the recovered shoot tips (Varghese *et al.* 2009). These abnormalities can appear in form of lateral calli because only the apical dome is expected to overcome the freezing stress due to the presence of totipotent cells (Panis *et al.* 1996). Then, callus formation is undesired because the probability of genetic variation increases, leading to a change in the phenotypic characteristics of the species (Sakai and Engelmann 2007).

Acclimation, which is the last step in a plant regeneration process, is necessary due to the artificial conditions in which plantlets are cultured, affecting their physiology. Therefore a conditioning period is required before plantlets are transferred to the soil. In the present study, 85% of the plantlets acclimatized successfully, as was previously reported for other Solanaceous species (Rascio *et al.* 2002).

We report for the first time, an efficient protocol for cryopreservation of *S. quitoense* based on the PVS2 vitrification technique. This protocol will allow the development of new strategies for long term conservation of this important Andean species.

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