



Cryoprotectants and X-ray analysis on *Passiflora* seeds cryopreserved

Crioprotetores e análise de raio-x em sementes de *Passiflora* criopreservadas

A. Faria^{1*}; S. Paiva Sobrinho¹; A. S. Oliveira¹; A. R. Tavares²; P. B. Luz¹

¹Departamento de Agronomia/Laboratório de Sementes e Plantas Ornamentais/Universidade do Estado de Mato Grosso, 78200-000, Cáceres-MT, Brasil

²Departamento de Fruticultura/Instituto Agronômico (APTA), 01037-912, Campinas-SP, Brasil

*petterson@unemat.br

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The study aimed to evaluate the internal morphology of *Passiflora eichleriana* Mast., *Passiflora nitida* Kunth. and *Passiflora mucronata* Lam. seeds cryopreserved with different cryoprotectants. The treatment were control (without cryoprotectant or cryopreservation), seeds cryopreserved without cryoprotectant, 1.73, 2.28, 2.60 or 2.71 M glycerol, 0.37, 0.46, 0.54 or 0.61 M sucrose and 0.37, 0.72, 1.04 or 1.35 M dimethylsulfoxide. The seeds were cryopreserved in liquid nitrogen (-196 °C) for seven days. The cryopreserved seeds were dipped in water bath (37 °C) for 5 minutes to defrost. The percentage of filled, empty, malformed and damaged seeds was determined. The seeds were analyzed by X-ray to verify damages caused by the cryopreservation or defrost. Our results demonstrate that X-ray is an efficient method to analyses cryopreserved seeds and the cryopreservation technique did not cause mechanical damage of *P. eichleriana*, *P. nitida* and *P. mucronata* seeds.

Keywords: *Passiflora eichleriana* Mast., *Passiflora nitida* Kunth., *Passiflora mucronata* Lam.

O estudo teve como objetivo avaliar a morfologia interna de sementes de *Passiflora eichleriana* Mast., *Passiflora nitida* Kunth. e *Passiflora mucronata* Lam. criopreservadas com diferentes crioprotetores. Os tratamentos foram controle (sem crioprotetor e criopreservação), sementes criopreservadas sem crioprotetor, criopreservadas com glicerol a 1,73, 2,28, 2,60 e 2,71 M; sacarose a 0,37, 0,46, 0,54 e 0,61 M; dimetilsulfóxido a 0,37, 0,72, 1,04 e 1,35 M. As sementes foram criopreservadas em nitrogênio líquido (-196 °C) por sete dias. As sementes criopreservadas foram imersas em banho-maria (37 °C) por 5 minutos para descongelamento. Foi determinada a porcentagem de sementes cheias, vazias, malformadas e danificadas. As sementes foram analisadas por raio-x para verificação de danos causados pela criopreservação ou degelo. Nossos resultados demonstram que a radiografia é um método eficiente para análise de sementes criopreservadas e que a técnica de criopreservação não causou danos mecânicos nas sementes de *P. eichleriana*, *P. nitida* e *P. mucronata*.

Palavras-chave: *Passiflora eichleriana*, *Passiflora nitida*, *Passiflora mucronata*.

1. INTRODUCTION

Passiflora is an important tropical commercial crop mainly used in juice industry and also for alimentary, medicinal and ornamental purposes [1]. *Passiflora* is commonly propagated by seeds, requiring maintenance during its storage in germplasm banks to preserve seeds with quality and minimal physiological activity until it is sown [2]. The cryopreservation technique is considered an ideal method for seed preservation, allowing seed preservation for long periods at extremely low temperatures (-196 °C) [3]. The main advantages of storing plant material in liquid nitrogen are the seed conservation for a long period and reduced space for seed storage. Despite the advantages of cryopreservation technique, the process can origin physical damage to the embryo during seed freezing and defrosting, compromising its viability [4]. The vitrification of cells is also a limitation of the technique, in which the cellular components pass from the liquid state to the amorphous solid avoiding ice crystals formation inside the cells, causing rupture of the membranes and cell death [5]. In order to minimize seed cells injuries, it is necessary to use cryoprotectants before cryopreservation [6]. Cryoprotectants maintain the viability of biological materials [7], protecting membranes from the harmful effects caused by ice crystals formed inside and outside of cells [4]. Sucrose is considered an excellent anti-vitrification agent and is highly efficient to stabilize cell

membranes during dehydration and freezing processes and is not toxic to plant cells even when large amounts accumulate in the cytoplasm [4]. Sugars act as external osmotic agents by removing the excess intracellular water through an osmotic gradient or replacing the water removed from biomolecules; thus, maintaining the hydrophilic structures even after removal of the water. The soluble sugars inside the cell form hydrogen bonds and thus replace water, keeping the hydrophilic structures in their orientation when hydrated [8].

Radiographic analysis is a technique that permit to visualize damages in cryopreserved seeds, examining their internal structure, existence of cracks that occur during the cryopreservation and defrosting processes. The X-ray technique allows to correlate seed morphology with seed germination, or seedling morphology of several agricultural and foresting species [9, 10]. In addition, it is possible to identify moisture in soybean seeds [11], as well as seed mechanical injuries [10, 12]. The technique is applied on native species, evaluating seed quality in seed banks for germplasm ex situ conservation; allowing to select well-formed seeds [13]. The rigidity of *Passiflora* seeds tegument difficult the visual analysis of damages in the embryo, requiring methodologies that permit the analysis of the internal morphology of cryopreserved seeds. Thus, the present study aims to evaluate the quality of *Passiflora eichleriana* Mast., *Passiflora nitida* Kunth. and *Passiflora mucronata* Lam. seeds cryopreserved by X-ray and correlate the images with the damages caused by the cryopreservation technique.

2. MATERIALS AND METHODS

The experiment was carried out in the Laboratory of Seeds and Ornamental Plants of the University Campus of Cáceres-MT, Mato Grosso State University (UNEMAT) and Central Laboratory of Seeds of Lavras Federal University (UFLA).

Passiflora eichleriana Mast., *Passiflora nitida* Kunth. and *Passiflora mucronata* Lam. seeds were obtained from the Active Germplasm Bank (AGB) of Cáceres University Campus, Mato Grosso State University (UNEMAT). Mature fruits were cut to remove the pulp together with the seeds, rubbed on a sieve with hydrated lime, and washed on tap water until complete removal of seed mucilage. The seeds were dried for 24 hours at room temperature and stored in a cold chamber (9 °C). The water contents in the seeds were measured by the air forced oven method at 105 ± 3 °C for 24 hours (n = 50), according to the Guidelines for Seed Analysis [14].

The seeds were submitted to different cryoprotectants and dosages before cryopreservation as follow 1.73, 2.28, 2.60 and 2.71 M glycerol; 0.37; 0.46; 0.54 and 0.61 M sucrose and 0.37; 0.72; 1.04 and 1.35 M DMSO (Dimethyl sulfoxide). The seeds were immersed for three hours in the cryoprotectants solutions [15], and were subsequently removed and naturally dried for 24 hours. In addition to the cryoprotectant treatments, one treatment without cryoprotectant with cryopreservation and one treatment without both cryoprotectant and cryopreservation (Control) were used, totalizing 14 treatments. The seeds (except for Control) were packed in canisters and immersed in liquid nitrogen (-196 °C) for 168 hours (7 days) in a cold room (9 °C). The defrosting was carried out in a water-bath at 37 °C for five minutes [14].

The seeds of the 14 treatments were fixed on transparent slides for radiographic tests. The seeds were exposed to X-ray equipment (Faxitron MX-20 DC 12), with automatic adjustment of exposure time and radiation intensity. The X-ray analyzed seeds were classified as filled (FS), empty (ES), malformed (MFS) and damaged (SD). A completely randomized design with four replicates of 25 seeds was used for each treatment.

The seeds from the X-ray analysis were used on germination test, first the seed dormancy was overcome as follows *P. eichleriana* and *P. mucronata* - soaking in KNO₃ solution (1%) for 24 hours in seed germination chamber at 25 °C in the dark; *P. nitida* - immersed in 1,000 mg L⁻¹ GA₃ (gibberellic acid) solution for 24 hours in germination chamber at 25 °C in the dark. The seeds were sown on "Gerbox" type acrylic boxes with paper moistened with distilled water as substrate. Sown was performed with 100 seeds per treatment, divided into four replicates of 25 seeds, and cultured in a B.O.D. germination chamber with 20-30 °C alternated temperature and 12-hour photoperiod for 30 days. The seeds were considered germinated (%) with 2 mm length emitted radicles. The germination rate index (GSI) was calculated according to Maguire (1962) [16].

The germination (GP) and germination speed index (GSI) results of each treatment and species were submitted to Dunnett test for variance analysis, using ASSISTAT statistical program, version 7.7 beta [17]. The germination rate (GR) and germination percentage (GP) data were transformed with the arc sin $\sqrt{(X\% / 100)}$ for data percentage. Arc sin transformation was used due to data binomial distribution and when the variables come from proportions such as: percentage of seed germination [18].

3. RESULTS AND DISCUSSION

The initial seed water contents were 7.2, 7.4 and 11.5% for *P. eichleriana* Mast., *P. nitida* Kunth. and *P. mucronata* Lam., respectively. The seeds used were suitable for cryopreservation due the low moisture contents, facilitating the visualization of seed structures during the radiographic analysis. Seed moisture can effect optical density, once seeds with lower humidity favors the differentiation of seed internal structures [19].

The X-ray do not show deleterious effects such as cracks on tegument or embryo caused by cryopreservation technique on *Passiflora eichleriana* Mast., *Passiflora nitida* Kunth. and *Passiflora mucronata* Lam. seeds (Table 1). The large percentage of malformed seeds of *Passiflora mucronata* can be attributed to the climatic conditions of cultivation. Because it is a typical species of sandbank and found mainly in the states of Bahia and Rio de Janeiro, cultivation in our climatic conditions may have caused such anomalies in the seed. The X-ray images showed filled, empty and malformed seeds for all *Passiflora* species (Figures 1, 2 and 3). The internal morphology of cryopreserved *P. eichleriana* seeds, displayed 82% full seeds, 9% empty seeds and 9% malformed seeds (Figure 1).

Table 1. Percentages of *Passiflora* spp. seeds cryopreserved and submitted to X-ray test and classified into full seed (FS), empty seed (ES), malformed seed (MFS) and damaged seed (SD).

Species	FS (%)	ES (%)	MFS (%)	SD (%)
<i>P. eichleriana</i>	82	9	9	0.0
<i>P. nitida</i>	68	4	29	0.0
<i>P. mucronata</i>	15	0.0	85	0.0

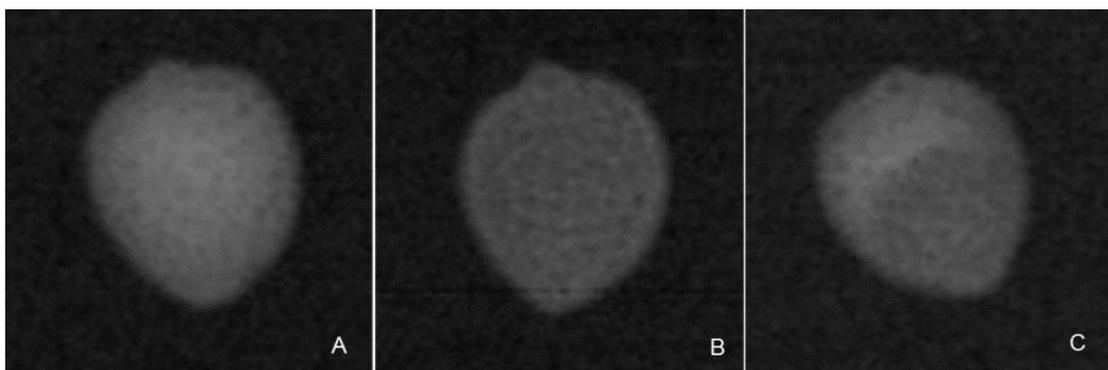


Figure 1. X-ray image of *Passiflora eichleriana* seeds classified into full seed - FC (A) and empty seed - ES (B) and malformed seed - MFS (C).

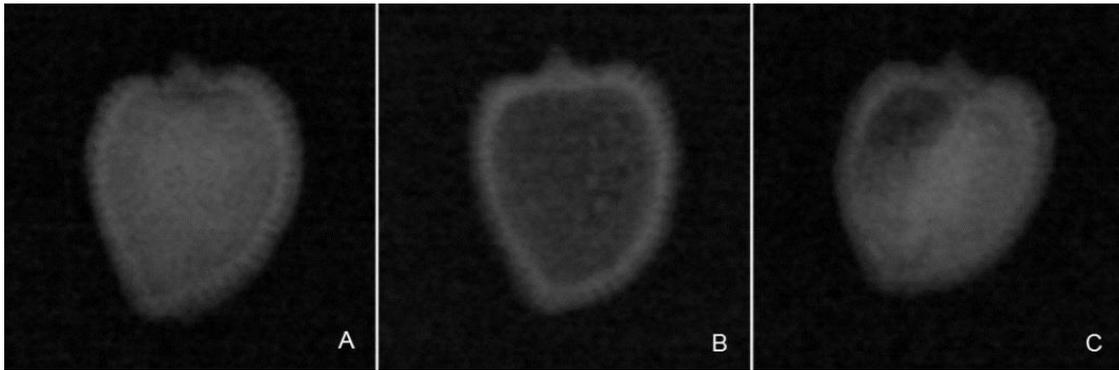


Figure 2. X-ray image of *Passiflora nitida* seeds classified into full seed - FS (A), empty seed - ES (B) and malformed seed - MFS (C).

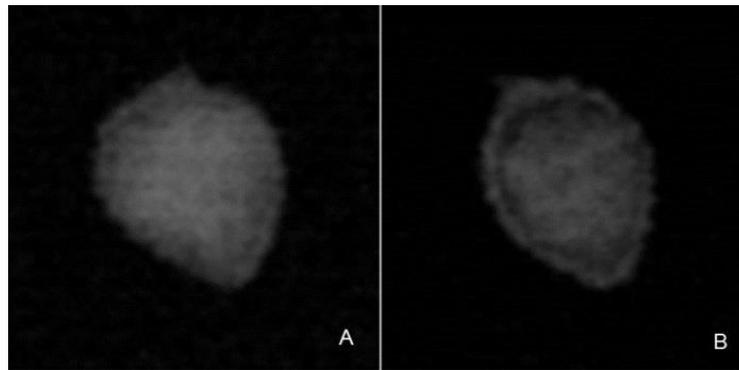


Figure 3. X-ray image of *Passiflora mucronata* seeds classified into full seed - FS (A) and malformed seed - MFS (B).

Passiflora eichleriana seed germination was reduced with 2.60 and 2.71 M glycerol and 1.04 M DMSO treatments. The germination rate was reduced on seeds cryoprotected with glycerol and, 0.72 and 1.35 M DMSO. This difference can be attributed to a toxicity effect of cryoprotectant glycerol and DMSO. The average percentage of germination (44%) compared to the full seeds percentage on X-ray analysis (82%) shows a divergence between the variables (Table 1), once full seeds theoretically characterize viable seeds. The highest germination percentage was 68% on treatment 0.46 M sucrose; also, lower than the percentage of filled seeds (82%). The lower percentage of germination of *P. eichleriana* seeds compared to the percentage of filled seeds is possibly due to the loss of viability and/or induction of dormancy triggered by the low temperature storage. *P. setacea* seeds had a lower germination rate after six months of storage and it was attributed to a dormancy induction by low temperature [20]. Marostega et al. (2017) [21] studying *P. eichleriana* seed dormancy observed that 66% of the seeds germinated indicating that the overcoming of seed dormancy method was not totally effective on this species. The difference between the percentage of filled seeds and germinated seeds is due to invisible infections, physiological damage, or seed dormancy [22].

The X-ray analyses did not show damage (cracks) in the embryo of *P. nitida* cryopreserved seeds (Figure 2), with 68% filled seeds, 4% empty seeds and 28% malformed seeds (Table 1). *P. nitida* seed germination was higher on seeds cryopreserved with sucrose (0.37, 0.46, 0.54 and 0.61 M), 0.37 M DMI and non-cryoprotected seeds and Control (Table 2). The seeds treated with glycerol don't germinate demonstrating the toxicity of this cryoprotectant on all concentrations; while DMSO was detrimental on higher concentrations. Wild *Passiflora* species such as *P. nitida* have a long period of dormancy; thus, low seed germination occurs probably due to a non-efficient treatment to overcome the dormancy [23].

Table 2. Germination percentage (GP, %) and germination speed index (GSI) of *Passiflora* spp. cryoprotected and cryopreserved.

Treatments	<i>Passiflora eichleriana</i>		<i>Passiflora nitida</i>		<i>Passiflora mucronata</i>	
	GP ⁽¹⁾	GSI ⁽²⁾	GP ⁽¹⁾	GSI ⁽²⁾	GP ⁽¹⁾	GSI ⁽²⁾
Control	58.0a*	2.689a*	29.0a*	0.331 a*	0.0 ^{ns}	0.000 ^{ns}
No cryopreservant	51.0a	1.876a	22.0a	0.394a	1.0	0.019
1.73 M glycerol	42.0a	1.319b	0.0c	0.000b	0.0	0.000
2.28 M glycerol	48.0a	1.466b	0.0c	0.000b	0.0	0.000
2.60 M glycerol	9.0b	0.266b	1.0c	0.000b	0.50	0.011
2.71 M glycerol	18.0b	0.617b	0.0c	0.000b	0.5	0.010
0.37 M sucrose	40.0a	1.922a	23.0a	0.276a	0.0	0.000
0.46 M sucrose	68.0a	2.668a	22.0a	0.196a	0.5	0.008
0.54 M sucrose	59.0a	2.012a	21.0a	0.329a	1.0	0.019
0.61 M sucrose	66.0a	3.025a	17.0a	0.216a	0.0	0.000
0.37 M DMSO	44.0a	2.078a	9.0b	0.252a	0.5	0.086
0.72 M DMSO	26.0b	1.055b	1.0c	0.028a	0.0	0.000
1.04 M DMSO	47.0a	2.289a	1.0c	0.000b	0.0	0.000
1.35 M DMSO	38.4a	1.370b	0.0b	0.000b	0.0	0.000
CV (%)	15.8	10.40	41.38	6.20	41.18	1.55
Média	43.88	1.760	9.69	0.144	0.28	0.011

*Means within same column followed by same letter in the column do not differ from each other by Dunnett's test at 5% probability; ^{ns} - not significant. Data transformed into $\sqrt{Y + 0,5}$ (1) and $\text{arc sen } \sqrt{(X| - |)/100}$ (2).

The internal morphology of *P. mucronata* seeds showed filled and malformed seeds (Figure 3). A large percentage of malformed seeds (85%) was observed (Table 1); no empty seeds and no damaged seeds were observed (with cracks). It is observed from these results that the seeds of *P. mucronata*, do not suffer damage during the cryopreservation process, freezing or thawing, as they did not present seeds damaged after the cryoprotection and cryopreservation processes (Table 2). The high percentage of *P. mucronata* malformed seeds is possibly due to an inadequacy on seed formation process as the pollination, fertilization, seed maturation, or even local climatic conditions unsuitable for the species cultivation. X-ray images of *Tecoma stans* (ipê-de-jardim) and *Tabebuia heptaphylla* (Vell.) (ipê-purple) seeds showed a diminutive embryo development and embryonic anomalies are probably originated during fruit maturation [24], as well as observed for *P. mucronata*. The high percentage of malformed seeds it is an indicative of low percentage of germination, which was efficiently observed on *P. mucronata* seeds, confirming that internal morphology of seeds can be an indicator of seed viability. However, there was no correspondence between the percentage of filled seeds and the percentage of seed germination with respectively 15% and 1%.

The correlation between GP and GSI of *P. eichleriana* seeds was positive and significant at 1% probability (Table 3). The GP correlates with filled seeds (FS) and weak positive with *P. eichleriana* empty seed (ES). Malformed seeds (MFS) and germination percentage correlated weakly ($p \leq 0.05$), associating low germination with malformed seeds. The correlation between filled (FS), empty (ES) and malformed seeds (MFS) is characterized as significant negative ($p \leq 0.01$); thus, as the percentage of filled seeds increases there is a reduction on malformed and empty seeds. A positive correlation was observed between percentage and germination rate and negative between filled and malformed seeds on *P. nitida*. A perfect negative correlation was also observed between the malformed seed (SMF) and full seed (SC) of *P. mucronata*.

Table 3. Pearson correlation matrix between germination percentage (GP), germination speed index (GSI), full seed (FS), empty seed (ES), malformed seed (MFS) of *Passiflora eichleriana*, *Passiflora nitida* and *Passiflora mucronata* submitted to X-ray analysis and germination test.

<i>Passiflora eichleriana</i>					
	GP	GSI	FS	ES	MFS
GP	1				
GSI	0.9249**	1			
FS	0.2127 ^{ns}	0.2079 ^{ns}	1		
ES	0.0708 ^{ns}	0.0915 ^{ns}	0.6704**	1	
MFS	-0.2848*	-0.2583 ^{ns}	0.7389**	0.1049 ^{ns}	1
<i>Passiflora nitida</i>					
	GP	GSI	FS	ES	MFS
GP	1				
GSI	0.9341**	1			
FS	-0.2119 ^{ns}	-0.1764 ^{ns}	1		
ES	0.3392 ^{ns}	0.4246 ^{ns}	-0.4047*	1	
MFS	0.2912 ^{ns}	0.2392 ^{ns}	0.9203**	0.0528 ^{ns}	1
<i>Passiflora mucronata</i>					
	GP	GSI	FS	MFS	
GP	1				
GSI	0.9494**	1			
FS	-0.6466 ^{ns}	-0.555 ^{ns}	1		
MFS	0.2448 ^{ns}	0.3093 ^{ns}	-1**	1	

** * Significant at 1 and 5% probability by t-test. ^{ns} - not significant

4. CONCLUSIONS

Our results demonstrate that X-ray is an efficient method to analyses cryopreserved seeds and the cryopreservation technique did not affect the quality of *P. eichleriana*, *P. nitida* and *P. mucronata* seeds.

The *Passiflora eichleriana*, *P. nitida* and *P. mucronata* seeds can be cryopreserved without the use of cryoprotective substances.

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