

## Notas Científicas

### Survival of sugarcane shoot tips after cryopreservation by droplet-vitrification

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**Abstract** – The objective of this work was to evaluate the phytotoxicity of a plant vitrification solution (PVS2), and the survival of shoot tips of the sugarcane variety SP716949, after cryopreservation by droplet-vitrification. Shoot tips were precultured for 24 hours in MS medium containing 0.3 mol L<sup>-1</sup> sucrose, and exposed to PVS2 for 0, 20 or 30 min. Shoot tips were then immersed in liquid nitrogen. Thawing was fast in concentrated sucrose solution (1.2 mol L<sup>-1</sup>). PVS2 is a nontoxic to shoot tips, which in turn are sensitive to liquid nitrogen. The best results occurred when shoot tips were maintained for up to 20 min in PVS2 solution, before freezing, with 20% survival.

**Index terms:** *Saccharum*, in vitro conservation, PVS2, regeneration.

### Sobrevivência de ápices caulinares de cana-de-açúcar após criopreservação por “droplet-vitrification”

**Resumo** – O objetivo deste trabalho foi avaliar a fitotoxicidade da solução de vitrificação de plantas (PVS2) e a sobrevivência de ápices caulinares de cana-de-açúcar, variedade SP716949, após criopreservação por “droplet-vitrification”. Ápices caulinares foram pré-cultivados por 24 horas em meio MS com sacarose a 0,3 mol L<sup>-1</sup> e expostos por 0, 20 ou 30 min a PVS2. Em seguida, os ápices foram mergulhados em nitrogênio líquido. O descongelamento em solução concentrada de sacarose (1,2 mol L<sup>-1</sup>) foi rápido. A PVS2 não tem efeito tóxico aos ápices que, no entanto, são sensíveis ao nitrogênio líquido. O melhor resultado ocorreu, quando os ápices foram mantidos por 20 min na solução PVS2, antes do congelamento, com 20% de sobrevivência.

**Termos para indexação:** *Saccharum*, conservação in vitro, PVS2, regeneração.

Preservation in liquid nitrogen at ultralow temperature (-196°C) is a potential technique for long-term conservation of plant germplasm. Recently, a new technique called droplet-vitrification cryopreservation has been developed, which combines the application of highly concentrated vitrification solutions with an ultrafast freezing and thawing rate (Panis et al., 2005). In this technique the duration for which explants are treated with cryoprotectant solutions (PVS2) is of extreme importance, since it determines the extent of cell dehydration and the number of components that will permeate the cells (Chen et al., 2011).

According to Panis et al. (2011), depending on the target species, the development of a technique involving proper protocol may take years of study, since it depends on previous information available in

the literature and on behavioral characteristics of the species, as for example, if it is tropical or temperate, woody or herbaceous, mono- or dicotyledonous.

Sugarcane is a species of great economic importance. It propagates vegetatively and needs germplasm banks to meet breeding programs; however, few reports on the cryopreservation of this species using shoot tips and droplet vitrification technique are found in the literature. Barraco et al. (2011a) observed that the apices of two varieties have shown a relatively high tolerance to dehydration by the vitrification solution; however, the recovery of explants after cryopreservation was about 28%. In Brazil, although several groups are working with this culture, including in vitro conservation, to date,

there are no reports on cryopreservation protocols developed for the species.

The objective of this work was to evaluate phytotoxicity by exposure time to the osmoregulator PVS2, and the survival of sugarcane shoot tips cryopreserved through droplet-vitrification technique.

Shoot tips of approximately 1-2 mm were extracted from in vitro sugarcane plants, variety SP716949, with the aid of a stereomicroscope, and pre-cultured for 24 hours in the dark in MS medium (Murashige & Skoog, 1962) supplemented with 0.3 mol L<sup>-1</sup> sucrose. After this time, tips were exposed to a loading solution (LS) containing 2.0 mol L<sup>-1</sup> glycerol plus 0.4 mol L<sup>-1</sup> sucrose for a period of 20 min at room temperature. Then, tips were transferred to a plant vitrification solution 2 (PVS2) (Sakai et al., 1990) for 0, 20 and 30 min at 0°C.

After the dehydration treating, shoot tips were transferred to a strip of aluminum foil containing one drop of 10 µL cooled PVS2, and were rapidly immersed in liquid nitrogen (+LN), where they remained for at least 40 min. They were, then, quick thawed by immersing the aluminum foil in a recovery solution containing 1.2 mol L<sup>-1</sup> sucrose, for 15 min, at room temperature (Panis et al., 2005).

The shoot tips were introduced into MS medium containing 0.3 mol L<sup>-1</sup> sucrose, where they remained for 24 hours and, then, they were transferred to MS multiplication medium supplemented with 30 g L<sup>-1</sup> sucrose, 0.10 mg L<sup>-1</sup> kinetin (KIN) in combination with 0.20 mg L<sup>-1</sup> 6-benzylaminopurine (BAP), and gelled with 2.3 g L<sup>-1</sup> Phytigel (Sigma, St. Louis, MO, USA). For comparison, apical segments were extracted and directly introduced into the multiplication medium, while others underwent the same procedures for pre-treatment and dehydration, but were not exposed to liquid nitrogen (-LN).

An experimental completely randomized design was used, with 10 replicates per treatment consisting of exposure times to PVS2 and immersion or not in LN. The regeneration was evaluated after forty days in culture medium, and the observed data were submitted to analysis of variance, followed by Tukey's post hoc test, at 5% probability. The percentage data were transformed by arcsine prior to analysis.

The vitrification solution provided no toxic effect nor osmotic injuries to sugarcane shoot tips, as 85% of the PVS2-treated and nonexposed to liquid nitrogen

explants showed a survival rate, in comparison to the control, in which the tips were not exposed to cryoprotectant solutions and exhibited 90% regeneration (Figure 1).

After treatment with the vitrification solution, the number of secondary shoots formed per explant did not differ among treatments. However, shoot height was significantly lower in both exposure times (average 1.3 cm) compared with the control (2.5 cm).

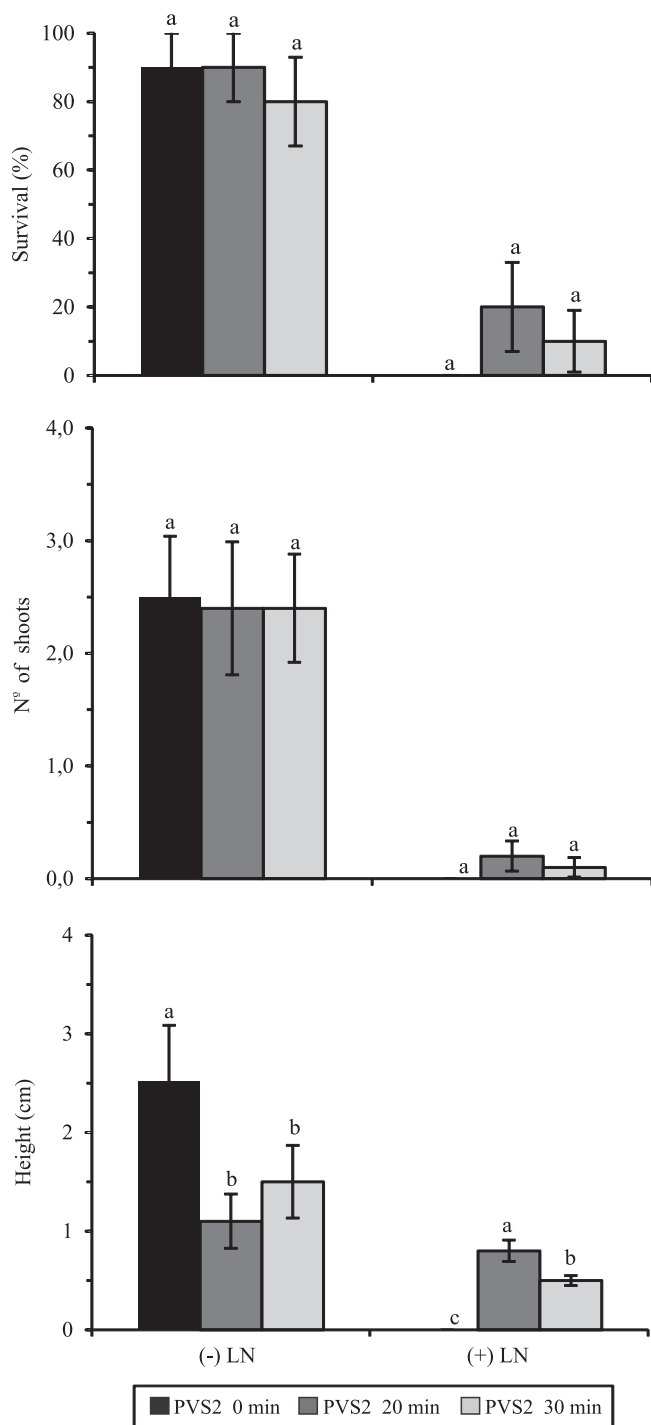
Cryopreservation of shoot tips of sugarcane, variety SP716949, resulted in a 20% rate of survival, for explants treated for 20 min with PVS2, and 10% for explants exposed for 30 min. These values were significantly lower than those of apices not exposed to LN.

Similar results were observed by Barraco et al. (2011b), in which apical meristems of *Limonium serotinum* (Rchb.) Pignatti showed a significant survival decrease following cryopreservation, and reached low to intermediate growth recovery rates of 0 to 37%. For *Prunus cerasifera* Ehrh., Vujovic et al. (2011) reported that growth recovery after cryopreservation was 20% at most. Preetha et al. (2013) observed a decrease in the viability of *Kaempferia galanga* L. cryopreserved shoot tips, since they become sensitive to PVS2 after 30 min exposure, unlike the control (50 min).

Based on Panis et al. (2011), the PVS2 treatment time varies considerably among species, but usually extended periods of exposure are eventually lethal to cells. The ideal exposure time was 20 min for *Rosa hybrida* L. (Halmagyi & Pinker, 2006), 30 min for *Byrsonima intermedia* A. Juss (Silva et al., 2013), 40 min for *Musa* spp. (Panis et al., 2005), and 60 min for *Malus domestica* Borkh. (Condello et al., 2011).

For sugarcane, Barraco et al. (2011a) found that shoot tips of two cultivars (H70-144 from Hawaii, and CP68-1026 from the USA) showed relatively high tolerance to two cryopreservation solutions PVS2 and PVS3, with an approximate average of 70% when exposed for 20 to 40 min to these solutions. However, the survival of explants, after freezing, reached maximum rates of 20 and 37%, respectively, which are values similar to those obtained in the present work, with the PVS2 solution. It is worth noting that the survival and regeneration percentages vary according to different varieties, even when using the same cryopreservation method (Panis et al., 2005; Chen et al., 2011).

Besides the viability after treatments, in the cryopreservation of germplasm it is also important



**Figure 1.** Effect of exposure time to vitrification solution (PVS2) on the survival of sugarcane shoot tips, and for number and height of shoots, after cryopreservation (+LN) or without immersion in liquid nitrogen (-LN). Means followed by the same lower case letter within each condition of liquid nitrogen (+LN or -LN), and among PVS2 treatments, belong to the same group and do not statistically differ among themselves by Tukey's test, at 5% probability.

that the apices or meristems are able to produce plants identical to the donor plant (Chen et al., 2011). In this work, regeneration occurred directly, without the intermediate calli stage, which is often undesirable for causing genetic instability of the regenerants.

As for growth parameters of the cryopreserved plants, at forty days of cultivation we noted a lower formation rate of secondary shoots and lower shoot length, an average of 0.7 cm (Figure 1). This smaller increase is probably due to the fact that the regeneration of tips, after immersion in LN, had been delayed about seven days following that of the explants of the control treatments (-LN). Guzmán-García et al. (2013) found that the cryoprotectant solutions caused some delay in reactivating the growth of embryogenic cultures of *Persea americana* Mill. after cryopreservation by the droplet method. Similar results were also observed for *Elaeis guineensis* somatic embryos, after immersion in LN in the presence of PVS2 (Suranthran et al., 2012). According to Preetha et al. (2013), this delay of growth recovery is due to the fact that frozen explants depend on the recovery of effects caused by the toxicity, as well as by the crystallization phenomenon, whereas the control only depends on the toxicity of PVS2.

This work represents an initial step and potential for long-term preservation of sugarcane germplasm in Brazil. To our knowledge, this is the first report using this technique for cryopreservation of Brazilian sugarcane varieties.

### Acknowledgment

To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), and to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), for financial support and fellowships.

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Received on July 29, 2013 and accepted on October 30, 2013