AGRONOMY (AGRONOMIA)

Revista Brasileira de Ciências Agrárias
ISSN (on line) 1981-0997
v.15, n.4, e8439, 2020
Recife, PE, UFRPE. www.agraria.pro.br
DOI:10.5039/agraria.v15i4a8439
Protocol 8439 - 07/04/2020 • Approved in 23/07/2020

Cryopreservation of seeds from endangered Brazilian bromeliads - *Dyckia brevifolia* Baker and *D. delicata* Larocca & Sobral

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ABSTRACT: This study aimed to evaluate the influence of different solutions that cryopreserve, in liquid nitrogen, seeds from Brazilian bromeliads species threatened with extinction, the *Dyckia brevifolia* Baker and *D. delicata* Larocca & Sobral. Ten treatments and four replicates per treatment were used, namely, without adding the cryoprotective solution and no immersion in liquid nitrogen; immersion in liquid nitrogen without adding cryoprotectant; 1 M glycerol; 2 M glycerol; 0.4 M sucrose; 0.8 M sucrose; PVS1; PVS2; PVS2 + 1% phloroglucinol and PVS3. The seeds remain frozen for 15 days until the evaluation of germination percentage, abnormal seedlings, germination speed index, and seedling length and dry mass took place. After cultivation in a greenhouse for six months, the length and the dry weights of the shoot and the root system were evaluated, as well as the total dry weight and the shoot/root ratio. Concerning the treatment without using cryoprotectants, *D. brevifolia* and *D. delicata* seeds attained, respectively, 92% and 79% of germination. Using cryoprotectants did not impair seed viability. *Dyckia brevifolia* seeds with 6.8% water content and *Dyckia delicata* with 8.9% can be cryopreserved in liquid nitrogen without needing cryoprotectant solutions.

Key words: cryoprotection solutions; liquid nitrogen; seed bank

Criopreservação de sementes das bromélias brasileiras ameaçadas de extinção - *Dyckia brevifolia* Baker e *D. delicata* Larocca & Sobral

RESUMO: O trabalho teve como objetivo avaliar a influência de diferentes soluções crioprotetoras na criopreservação em nitrogênio líquido de sementes das espécies de bromélias brasileiras ameaçadas de extinção *Dyckia brevifolia* Baker e *D. delicata* Larocca & Sobral. Foram utilizados dez tratamentos e quatro repetições por tratamento, isto é, sem adição de solução crioprotetora e sem imersão em nitrogênio líquido; imersão em nitrogênio líquido sem adição de crioprotetor; glicerol 1 M; glicerol 2 M; sacarose 0,4 M; sacarose 0,8 M; PVS1; PVS2; PVS2 + 1% floroglucinol e PVS3. As sementes permaneceram congeladas por 15 dias quando se avaliaram a porcentagem de germinação, plântulas anormais, índice de velocidade de germinação e comprimento e massa seca das plântulas. Após o cultivo em casa de vegetação durante seis meses, comprimento e massa seca da parte aérea e do sistema radicular, além da massa seca total e a razão parte aérea/raiz foram determinados. No tratamento sem crioprotetores, as sementes de *D. brevifolia* e *D. delicata* apresentaram, respectivamente, 92% e 79% de germinação. O uso de crioprotetores não prejudicou a viabilidade das sementes. Sementes de *Dyckia brevifolia* com 6,8% de teor de água e *Dyckia delicata* com 8,9% podem ser criopreservadas em nitrogênio líquido sem a necessidade de soluções crioprotetoras.

Palavras-chave: soluções crioprotetoras; nitrogênio líquido; banco de sementes

Introduction

The Bromeliaceae family is grouped into 58 genera and 3,140 species (Givnish et al., 2011). In Brazil, it is possible to find 83% of the species from the genus *Dyckia*, having the species *Dyckia brevifolia* Baker and *Dyckia delicata* Larocca & Sobral as endemic in the southern region (Leme et al., 2012). Bromeliads play an important role in the ecosystem and in maintaining the biodiversity. Due to human action as well as to the changes in their habitat, bromeliads species such as those abovementioned are faced with extinction (Martinelli & Moraes, 2013).

In order to maintain these and other species, cryopreservation rises as a tool for preserving plant material. This is a preservation method in which the biological material is subjected to extremely low temperatures (-196 °C) in nitrogen, in its liquid or vapor forms (-150 °C) and, after the thawing, the material still maintains its characteristics intact. As such, the preserved plant material does not suffer damage and can develop normally after thawing (Santos, 2000).

During the cryopreservation process, it is essential avoiding the formation of intracellular ice and ice crystals that would cause cell damage and even death of preserved cells. For this, during the process, cryoprotective substances are used. They act on the cell during the storage period at low temperatures and prevent the formation of intra and extracellular ice, besides preventing possible damage caused by dehydration (Han et al., 2009).

In order to minimize the possible osmotic and toxic damage when using these substances, there are the combined use of several cryoprotectants or the association between intracellular cryoprotectants and those with extracellular action. This varied cryoprotectants composition is found in PVS solutions (plant vitrification solutions) and, because of that it has great use in the cryopreservation process (Denniston et al., 2000).

However, some cryoprotectants, especially those with intracellular action, are said to be moderately toxic. For that purpose, knowing the action mechanism is essential, as well as the possible damage that these substances cause in the biological material to be frozen (Silveira, 2015).

Some species have internal mechanisms that enable freezing the seed without any kind of protection. Results found by Ferrari et al. (2016), using seeds from the bromeliad *Encholirium spectabile* Martius ex Schultes, indicated that using protective solutions is not necessary. Other six species from the genus *Encholirium* and two of *Dyckia* also did not have a negative effect on the seed germination percentage after immersion in liquid nitrogen without cryoprotectants (Tarré et al., 2007).

Among the cryopreservation methods, vitrification is nowadays the most used for preserving different biological materials, not requiring any specific equipment, thus reducing the cost in addition to having a high restoration rate of the frozen material. This method has as its principle the high concentration of substances, thus triggering the cell to change into a vitreous state before the formation of ice crystals inside it, decreasing their quantitiy (Saragusty & Arav, 2011).

With this in mind, the present study aimed to evaluate the influence of different cryoprotective solutions on the cryopreservation in liquid nitrogen of seeds from Brazilian bromeliad species faced with extinction, *D. brevifolia* and *D. delicata*.

Materials and Methods

The experiment was conducted at the Department of Agronomy of the State University of Londrina (UEL). Seeds of *Dyckia brevifolia* Baker (Figure 1) and *Dyckia delicata* Larocca & Sobral (Figure 2) were collected from stock plants part of the property of a collector , located in the municipality of Londrina, PR (23° 22' South latitude and 51° 11' West longitude). As according to the Koppën classification, the climate in this region is of the Cfa type (humid subtropical), with average temperature of 17.0 °C in the coldest month and of 24.0 °C in the hottest month. The average annual precipitation is 1632 mm and the average annual temperature is 21.2 °C (Iapar, 2019).

The seeds were manually extracted from mature capsules and stored inside Kraft paper bags at 10 ± 2 °C, for two weeks. For the characterizing the lot, the water content of the seeds was determined after their collection, as well as their viability

Figure 1. Aspects of the plant (A), inflorescence (B) and seeds (C) of *Dyckia brevifolia.*

Figure 2. Aspects of the plant (A), inflorescence (B) and seeds (C) of *Dyckia delicata.*

by the tetrazolium test, both following the Brazilian Rules for Seed Testing (Brazil, 2009).

In order to determine the water content, 0.2 g seeds of each species were placed in an oven at 105 ± 3 °C for 24 hours. When performing the tetrazolium test, 4 replicates cotaning 50 seeds each were used. For each species, the seeds were placed in cryotubes with a capacity for 2 mL, filled to the completion with distilled water. The water was removed after a period of 24 hours at 25 °C in a germination chamber and a tetrazolium salt solution at 1% was added, with the seeds then were kept inside a B.O.D. in absence of light for 24 hours at 30 °C. After this period, the viable seeds percentage was evaluated with a magnifying glass and, for its calculation, the empty seeds, that is, those without an embryo, were not taken into account.

At the first part of the study, when setting the experiments up, the seeds were placed in cryotubes with volumetric capacity for 2 mL and 4 cryotubes with 50 seeds per treatment were used, totaling 2,000 seeds for each evaluated species. The treatments consisted of 1 M glycerol; 2 M glycerol; 0.4 M sucrose; 0.8 M sucrose; PVS1; PVS2; PVS2 + 1% phloroglucinol and PVS3, in addition to a treatment with seeds immersed in liquid nitrogen without adding cryoprotective substances. A control was also used, without adding cryoprotectants or having immersion in liquid nitrogen.

The PVS1 solution was composed of 19% glycerol (v/v), 13% ethylene glycol (v/v), 6% dimethyl sulfoxide (v/v) and 0.5 M sorbitol (Sakai et al., 1990) diluted in a MS medium (Murashige & Skoog, 1962) modified with half the concentration of macronutrients. The PVS2 solution contained 30% glycerol (v/v), 15% ethylene glycol (v/v), 15% dimethyl sulfoxide (v/v) and 0.4 M sucrose, diluted in MS medium with half the concentration macronutrients (Vendrame & Faria, 2011), and the PVS3 solution was composed of 50% glycerol (v/v) and 50% sucrose (v/v) diluted in distilled water (Teixeira et al., 2014). All solutions were prepared by using a volume of 100 ml.

In the control treatment, the seeds were stored inside cryovials at 10 ± 2 °C, until their transference to the germination chamber. In the unprotected treatment, seeds were immersed in liquid nitrogen without adding a cryoprotective substance. The other treatments consisted of adding 2 mL of different cryoprotectant solutions in the cryotubes with a Pasteur pipette.

The treatments with 1 M glycerol, 2 M glycerol, 0.4 M sucrose and 0.8 M sucrose were exposed to the solutions for 20 minutes at room temperature (25 ± 2 °C) and then immersed in liquid nitrogen. Treatments with PVS1, PVS2, PVS2 + 1% phloroglucinol and PVS3, that had seeds with the solutions, were left for 10 minutes in an ice bath (0 °C) and then immersed in liquid nitrogen.

Cryotubes remained stored in liquid nitrogen (-196 °C) for 15 days. After the removing the seeds, a rapid defrosting was performed in an Evlab® water bath, model EV:015-D, with precision of 0.1 ºC, at the temperature of 40 °C for one minute and a half. The cryoprotective solutions were removed from the cryotubes using a Pasteur pipette. Afterwards, the seeds were washed with autoclaved water three times in a row and sterilized in sodium hypochlorite solution at 1% for another minute and washed once again with autoclaved water, subsequently finally having been subjected to the germination test.

The germination test was conducted on germitest paper moistened with distilled water in an amount of 2.5 times the mass of the non-hydrated paper, and the seeds packed in 11 cm x 11 cm x 3 cm polystyrene crystal boxes (Gerbox®). The boxes were kept in a growth chamber (B.O.D. type) at a temperature of 25 ± 2 °C, under constant light, according to the recommendations from the Brazilian Rules for Seed Testing (Brazil, 2009) for other small-seed species.

The germination reading started from the third day after the sowing until the tenth day. Its percentage was quantified by computing all normal seedlings (Brazil, 2009), considered as those that demonstrated potential to continue their development and give rise to plants when under favorable conditions. A normal seedling was considered to have a 1:2 ratio (one shoot part to two root parts).

Simultaneously with the germination test, the number of germinated seeds was counted to establish the germination speed index (GSI), obtained through Equation 1, as described by Maguire (1962).

$$GSI = \frac{G1}{N1} + \frac{G2}{N2} + \ldots + \frac{Gn}{Nn} \quad (1)$$

in which: G1, G2 and Gn = number of normal seedlings, computed in the first, second and last counts; N1, N2, Nn = number of sowing days at the first, second and last counts.

On the tenth day of the germination test, the percentages of germination and abnormal seedlings were determined, and the result expressed as percentage, in addition to determining the seedlings length (mm) aided by a caliper, through the random selection of twenty seedlings. Subsequently, the seedlings dry weight was determined, which were placed in Kraft paper bags and kept in an oven with forced air circulation at 65 °C, until reaching a constant weight (period of five days). The seedlings were weighed on an analytical balance (precision ± 0.0001 g) and the result expressed in milligrams.

At the end of the germination test, the second part of the experiments was conducted by acclimatizing the seedlings in a growth chamber for a period of thirty days, when they were randomly selected and transplanted in polypropylene trays with 200 cells, using a Carolina Soil® as substrate. The seedlings were kept in an acclimatized greenhouse, model Van der Hoeven® covered with transparent polycarbonate plates and diffuser, having 50% luminous retention due to the Aluminet® shade screen and the controlled temperature at 28 ± 3 °C. Irrigation was manual, by adding a 6 mm water depth daily, during the mornings. After 180 days, the length of the shoot and of the root system (cm) were evaluated by considering the largest root length, in addition to the determination of dry weights of the shoot and root parts (mg). With the obtained results, we determined the total dry weight (shoot + root) and the shoot/root ratio.

The completely randomized experimental design was adopted for the germination test of cryopreservatives-treated seeds and the acclimatization of seedlings in a greenhouse for evaluating growth and development, assessing biometric variables and dry weight production.

The statistical analysis for the germination test consisted of 10 treatments with 4 replicates and 50 seeds per Gerbox®, totaling 2,000 seeds per species, and concerning the biometric and dry weight measurements, 10 seedlings were randomly chosen per plot, totaling 400 seedlings by species.

For the statistical analysis of the seedlings acclimatization in a greenhouse, 10 treatments with 5 replicates and 5 seedlings per plot were considered, totaling 200 seedlings per species.

The assumptions of normality and homogeneity of variances were tested by Shapiro-Wilk and Hartley ($p < 0.05$), respectively. For all variables, both in the germination experiment and in acclimatization, the data were not transformed, except for the variable ratio (SDW/RDW) in which, for both species, the data were transformed to $1/\sqrt{x}$ as according to the methodology of Box & Cox (1964). Subsequently, the data were subjected to analysis of variance ($p < 0.05$) by employing the R software (R Core Team, 2019) and the means were compared using the Tukey test, considering a significance level of 5%error probability.

Results and Discussion

The seeds of *Dyckia brevifolia* and *Dyckia delicata* had 6.8% and 8.9% of water content and 98% and 94% of viability, respectively (Figure 3), just before they were subjected to the cryopreservation process.

The low water content in the seeds is a fundamental factor for their survival after removal from the liquid nitrogen (Stegani et al., 2017), with this as one of the main factors that influence the cryopreservation process (Walters et al., 2004). Concerning the studied species, the water content is in accordance with the recommendation of Silva et al. (2011), below 10%; however, the ideal humidity range for freezing differs between species. Cryopreservation thus becomes an alternative in storing biological material for an indefinite period, being advantageous due to its simple and efficient nature as a method, when in comparison to traditional preservation methods (Vendrame et al., 2014).

Among the used materials, seeds have an advantage in relation to survival and regeneration when cryopreserved, since dehydration and freezing occur more quickly and uniformly. As it is a young and small material, with small cells and dense cytoplasm with few vacuoles, there is little free water available for freezing (Santos, 2000), which favors the process of cryopreservation and maintenance of viability.

For *Dyckia brevifolia*, the germination percentage remained high after cryopreservation, varying from 87 to 94%, with no statistical difference between treatments (Table 1).

In the treatment without the cryopreservation process, the germination percentage was 95% for *D. brevifolia*. According to Nikishina et al. (2007), the seed germination rates after exposure to liquid nitrogen vary according to the species and can be lower or higher than the control, with the cryoprotectants efficiency variating depending on the structure (cell or tissue) to be cryopreserved, in addition to the used chemical substance, concentration and exposure time prior the cryopreservation process.

The germination speed index (GSI) when using 2 M glycerol (2.81) was lower than the other treatments, which may indicate a toxic effect on the seeds. In studies available in the literature, results have been reported in which glycerol can affect the cytoplasmic organization and alter the permeability and stability of the lipid bilayer (Hammerstedt & Graham, 1992).

No influence of the treatments used was found in relation to the percentage of abnormal seedlings (AS), length (L)

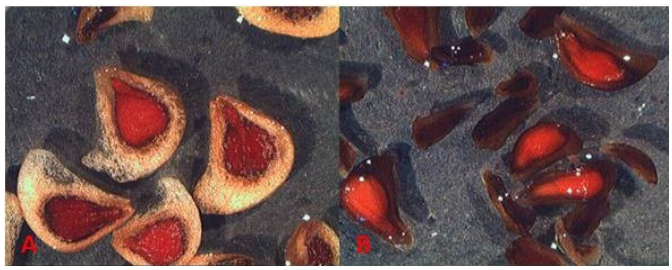

Figure 3. Seeds of *Dyckia brevifolia* (A) and *Dyckia delicata* (B) subjected to the test before the cryopreservation in liquid nitrogen.

Table 1. Percentages of germination (GERM) and abnormal seedlings (AS), germination speed index (GSI), length (L) and dry weight of seedlings (DWP) of *D. brevifolia*.

Treatment	GERM (%)	AS (%)	GSI	L (mm)	DWP (mg)
No cryoprotector and no liquid nitrogen (control)	95.50 a*	1.50 a	6.68 a	6.59 a	3.73 a
No cryoprotector	92.00 ab	3.50 a	6.83 a	6.26 ab	3.22 abcd
1 M glycerol	88.00 ab	2.50 a	7.04 a	5.83 ab	3.04 bcd
2 M glycerol	87.00 b	2.00 a	2.81 b	5.85 ab	2.98 cd
0.4 M sucrose	93.50 ab	2.50 a	7.11 a	6.01 ab	3.54 ab
0.8 M sucrose	87.50 b	4.00 a	7.04 a	5.78 ab	2.74 d
PVS1**	91.00 ab	3.00 a	6.83 a	5.95 ab	3.41 abc
PVS2**	91.00 ab	5.00 a	6.88 a	5.72 b	3.27 abcd
PVS2 + 1% F**	94.00 ab	1.50 a	7.68 a	6.12 ab	3.47 abc
PVS3**	88.00 b	2.00 a	6.20 a	6.00 ab	3.18 bcd
CV (%)	3.34	72.97	10.31	5.65	6.86

*Means followed by the same letter in the column do not differ from each other by the Tukey test ($p \geq 0.05$)
**PVS1 [19% glycerol (v/v), 13% ethylene glycol (v/v), 6% dimethyl sulfoxide (v/v) and 0.5 M sorbitol, diluted in ½ half MS]; PVS2 [30% glycerol (v/v), 15% ethylene glycol (v/v), 15% dimethyl sulfoxide (v/v) and 0.4 M sucrose, diluted in ½ half MS]; PVS2 + 1% phloroglucinol and PVS3 [50% glycerol (v/v) and 50% sucrose (v/v) diluted in distilled water].

and seedling dry weight (SEDW). Other studies with seeds of *Encholirium heloisae*, *E. pedicellatum*, *E. magalhaesii*, *E.reflexum*, *E. subsecundum*, *E. scrutor*, *Dyckia sordida* and *D. ursina* demonstrated no negative effect on the germination percentage, after immersion of seeds in liquid nitrogen (Tarré et al., 2007).

Endemic bromeliads species from the Atlantic Forest (*Alcantarea imperialis* (Carrière) Harms, *Nidularium ferdinandocoburgi*i Wawra, *Pitcairnia albiflos* Herb., *Pitcairnia encholiriodes* LBSm., *Pitcairnia flammea* Lindl., *Vriesea cacuminis* LBSm., *Vriesea fribant*. Baker) Leme had their seeds preserved with a low water content (5-7%) after freezing in liquid nitrogen, maintaining viability for one year without using cryoprotective substances (Alba et al., 2014). Pereira et al. (2010), researching the *Pitcairnia albiflos* bromeliad, witnessed the maintenance of viability for one year of seeds with 5-7% of water content after the cryopreservation process (-196 °C); however, they also highlight that when the water content was in 3%, there was a detrimental effect on seed longevity.

D. delicata seeds had a similar responses (Table 2), maintaining viability with low water content; however, no particular treatment stood out during the freezing process.

The germination percentage in the control was 92%, differing only from the treatment subjected to freezing without cryoprotectant. The higher water content in *D. delicata* seeds in comparison to *D. brevifolia* may have led to a reduction in the seed quality after the cryopreservation process, when cryoprotectants were not used. No significant difference in germination between the other treatments occurred, with variation of 79% for the treatment subjected to unprotected liquid nitrogen and 88% for the treatment with PVS2+1%F.

On the variable abnormal seedlings percentage (AS), the treatment without cryoprotectant stood out in comparison to other treatments and differed significantly from the control (without any cryoprotectant and liquid nitrogen).

For the germination speed index (GSI), the treatment without cryoprotectant did not differ from the treatments with 1 M glycerol and PVS2, showing a significant difference from the other treatments. The control showed the best result for the seedling length (L) variable, differing significantly from the treatment without cryoprotectant.

The seedling dry weight had the highest values in the control (without cryoprotectant and neither subjected to liquid nitrogen) with 9.26 mg, not differing only from the treatment with the solution of PVS2+1%F (8.96 mg), presenting these higher dry weight values compared to the others.

The studied species seeds maintaining their physiological quality when in liquid nitrogen (-196 °C) is explained by

Table 2. Percentages of germination (GERM) and abnormal seedlings (AS), germination speed index (GSI), length (L) and dry weight of seedlings (SEDW) of *D. delicata.*

Treatment	GERM (%)	AS (%)	GSI	L (mm)	SEDW (mg)
No cryoprotector and no liquid nitrogen (control)	92.50 a*	1.50 b	4.06 a	6.23 a	9.26 a
No cryoprotector	79.50 b	5.00 a	2.13 b	5.32 b	5.97 f
1 M glycerol	86.50 ab	4.00 ab	3.46 ab	5.42 ab	7.68 bc
2 M glycerol	82.00 ab	5.50 ab	4.28 a	5.75 ab	7.53 bc
0.4 M sucrose	88.00 ab	3.50 ab	4.09 a	6.18 ab	6.24 ef
0.8 M sucrose	87.00 ab	4.00 ab	4.04 a	6.10 ab	8.01 b
PVS1**	87.00 ab	2.50 ab	4.31 a	5.54 ab	7.94 b
PVS2**	86.50 ab	3.50 ab	3.94 ab	5.94 ab	6.70 de
PVS2 + 1% F**	88.50 ab	2.00 ab	4.59 a	6.04 ab	8.96 a
PVS3**	86.50 ab	2.50 ab	4.17 a	5.59 ab	7.13 cd
CV (%)	7.48	57.51	19.33	6.95	3.93

*Means followed by the same letter in the column do not differ from each other by the Tukey test ($p \geq 0.05$)
**PVS1 [19% glycerol (v/v), 13% ethylene glycol (v/v), 6% dimethyl sulfoxide (v/v) and 0.5 M sorbitol, diluted in ½ half MS]; PVS2 [30% glycerol (v/v), 15% ethylene glycol (v/v), 15% dimethyl sulfoxide (v/v) and 0.4 M sucrose, diluted in ½ half MS]; PVS2 + 1% phloroglucinol and PVS3 [50% glycerol (v/v) and 50% sucrose (v/v) diluted in distilled water].

Stanwood & Roos (1979). According to these authors, orthodox seeds, as the species studied, can be dehydrated to a very low degree of humidity without damage by freezing or by the formation of ice crystals, and without prejudice to viability.

The seeds of both species showed high germination and normal initial development, even without using cryoprotectants (Figure 4).

The low free-water content in the seeds was essential in avoiding the formation of intracellular ice crystals during the freezing, which could cause the endomembrane system to rupture and result in losing both the semipermeability and the cell compartmentalization (Kaviani et al., 2009). Moreover, the seeds, especially orthodox ones, have protective mechanisms capable of maintaining cell membrane systems, macromolecule structures and reserve substances in conditions to restore their physiological functions after a period of dehydration (Walters et al., 2001).

Figure 4. Seedlings of *Dyckia brevifolia* (A) and *Dyckia delicata* (B) after 30 days in a germination chamber observed in the control treatment.

However, using cryoprotectants was also efficient in maintaining the viability and a high germinative potential. This is due to the hydrogen bonds between the cryoprotectants and the water molecule decreasing the formation of ice crystals, in addition to promoting the stabilization of the quaternary structure of membrane proteins, preserving them from dehydration (Sakai, 1995). Nonetheless, these cryoprotectants, especially intracellular ones, can be toxic or resulting in osmotic stress, causing cells to die or changing their morphogenetic response (Sakai, 1995). For this, adjustments are always needed in relation to the cryoprotectant type and the plant material under study; although in this case there was no toxic effect for these species.

In order to ensure that after thawing the seedlings will develop normally, studies after the germination are essential. The results indicated that, after six months of cultivation, for both species, there was no influence of adding or not cryoprotective substances, and there was also no change in the development of roots, shoot and dry weight, total dry weight and SDW/RDW ratio among the used cryoprotectants. Hence, in all treatments, the seedlings demonstrated satisfactory growth and development, both for the biometric and weight variables (Table 3).

Table 3. Lengths of the shoot (SL) and root (RL), dry weights of shoot (SDW) root (RDW) and total (TDW), and SDW/RDW ratio of *D. brevifolia* and *D. delicata* after 180 days of cultivation in a greenhouse.

Treatment	SL (cm)	RL (cm)	SDW (mg)	RDW (mg)	TDW	SDW/RDW[1]
			D. brevifolia			
No cryoprotector and no liquid nitrogen (control)	3.64 a*	4.74 a	108.66 a	127.74 a	236.40 a	0.85 a
No cryoprotector	3.20 ab	4.82 a	104.30 a	118.04 ab	222.34 ab	0.94 a
1 M glycerol	2.90 b	4.87 a	84.82 a	99.14 ab	183.96 ab	0.88 a
2 M glycerol	2.86 b	4.80 a	103.28 a	99.66 ab	202.94 ab	1.13 a
0.4 M sucrose	2.95 b	4.87 a	93.94 a	84.82 ab	178.76 ab	1.28 a
0.8 M sucrose	2.97 b	4.73 a	86.40 a	79.96 ab	166.36 ab	1.09 a
PVS1**	3.15 ab	5.05 a	85.30 a	76.02 ab	161.32 b	1.14 a
PVS2**	2.94 b	4.91 a	102.80 a	85.40 ab	188.20 ab	1.42 a
PVS2 + 1% F**	3.18 ab	5.02 a	95.02 a	75.84 ab	170.86 ab	1.31 a
PVS3**	2.82 b	4.91 a	99.38 a	75.30 b	174.68 ab	1.44 a
CV (%)	9.71	5.42	22.13	26.77	17.99	16.67 a
			D. delicata			
No cryoprotector and no liquid nitrogen (control)	3.04 a*	5.25 a	114.08 a	88.16 a	202.24 a	1.48 a
No cryoprotector	3.10 a	5.13 a	110.08 a	77.28 a	187.36 ab	1.43 a
1 M glycerol	2.79 a	4.73 a	98.38 a	70.96 a	169.34 ab	1.39 a
2 M glycerol	2.89 a	4.78 a	86.36 a	65.82 a	152.18 b	1.33 a
0.4 M sucrose	2.86 a	4.72 a	84.64 a	64.92 a	149.56 b	1.32 a
0.8 M sucrose	2.83 a	4.69 a	82.56 a	66.42 a	148.98 b	1.30 a
PVS1**	3.06 a	5.09 a	97.32 a	69.94 a	167.26 ab	1.48 a
PVS2**	2.87 a	5.07 a	107.22 a	65.98 a	173.20 ab	1.70 a
PVS2 + 1% F**	2.97 a	5.06 a	104.48 a	68.98 a	173.46 ab	1.52 a
PVS3**	2.85 a	4.91 a	97.08 a	66.82 a	163.90 ab	1.47 a
CV (%)	5.08	6.46	18.18	19.30	12.60	13.99 a

[1] Data from this variable were transformed for 1√x

*Means followed by the same letter in the column do not differ from each other by the Tukey test ($p \geq 0.05$)

**PVS1 [19% glycerol (v/v), 13% ethylene glycol (v/v), 6% dimethyl sulfoxide (v/v) and 0.5 M sorbitol, diluted in ½ half MS]; PVS2 [30% glycerol (v/v), 15% ethylene glycol (v/v), 15% dimethyl sulfoxide (v/v) and 0.4 M sucrose, diluted in ½ half MS]; PVS2 + 1% phloroglucinol and PVS3[50% glycerol (v/v) and 50% sucrose (v/v) diluted in distilled water].

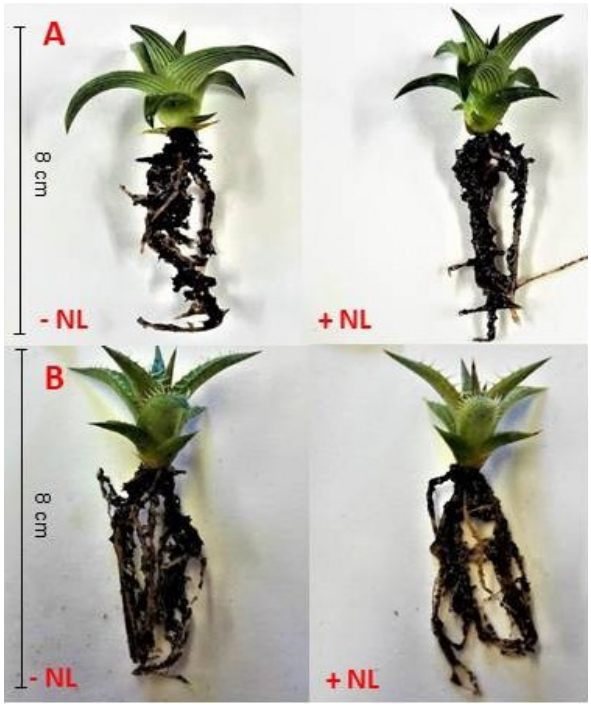

Figure 5. Growth and development of *Dyckia brevifolia* (A) and *Dyckia delicata* (B) plants after 180 days, when (+NL) or not (-NL) to cryopreservation in liquid nitrogen.

The seeds from both species demonstrated that there is no need for cryoprotectants. Figure 5 illustrates the satisfactory plant growth when comparing the treatment with and without using liquid nitrogen. The results demonstrate that the cryopreservation technique can be performed in a simple way and on a low cost (Stegani et al., 2017).

Conclusions

Using cryoprotector did not impair the viability of the seeds.

Seeds of *Dyckia brevifolia* with 6.8% water content and *Dyckia delicata* with 8.9% can be cryopreserved in liquid nitrogen, having no need for cryoprotector solutions.

Acknowledgements

To the researcher Walter Miguel Kranz for donating the seeds and collaborating in developing this study. The authors would also like to thank CAPES (Coordination for the Improvement of Higher Education Personnel) for the scholarship granted to the first author (no. 88882.448347/2019-01) and CNPQ (Brazilian National Council for Scientific and Technological Development) for financial supporting the last author (no. 301684/2017-0).

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