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RESEARCH NOTE

Seed cryopreservation of Passiflora species[1]

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ABSTRACT - The aim of this study was to evaluate the cryopreservation of seeds of Passiflora species, using different cryoprotectants. The completely randomized design was used with four treatments (T1 - DMSO at 7%; T2 - 0.3 M sucrose; T3 - storage in NL_2 without cryoprotectants; T4 - control) and five replications of 50 seeds. The seeds were stored for 120 hours in NL_2 (-196 °C), and thawing was conducted in a water bath (37 °C) for 20 minutes, proceeding with the germination and vigor tests. The data were submitted to ANOVA and Tukey's test (5% of probability) using the SISVAR program. In *P. mucronata, P. suberosa* and *P. edulis* seeds, the responses obtained were favorable to cryopreservation without the use of cryoprotectants in regards to *P. micropetala,* there was a considerable loss of germination potential after storage in NL_2. Given these circunstances, cryopreservation can be used for long-term conservation of *Passiflora mucronata*, *P. suberous* and *P. edulis*, without the use of cryoprotectants.

Index terms: passion fruit, storage, germination.

Criopreservação de sementes de espécies de *Passiflora*

RESUMO: Objetivou-se avaliar a criopreservação de sementes de espécies de *Passiflora,* utilizando-se diferentes crioprotetores. Foi utilizado o Delineamento Inteiramente Casualizado, com quatro tratamentos (T_1 – DMSO a 7%; T_2 – sacarose a 0,3 M; T_3 - armazenamento em NL_2 sem crioprotetor; T_4 – controle) e cinco repetições de 50 sementes. As sementes foram armazenadas durante 120 horas em NL_2 (-196 °C), realizou-se o descongelamento em banho-maria (37 °C) durante 20 minutos, e prosseguiram-se os testes de germinação e vigor. Os dados foram submetidos à análise de variância e teste de Tukey (5% probabilidade) pelo programa SISVAR. Em sementes de *P. mucronata, P. suberosa* e *P. edulis* foram obtidas respostas favoráveis à criopreservação sem o uso de crioprotetores, e em *P. micropetala* houve perda considerável do potencial germinativo após armazenamento em NL_2. Diante disso, a criopreservação pode ser utilizada para conservação em longo prazo de sementes de *Passiflora mucronata, P. suberosa* e *P. edulis*, sem o uso de crioprotetores.

Termos para indexação**:** maracujá, armazenamento, germinação.

Introduction

The Passifloraceae family is represented in South America by four genera, among which is *Passiflora*, constituted by over 500 species, with approximately 150 of them originating from Brazil; this is the most important genus in terms of quantity and economically (Hansen et al., 2006).

Several wild species of *Passiflora* have shown a major potential for use in genetic improvement programs, with nutritional, ornamental, and medicinal characteristics, as a source of resistance to diseases, as rootstock, in terms of the quality of the fruits and its adaptability for culture (Faleiro et al., 2011).

In Brazil, the genetic erosion in *Passiflora* species has occurred in a significant manner, due to factors such as the growing use of new areas of the Central-Northern regions for agricultural and industrial purposes (Faleiro et al., 2011). Therefore, the preservation of the germplasm of local species,

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domestic cultivars and wild relatives of agronomic species has been considered as an important research area (Carvalho and Otoni, 2010).

According to Delanoy et al. (2006), several authors have shown that the germination of passion fruit trees may go from ten days to three months, showing a low germination percentage and irregularity in the formation of seedlings. In that sense, Fowler and Bianchetti (2000) observed that some species show dormant seeds, that may be related to physical factors (tegument impermeability to water and gas). The thick tegument of the seeds is mentioned as the limiting factor to permeability. On studies for different species, it was observed that tissues that surround the embryo, such as the endosperm and perisperm, may restrict the radicular growth (Welbaum and Bradfort, 1990).

Traditionally, genetic resources from most *Passiflora* species have been preserved in an *ex situ* manner on field collections; however, the in field preservation has some disadvantages that limit its efficiency and threaten the safety of vegetable genetic resources preserved in such a way (Engelmann, 2004).

Cryopreservation has stood out as a preservation method of biological material at ultra-low temperatures (up to -196 °C) on Liquid Nitrogen (NL_2) or during its vapor phase (between -154 °C and -196 °C), which allows taking the material to a state of lack of cellular division and drastically reduced metabolism, keeping its feasibility for a long term (Carvalho and Otoni, 2010; Kaviani, 2011).

For the cryopreservation, it is essential to avoid freezing the intracellular water, which occurs during the quick cooling in liquid nitrogen, in case the tissues are hydrated (Kami, 2012). Due to this reason, the water content is probably the most critical factor in order to define a cryopreservation protocol for seeds (Ospina et al., 2000).

Cryoprotectants are chemical substances that cause the osmotic dehydration of the tissues, taking them to a vitreous state, which reduces the damage to the cells during their freezing and/or thawing (Benson, 2008; Carvalho and Otoni, 2010). The most commonly used cryoprotectants for the cryopreservation of plants are glycerol, sucrose, dimethyl sulfoxide (DMSO - Me_2SO), methanol and glycols with lower molecular weight (Benson, 2008).

The development of cryopreservation protocols for native plants from Brazil is still limited, with most of the efforts directed toward species that are economically important (Pilatti et al., 2011). Given these circumstances, the aim of this study was to evaluate the cryopreservation of Passiflora seed species, using different cryoprotectants.

Material and Methods

The experiment was conducted at the Laboratory of Seeds and Ornamental Plants of Universidade do Estado de Mato Grosso, Cáceres Campus – MT. Yellow mature fruits were obtained from 12 mother plants, from each species, in the experimental field of the campus, located at 16º11‟42” of South latitude and 57º40‟51” of West longitude, with annual mean temperature of 26.24 ºC, total annual rainfall of 1,333 mm and altitude of 118 m (Neves et al., 2011). The fruits were collected when matured and submitted to the Laboratory of Seeds and Ornamental Plants, where they were transversally cut, and the pulp that contains the seeds was removed. The method used to remove the aril was manual, with friction of the seeds on a sieve with mesh that was smaller than its size, with the help of lime and running water until the mucilage had been completely cleaned.

Four species from the *Passiflora* genus were evaluated: *P. mucronata, P. micropelata, P. suberosa* and *P. edulis* (UNEMAT/UFV/UENF 50). The seeds were distributed on trays for two days at room temperature in the laboratory (27.7 °C ± 1.9) to dry and then they were stored on glass containers and placed on the refrigerator (± 7 °C) for two days.

Initial seed water content: it was determined by the oven method at 105 °C (± 3 °C), according to Brasil (2009), using two subsamples of 50 seeds kept on a forced air circulation oven for 24 hours. The results were expressed in percentage.

Seed cryopreservation: four treatments were conducted with five replications for 50 seeds on a completely randomized design, knowingly: T_1 – cryopreserved seeds with the addition of DMSO at 7% of concentration; T_2 – cryopreserved seeds with the addition of sucrose at a concentration of 0.3 M; T_3 – cryopreserved seeds without cryoprotectants; T_4 – control: seeds that were not stored on NL_2 and with no cryoprotectants. Before submitting the seeds to the treatment with cryoprotectants, an assay was conducted to determine the seed water content (b.u. %) to be used for the cryopreservation of the seeds on liquid nitrogen (NL_2). The initial water contents varied from 6.12% to 12% for the different studied species (Table 1). The seeds were immersed on the cryoprotecting solutions for three hours, according to Vendrame et al., (2007), and maintained at room temperature of the laboratory (27.7 °C ± 1.9) for one day to dry; then, the desired water content was adjusted, as described on Table 1, according to the methodology by Cromarty et al. (1985). The seed samples were placed on aluminum packages, which were stored on a liquid nitrogen tank (-196 °C) for a period of 120 hours.

Thawing: the method used was a water bath at a temperature of 37 °C for five minutes, according to Molina et al. (2006). For such, the seed samples on the aluminum packages were removed from NL_2 and placed on polyethylene

bags, with the purpose of avoiding any direct contact between the seeds and the water.

Overcoming of dormancy: following tests conducted in the laboratory, the seeds of some species underwent treatment to overcome the dormancy. For *Passiflora mucronata*, the treatment was conducted after thawing, and consisted in the immersion on potassium nitrate (KNO_3) at a concentration of 1%, for twenty-four hours. For *Passiflora micropetala*, the treatment was conducted before thawing, by mechanical scarification at the distal region in relation to the embryonic axis of the seed, rubbing it for three seconds with a sandpaper number 120.

Germination test: the seed samples were distributed on acrylic boxes ("Gerbox"), containing a blotting paper substrate previously moistened with distilled water at a proportion of 2.5 times the weight of the paper. They were then placed on BOD with constant temperature of 25 °C, photoperiod of 12 hours, and evaluated for 30 days, considering as germinated the seeds that had a radicle with at least 2 mm in length (Hadas, 1976).

Seedling emergence test: in each treatment, four replications with 50 seeds were used, distributed on acrylic boxes ("Gerbox") containing the commercial substrate "Vermiculita" at an amount of 0.040 grams, and moistened with distilled water. The boxes were maintained in the laboratory, under room temperature (19.9 to 35.7 °C). The evaluation was conducted 30 days after sowing, calculating the normal emerged seedlings, and the result was expressed as percentage. The following variables were determined:

Germination percentage of the seeds (G%) and Emergence percentage of seedlings (E%); Germination Speed Index (GSI) and Emergence Speed Index (ESI): calculated according to Maguire (1962); Dry matter mass of the seedlings (DM): the normal seedlings obtained from the emergence test were weighted to determine the fresh mass. Then, they were stored on paper bags and taken to the air circulation oven at 70 °C for 72 hours and were weighted to determine the dry matter mass: the results were expressed as $mg.seedling^{-1}$; Length of the above-ground part of the seedling (APL): the seedlings from the emergence test obtained at 30^{th} day after sowing were used, and their above-ground length was measured with a millimeter ruler. The mean length of the seedlings from each replication was obtained by dividing the sum of the measurements taken from the subsamples by the number of normal seedlings measured, and the results were expressed as $cm.seedling^{-1}$; Length of the radicle (LR): it was calculated using the normal seedlings from the emergence test. The evaluation was conducted on the thirtieth day after sowing, measuring the normal seedlings with a millimeter ruler, and the result was expressed in $cm.seedling^{-1}$.

The data were subjected to the normality test by Shapiro-Wilk using the statistical program R version 2.15.2. Therefore, the results were subjected to analysis of variance through the F test, with the means compared using Tukey's test, at a 5% probability level, using the computer program Sistema para Análise de Variância - SISVAR (Ferreira, 2008), considering that the data were transformed into to square root of X+0.5, since they did not show a normal distribution.

Results and Discussion

The water content of the seeds used for the cryopreservation varied from 6.2% (moist basis) for *P. micropetala* to 12% for the *P. mucronata* and *P. edulis* species. Martins et al. (2009) verified that, for *Tabebuia impetiginosa* (Mart. ex DC.) Standl. seeds, the humidity degree of 12.5 at 4.2% assured an adequate physiological performance of the seeds under liquid nitrogen. For orthodox seeds, contents with humidity below 10% are recommended; however, the favorable humidity interval for freezing differs according to the species (Silva et al., 2011).

There were significant results ($P<0.05$) for the treatments applied in all evaluated species (Table 1). The results obtained indicated positive effects of cryopreservation on some Passiflora species without the use of the cryoprotectants.

Regarding the *P. mucronata* species, significant effects of the cryopreservation were observed regarding most variables, given that for the IVG, germination and root length variables no significant differences were observed between the control and cryopreservation without the use of cryoprotectants (Table 1).

The results obtained regarding the emergency of *P. mucronata* seedlings indicated losses on the vigor of seedlings after their storage on liquid nitrogen (Table 1). On this regard, some authors state that after the cryopreservation, abnormal germination may occur, or even death due to internal injuries, which may be related to the characteristics of certain seeds, such as size, water content and chemical composition (Goldfarb et al., 2010; Silva et al., 2011). However, sucrose was promising regarding the reduction of the injury caused to the seeds by the ultra-low temperatures, maintaining the emergency percentage of seedlings on the same levels than the control treatment. The development of the above-ground part of the seedlings (APL) and the dry matter mass (DM) was also favored, with the same means as the control.

Studies confirm that sugars, especially sucrose, have the ability to stabilize the phospholipid bilayer, acting as an external osmotic agent. In addition, in the absence of water, sucrose maintaining the ability to transport calcium through the membranes, in addition of inhibiting their merger, maintaining the lipids on a fluid phase, stabilizing proteins under freezing conditions, and they are free from cytotoxicity even when under high concentrations (Woelders et al.,1997).

Table 1. Germination Speed Index (GSI), Germination (G), Emergence Speed Index (ESI), Emergence (E), Radicle Length (RL), Above-ground Part Length (APL) and Dry Matter Mass (DM) of seedlings obtained from the cryopreservation evaluation on Liquid Nitrogen (NL_2) involving six *Passiflora* species.

Species (Seed moisture content)	Treatment	Variables						
		GSI	G (%)	ESI	E (%)	RL (cm.seedling$^{-1)}$	APL (cm.seedling^{-1})	DM (g.seedling^{-1})
P. mucronata (12%)	DMSO	0.174a	8a	0.212c	9b	5.4a	3.7c	0.014b
	Sucrose	0.698a	22a	0.938b	36a	3.6b	5.9a	0.066a
	wto/ cryoprotectants	1.250a	30a	0.389c	16b	4.2ab	5.3b	0.018b
	Control	1.088a	30a	1.765a	57a	3.4b	6.4a	0.052a
	CV%	25.32	38.25	9.88	1766	8.63	2.75	35.28
P. micropetala (6.2%)	DMSO	0.463b	26b	0.221a	17a	1.0a	2.3a	0.009a
	Sucrose	0.321b	16b	0.109a	8a	1.7a	3.7a	0.005a
	wto/ cryoprotectants	0.473b	28ab	0.173a	13a	1.9a	3.5a	0.005a
	Control	0.980a	56a	0.095a	7a	1.2a	2.2a	0.003a
	CV%	10.74	27.75	12.53	62.45	28.95	36.78	0.65
P. suberosa (9.5%)	DMSO	2.561a	48a	1.395a	34a	2.6c	4.5a	0.029a
	Sucrose	2.447a	47a	1.491a	34a	3.1ab	4.3ab	0.024a
	wto/ cryoprotectants	3.393a	62a	1.127a	28a	3.5a	4.2ab	0.019a
	Control	2.556a	52a	1.892a	39a	2.9bc	3.8b	0.020a
	CV%	10.85	11.85	11.76	17.11	3.37	3.26	0.77
P. edulis (12%)	DMSO	0.000c	0.0c	0.479bc	18b	4.1a	6.4a	0.094bc
	Sucrose	1.173b	49b	0.384c	13b	3.9a	6.4a	0.048c
	wto/ cryoprotectants	3.301a	78a	0.837b	29a	3.6a	5.5a	0.136ab
	Control	2.899a	70a	1.776a	41a	3.8a	5.7a	0.180a
	CV%	6.99	10.50	8.48	13.57	5.03	6.53	2.89

The means inside each column followed by the same letter do not differ from each other according to Tukey's test, at 5% of probability. Data transformed into the root of x + 0.5.

The germination of *P. micropetala* seeds was affected by the exposure to liquid nitrogen on the IVG and Germination Percentage variables, for which the "without cryoprotectants" (0.473 and 28% respectively), sucrose (0.321 and 16%) and DMSO (0.463 and 26%) treatments had similar means; however, they were lower than the control treatment (0.980 and 56%) respectively.

According to Mullen and Crister (2007), several of the factors that may contribute for the damages on the cell resulting from freezing are interdependent. Martínez-Montero et al. (2002) reported possible damages that may have been caused by the loss of cellular integrity due to the formation of ice crystals and the use of the cryoprotectants.

Aguiar et al. (2012) state that the deleterious effects to the biological materials caused by the cryopreservation process are related to the formation of intracellular ice crystals, to the water flow outside the cell (dehydration) and the increase of intracellular concentration of solutes.

The emergency and development of *P. micropetala* seedlings remained the same after the storage. Martins et al. (2009) observed that on pink trumpet tree seeds (*Tabebuia impetiginosa* (Mart. ex DC.) Standl.) with humidity degrees of 12.5, 8.4 and 4.2%, their physiological performance was maintained after immersed on liquid nitrogen.

The results of the cryopreservation for the *P. suberosa* species were satisfactory for most variables, except for the Above-ground Length (APL). For the APL variable, the highest means were obtained when the DMSO cryoprotectant was used in comparison to the control treatment (Table 1).

The variations obtained reflect the difficulty in determining the most adequate treatment to cryopreservation. According to Galdiano Jr. et al. (2012), one of the main factors for a successful vitrification is the adequate selection of cryoprotectants and the exposure time of the tissue to the vitrifying solutions, in order to reduce the potential toxicity on vegetable cells and increase the recovery after storage.

Some cryoprotecting agents, such as dimethyl sulfoxide (DMSO), have low toxicity and are highly permeable to cells, while several sugars show low permeability of the membrane. However, it is not yet clear how these cryoprotecting solutions act to reduce damages on tissues and cells during the freezing/thawing, nor whether there are common cryoprotection

mechanisms on both compound classes (Suzuki et al., 2005).

Although the awareness on the mode of action of cryoprotecting agents is still incomplete, it is likely that the cryoprotecting effects are multifactorial, and that cryoprotectants of different classes (for example, alcohols, sugars, diols, amides, large polymers) act through different mechanisms (Mullen and Crister, 2007).

For *P. edulis*, the highest means of the Germination Speed Index and Germination Percentage corresponded to the treatment "without cryoprotectants" (3.300 and 78%) and the control treatment (2.899 and 70%), with a slight increase on the germination of the treatment "without cryoprotectants" (8%), indicating a possible dormancy overcoming event of the seeds.

When evaluating the effect of cryopreservation on seeds of rare tropical orchids, Nikishina et al. (2007) concluded that, after the storage on liquid nitrogen, the germination capacity of the seeds increased for two species and decreased for other two species.

For *P. edulis*, the worst results were observed on the treatment with dimethyl sulfoxide (0%). According to Hubálek (2003), due to its high solubility in water, DMSO may cause changes to the cellular membrane, which damage the cells and make them not viable. Therefore, even at very low concentration, it may be toxic to some biological systems, mainly when kept under temperatures above 5 °C for a long period.

In the case of the other treatments, even when there are significant losses after the storage, considering that the useful life of *P. edulis* seeds has lasted for only one year (Meletti and Bruckner, 2001), cryopreservation becomes an advantageous method to protect the interesting genes of this commercial species for a long term, without the need for the periodical renewal of the seed bank in the field.

The dry matter mass of *P. edulis* was more expressive on the cryopreservation without the use of cryoprotectants, such as occurred for the Germination Speed Index and Germination Percentage (%) variables. According to Nakagawa (1994), vigorous seeds offer greater dry mass transfer from its reserve tissues to the embryonic axis, originating seedlings with greater weight, due to the greater matter accumulation.

Given the above-mentioned, it is possible to infer that the behavior of the seeds after storage on nitrogen varied across the evaluated species. As shown on other studies, it is not possible to predict the behavior of the seeds of a species after cryopreservation considering its size or due to taxonomic relationship (Touchell and Dixon, 1994).

According to Towill (2002), the critical point for the cryopreservation of orthodox seeds is to define the ideal water content before immersing them on liquid nitrogen. Yet, cryopreservation is an advantageous method, since it requires a limited space, protects the materials from contamination, requires very little maintenance and it is considered as cost-efficient (Rao, 2004).

Further research is suggested for the definition of the most adequate cryoprotectants/concentrations for the cryopreservation process of these and other *Passiflora* species, which may contribute for subsequent uses in vegetable genetic improvement programs and also as a conservation method for the species.

Conclusions

Cryopreservation may be used for the long-term conservation of *Passiflora mucronata*, *P. suberosa* and *P. edulis* seeds, without the use of cryoprotectants.

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