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CRYOPRESERVATION OF SUGARCANE SPECIES BY DROPLET-VITRIFICATION

CRIOPRESERVAÇÃO DE ESPÉCIES DE CANA-DE-AÇÚCAR POR VITRIFICAÇÃO EM GOTAS

CRIOCONSERVACIÓN DE ESPECIES DE CAÑA DE AZÚCAR MEDIANTE VITRIFICACIÓN POR GOTAS

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ABSTRACT
Sugarcane (Saccharum spp.) is considered one of the major crops for energy purposes in tropical, semi-tropical and subtropical regions worldwide. The in vitro maintenance of the accessions available in field bank germplasm, has been considered a complementary and security strategy and the cryopreservation methods are used by world germplasm banks to preserve the biodiversity of plant species. The aim of this study was to evaluate the efficacy of the droplet-vitrification technique in two species of Saccharum spp. Shoot tips from MIA 35301 (S. robustum L.) and NSL 291979 (S. spontaneum L.) were excised from in vitro culture in the 2nd subculture. First, different concentrations of sucrose were used on the preculture step for MIA 35301'. After that, the best concentration was used (0.3 M sucrose) and the MIA 35301 shoot tips were exposure to PVS3 and PS3 solutions at different times. For the third experiment, Shoot tips from MIA 35301 and NSL 291979 accessions were precultured and dehydrated in PVS2 and PVS3 at 15, 30 and 45 min, cooled and rewarming in loading solution and transferred to regeneration medium. Preculture in MS culture medium with 0.3 M sucrose and exposure to PVS2 for 45 min is promising for the cryopreservation of MIA 35301 (S.

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robustum L.) by droplet-vitrification.

Keywords: Saccharum spp; cryopreservation; PVS2.

RESUMO
A cana-de-açúcar (Saccharum spp.) é considerada uma das principais culturas para fins energéticos em regiões tropicais, semitropicais e subtropicais em todo o mundo. A manutenção in vitro de acessos disponíveis em bancos de germoplasma de campo, tem sido considerada uma estratégia complementar e segura. E a criopreservação tem sido utilizada em bancos de germoplasma para preservar a biodiversidade de várias espécies. O objetivo deste estudo foi avaliar a eficácia da técnica de gotícula-vitrificação em duas espécies de Saccharum spp. Meristemas apicais dos acessos MIA 35301 (S. robustum L.) e NSL 291979 (S. spontaneum L.) foram excisados de culturas in vitro no 2º subcultivo. Primeiro, diferentes concentrações de sacarose na etapa de pré-cultura foram avaliadas para o acesso MIA 35301. Em seguida, a melhor concentração de sacarose foi utilizada na fase de pré-cultura e meristemas apicais de MIA 35301 foram desidratados em soluções de vitrificação PVS2 e PVS3 em diferentes tempos. Para o terceiro experimento, meristemas apicais dos acessos MIA 35301 e NSL 291979 após pré-cultura, foram expostas às soluções de PVS2 e PVS3 por 15, 30 e 45 min, submetidos ao nitrogênio líquido, reaquecidos em solução de carregamento e transferidos para meio de regeneração. A pré-cultura de meristemas apicais em meio de cultura MS com 0,3 M sacarose e a exposição ao PVS2 por 45 min são promissoras na criopreservação por vitrificação em gotas do acesso MIA 35301 (S. robustum L.).

Palavras-chave: Saccharum spp; criopreservação; PVS2.

RESUMEN
La caña de azúcar (Saccharum spp.) es considerada uno de los principales cultivos con fines energéticos en las regiones tropicales, semitropicales y subtropicales de todo el mundo. El mantenimiento in vitro de accesiones disponibles en bancos de germoplasma de campo se ha considerado una estrategia complementaria y segura. Y la criopreservación se ha utilizado en bancos de germoplasma para preservar la biodiversidad de varias especies. El objetivo de este estudio fue evaluar la efectividad de la técnica de vitrificación por gotitas en dos especies de Saccharum spp. Los meristemas apicales de las accesiones MIA 35301 (S. robustum L.) y NSL 291979 (S. spontaneum L.) se extirparon de cultivos in vitro en el segundo subcultivo. Primero, se evaluaron diferentes concentraciones de sacarosa en el paso de precultivo para la accesión MIA 35301. Luego, se utilizó la mejor concentración de sacarosa en el paso de precultivo y los meristemos apicales de MIA 35301 se deshidrataron en soluciones de vitrificación PVS2 y PVS3 en diferentes tiempos. Para el tercer experimento, los meristemas apicales de las accesiones MIA 35301 y NSL 291979 después del precultivo se expusieron a soluciones PVS2 y PVS3 durante 15, 30 y 45 min, se sometieron a nitrógeno líquido, se recalentaron en solución de carga y se transfirieron a regeneración. El precultivo de meristemos apicales en medio de cultivo MS con sacarosa 0,3 M y la exposición a PVS2 durante 45 min son prometedores para la crioconservación por vitrificación en gotas de la accesión MIA 35301 (S. robustum L.).

Palabras clave: Saccharum spp; criopreservación; PVS2.

1. Introdution

Sugarcane (*Saccharum* spp.) is considered one of the major crops for energy purposes in tropical, semi-tropical and subtropical regions worldwide. Cultivated in tropical and subtropical regions, it has an impact on the energy matrix of consumption due to its capacity for biomass generation [1]. Brazil is its largest producer, with a harvest estimated in 628.1 million tons for 2021/22 [2]. The development of new varieties has contributed to expand of sugarcane cultivation.

The current varieties of sugarcane are hybrids from the cross between *Saccharum spontaneum* (wild material) x *Saccharum officinarum* (noble canes), with subsequent successive backcrosses for the parental *S. officinarum*, with this strategy the breeding programs have developed varieties with high sugar production, low fiber content and high resistance to diseases [3]. On the other hand, the sugarcane with the ideotype for energy called "energy cane" seeks in it the existing variability of the material high production of biomass, high fiber content and greater adaptation to adverse cultivation conditions, favoring the exploitation in restrictive environments to conventional sugarcane cultivation. To promote and increase fiber in sugarcane energy it is necessary to access wild germplasm [3].

The germplasm maintained in the field are a source of genetic diversity available to breeding programs. The Sugarcane Complex Active Germplasm Bank (Saccharum Complex BAG) from Embrapa Tabuleiros Costeiros is located at the Jorge Prado Sobral Experimental Field, in Nossa Senhora das Dores, SE, Brazil. Approximately, 120 accessions, including the genus Saccharum (*S. officinarum*, *S. spontaneum* and *S. robustum*), *Erianthus* and *Miscanthus* have been kept in the field since 2010. Among these, *S. spontaneum* stands out because in addition to having resistance to various diseases, it is the species that has the highest fiber content of the genus *Saccharum* [4]. The in vitro maintenance of the respective accessions at the Plant Tissue Culture Laboratory, has been considered an alternative strategy for the security of the collection [5].

Conservation at ultra-low temperatures, usually in liquid nitrogen (-196°C), is the only method ensuring long-term storage [6]. Cryopreservation methods are

used by world germplasm banks to preserve the biodiversity of plant species of agronomic importance [7]. In vitrification techniques, the solidification of plant tissue solutions (both intracellular and extracellular) occurs by an extreme increase in their viscosity during the cryoprotective and ultra-fast cooling processes [8]. In this state, the formation of ice crystals is inhibited or minimized. Contemporary cryopreservation protocols have been based on the droplet-vitrification technique, which consists in the treatment of shoot tips with Plant Vitrification Solution 2-PVS2 [9] or Plant Vitrification Solution 3-PVS3 [10] cryoprotectant solutions under an aluminum foil strip. Besides this, according to González-Arnao et al. (2020) [11] the cryotherapy has potential to remove sugarcane mosaic virus from sugarcane. Cryopreservation protocols have already been developed for different sugarcane explants. However, the use of shoot tips might ensure a better genetic stability of regenerated plants [12].

This cryopreservation technique has been the target of studies with variations in the results as a function of the pre-culture medium, vitrification solution, exposure time, among other factors [13, 14, 15,16, 17]. Most of these results were obtained for *S. officinarum* varieties and responses from other species of the genus *Saccharum* are scarce in the literature. In addition, variations in the responses between species and genotypes of the same species have been reported as a difficulty in applying a standard cryopreservation protocol for the *Saccharum* genus [17, 18].

The aim of this study was to study the responses of a shoot tip cryopreservation applied to the *S. robustum*, MIA 35301 and *S. spontaneum*, NSL 291979 accessions maintained at our facilities.

2. Material and Methods

Accessions of *S. robustum*, MIA 35301 and *S. spontaneum*, NSL 291979 from the Saccharum Complex BAG of Embrapa, previously in *vitro* established (2nd subculture) were used as a source of explants. Shoot tips were individualized and multiplied in MS basal salts and vitamins [19] (M519, PhytoTechnology®, KS) supplemented with 30 g L^{-1} sucrose, 2.22 µM BAP (6-benzylaminopurine, Sigma-Aldrich, MO) and gelled with 3.5 g L^{-1} of Phytagel™ (Sigma-Aldrich, MO).

The pH was adjusted to 5.8 and autoclaved at 12 °C for 20 min. The cultures were kept in a growth room at 25 ± 2 °C with a 12 h photoperiod under a light intensity of 52 µmol m^{-2} s^{-1} provided by white LEDs.

For the first experiment, were studied the effect of sucrose concentration in a preculture medium on the MIA 35301 shoot tips and its exposure to Plant Vitrification Solution 2 cryoprotectant (PVS2) [9] composed by MS basal salts with 15% w/v ethylene glycol, 15% w/v DMSO, 30% w/v glycerol, and 13.7% w/v sucrose and Plant Vitrification Solution 3 (PVS3) [10] cryoprotectant composed by MS basal salts with 50% w/v glycerol, and 50% w/v sucrose, at different times. The shoot tips measuring 1.0-1.5 mm were excised and inoculated in Petri dishes (60 x 15 mm) MS preculture medium with 0.3 M, 0.5 M and 0.625 M of sucrose grade II (Sigma-Aldrich, MO) for 24 h. After that, the shoot tips were exposure to PVS2 or PVS3 solutions at different times: 10, 20, 30, 40, 50 or 60 min.

To evaluate the best conditions obtained in the first experiment, the second assay was performed. The shoot tips were cultivated in MS preculture medium with 0.3 M of sucrose grade II (Sigma-Aldrich, MO) for 24 h. After that, the shoot tips were exposure to PVS2 or PVS3 solutions for 15, 30 and 45 min, at 0 °C, and plunged in liquid nitrogen, where they remained for at least 30 min (LN+).

For the third experiment, shoot tips from MIA 35301 and NSL 291970 accessions pre-cultured in MS culture medium with 0.3 M sucrose for 24 h were placed on aluminum foil strips (1.5 × 0.5 cm) and exposure to PVS2 (5 µL/drop) for 30, 45 or 60 min and plunged in liquid nitrogen, where they remained for at least 30 min (LN+).

Rewarming was performed at room temperature by immersion of the aluminum foil strip with the explants in 1.2 M sucrose solution for 15 min at room temperature [20]. After that, the explants were recovery in steril Petri dishes with the half of basal salts of MS (M519, PhytoTechnology®, KS) with 30 g L^{-1} sucrose, 3.0 g L^{-1} activated charcoal (Sigma-Aldrich, MO), 4.44 µM BAP and gelled with 3.5 g L^{-1} Phytagel. Explants (10 units) pre-cultured and dehydrated in PVS2 but not cryopreserved were used as a control (LN-). Cultures were kept in the dark for 15 days and transferred to growth room under the conditions described above.

At 15 days, survival was estimated for the presence of living tissues. At 60 days, the number of explants emitting shoots were considered regenerated. Each experiment was performed with two replicates, and each repetition with three Petri dish with five to seven shoot tips. Percentage data were transformed into arc sen (√x / 100) before statistical analysis. Means were submitted to analysis of variance and compared with Tukey at 5% probability through SISVAR [21].

3. Results and Discussion

Shoot tips from MIA 35301 precultured for 24 h did not show statistical different sensitivities to sucrose concentrations. When precultured in MS with 0.3M sucrose, the growth was 80%; in the presence of 0.5 M sucrose, 50%; and, 0.625 M sucrose, 70% (data not showed). Barraco et al. (2011) [12] reported that the optimal conditions included preculture of encapsulated shoot apices for 24 h in MS liquid medium with 0.75 M sucrose. For other hand, Rafique et al. (2015) [14] precultured shoot tips in the presence of 0.3 M sucrose. Preculture on medium with high sucrose concentration was important to induce tolerance to vitrification solutions [7, 17].

After precultured of shoot tips from MIA 35301 in 0.3 M sucrose, the effect of exposure to PVS2 or PVS3 vitrification solutions, but not cooled in LN at 10, 20, 30, 40, 50 or 60 min was evaluated. The growth of explants exposure to PVS3 (33.3%) differed statistically from those to PVS2 (11.7%) (Table 1). The exposure time and the interaction between vitrification solutions and time did not differ significantly ($p>0.05$).

Table 1: Growth (%) of shoot tips from MIA 35301 (*S. robus*tum) exposure to PVS2 and PVS3 solutions at different times but not cooled in liquid nitrogen (LN-).

Time	**Growth (%)**			
	PVS2		**PVS3**	
10 min	30	a	40	a
20 min	20	a	30	a
30 min	10	a	20	a
40 min	0	b	50	a
50 min	10	a	30	a
60 min	0	a	30	a
Means	11.7	b	33.3	a

Means followed by the same lowercase letter in the row do not differ significantly by Tukey ($p < 0.05$).

Source: Annie Carolina Araújo de Oliveira (2022)

In order to test these conditions, a new assay was performed. Shoot tips from MIA 35301 pre-cultured in 0.3 M sucrose for 24 h were exposure to PVS2 or PVS3 vitrification solutions for 15, 30 or 45 min and plunged in liquid nitrogen (Table 2). The survival of cryopreserved explants varied according to interaction between the vitrification solutions and the exposure time with an average of 30% for PVS2 and 10%, for PVS3. Considering the non-cryopreserved explants these values reached 36.67% and 46.67% for PVS2 and PVS3, respectively. The highest level of regrowth after cooled in liquid nitrogen was obtained for explants dehydrated by vitrification solutions for 45 min (25%).

Table 2: Survival (%) and regrowth (%) of shoot tips from MIA 35301 (S. robustum) exposure to PVS2 and PVS3 solutions at different times not cooled (LN-) and cooled (LN+) in liquid nitrogen.

Time	**LN-**							
	Survival (%)				**Regrowth (%)**			
	PVS2		**PVS3**		**PVS2**		**PVS3**	
15 min	40	aA	30	aA	40	aA	30	aA
30 min	30	aA	50	aA	30	aA	40	aA
45 min	40	aA	60	aA	30	aA	50	aA
Time	**LN+**							
	Survival (%)				**Regrowth (%)**			
	PVS2		**PVS3**		**PVS2**		**PVS3**	
15 min	0	aB	0	aA	0	aA	0	aA
30 min	50	aA	10	bA	30	aA	10	aA
45 min	40	aA	20	aA	30	aA	20	aA

Means followed by the same lowercase letter in the row and uppercase letter in the column do not differ significantly by Tukey ($p < 0.05$); non-cryopreserved (LN-) and cryopreserved (LN+)
Source: Annie Carolina Araújo de Oliveira (2022)

Dehydration of shoot tips with PVS3 provided higher recovery rates compared to PVS2 for controls (NL-). In contrast, PVS2 was more effective in cryoprotection of explants immersed in liquid nitrogen (NL+). Vitrification solutions may have protected plant cells from intracellular freezing that occurred during rapid cooling [22]. The efficiency in permeability between the different substances that composed them as well as toxicity limits their composition [23]. At this point, the knowledge about factors such as cryoprotectant concentration and exposure time are important for successful cryopreservation.

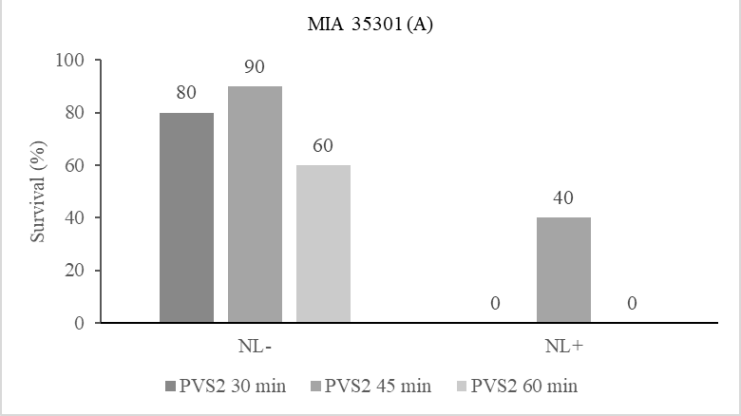
For the droplet-vitrification, MIA 35301 and NSL 291970 shoot tips were precultured in 0.3 M sucrose for 24 h and exposure to PVS2 for 30, 45 or 60 min prior plunged in liquid nitrogen. The average survival of cryopreserved explants was 25%, 15% and 10% when dehydrated with PVS2 for 60, 45 and 30 min,

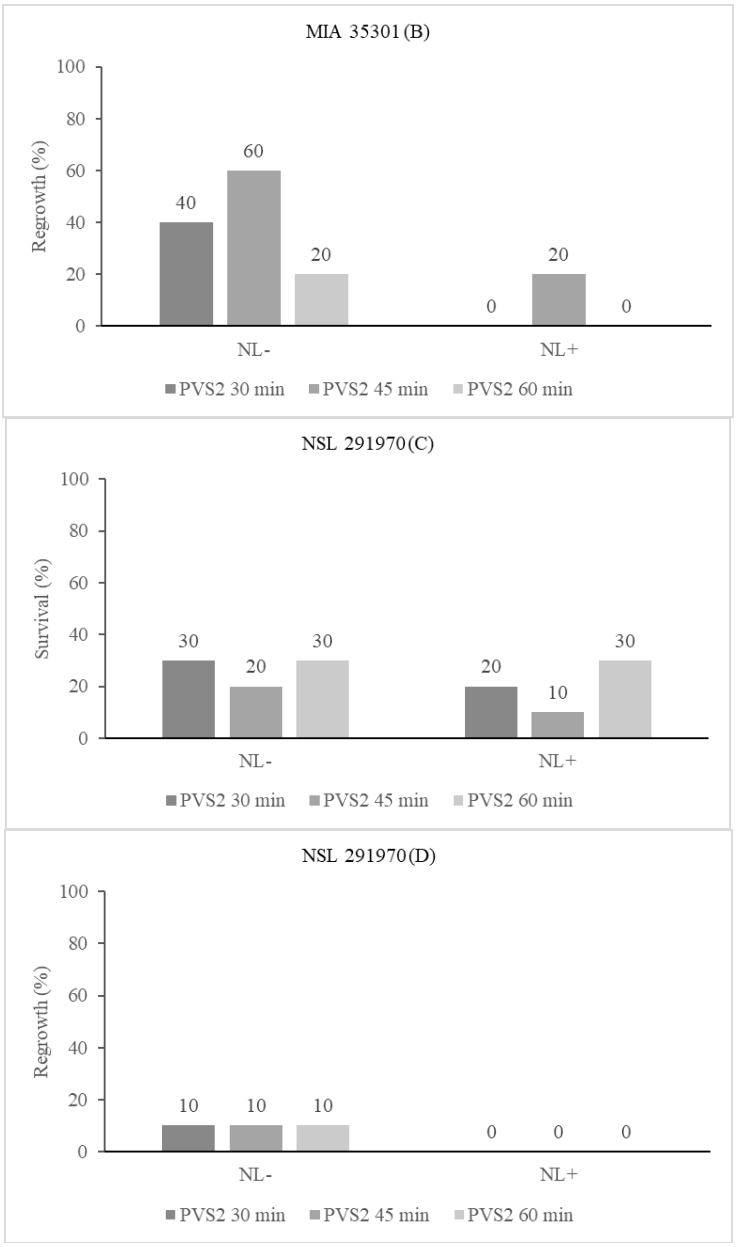
respectively (Figure 1a-c). The MIA 35301 accession reached 20% of regrowth after the cooled, when exposure to PVS2 for 45 min, (Figure 1b, 2).

Explants from NSL 291970 oxidized and did not survive after the cryopreservation procedure (Figure 1d). According to Ren et al. (2021) [24] Reactive oxygen species (ROS)-induced oxidative stress results in low success or even total failure of cryopreservation.

Some authors reported similar results for other sugarcanes varieties. Barraco et al. (2011) [13] achieved 20% regeneration for clone H70-144 (*Saccharum* spp.) with 20 min in PVS2 and 37% for clone CP68-1026 in PVS2 for 40 min. Nogueira et al. (2013) [14] reported 20% survival of the SP716949 variety (*S. officinarum*) when the shoot tips were treated for 20 min with PVS2. Volk et al. (2019) [17] observed regrowth ranged from 15 to 18% in shoot tips of cultivars 613 and 961 treated with PVS2 for 40 min. However, Rafique et al. (2015) [15] obtained regrowth ranging from 56.7% to 100% of 11 varieties cryopreserved using the V cryo-plate technique. Kaya and Souza (2017) [16] observed that shoot tips of *S. officinarum* varieties Q44830 and Q45251 reached 70.9% and 63.3% of regrowth, respectively, after dehydration with PVS2 for 45 min. For variety Q42431, the highest level (76.3%) was observed after dehydration with PVS2 for 30 min. Some factors play a significant role in the success of the sugarcane cryopreservation, and the age and quality of the in vitro cultures should be considered [17].

Figure 1: Survival (%) and regrowth (%) of sugarcane shoot tips from (a) e (b) MIA 35301 and (c) e (d) NSL 291970 accessions exposure to different times in PVS2, non-cryopreserved (LN-) and cryopreserved (LN+).





Source: Annie Carolina Araújo de Oliveira (2022)

Figure 2: Survival (%) and regrowth (%) of sugarcane shoot tips from MIA 35301 accession after cryopreserved (LN+).

Source: Ana da Silva Lédo (2022)

Contamination by endophytic microorganisms was observed. Volk et al. (2019) [17] reported the appearance of bacteria during regeneration of sugarcane cryopreserved shoot tips. Despite the use of antibiotics was effective in controlling bacterial growth, this problem represents a new challenge in the area, since it directly affects post-freezing recovery.

The results obtained in this work emphasize the high level of specificity in the behavior of sugarcane varieties when submitted to cryogenic procedures. Additional studies should be conducted to optimize the different steps of the droplet-vitrification protocol.

4. Conclusion

Preculture in MS with 0.3 M sucrose and exposure to PVS2 for 45 min is promising for the shoot tips cryopreservation of *S. robustum*, MIA 35301 by droplet-vitrification.

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