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Cryopreservation of *Pyrostegia venusta* (Ker Gawl.) Miers seeds

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Abstract – (Cryopreservation of *Pyrostegia venusta* (Ker Gawl.) Miers seeds). Seeds of *Pyrostegia venusta* (Ker Gawl.) Miers) were desiccated at 25 °C on silica gel for 0 h (T_0), 7 h (T_1) and 24 h (T_2), reaching moisture contents of 6.5%, 4.4% and 3.8%, respectively. Seeds were frozen rapidly in liquid nitrogen (+LN) at -263 °C.min.$^{-1}$ and after 72 h, they were thawed slowly at room temperature (25 ± 2 °C) at a speed of 5 °C.min.$^{-1}$. Seed germinability evaluation before (-LN) and after freezing (+LN) was conducted at 25 °C, on paper roll substrate, and germinated at 16 hrs light/8 hrs dark, normal seedling counts for 38 days. The germinative percentages were 88% (T_0-LN), 98% (T_0+LN), 61% (T_1-LN), 95% (T_1+LN), 78% (T_2-LN) and 89% (T_2+LN). Mean days for seedling formation were 23 (T_0-LN), 22 (T_1-LN and T_2-LN) and 30 days for seeds exposed to LN. Fast freezing, slow thawing, and the three tested moisture contents, were suitable for cryopreservation of *Pyrostegia venusta* seeds.
Keywords: desiccation, germination, orange trumpet vine

Resumo – (Criopreservação de sementes de *Pyrostegia venusta* (Ker Gawl.) Miers). Sementes de *Pyrostegia venusta* (Ker Gawl.) Miers) foram dessecadas a 25 °C, sobre sílica gel por 0 h (T_0), 7 h (T_1) e 24 h (T_2), atingindo teores de água de 6,5%, 4,4% e 3,8%, respectivamente. As sementes foram congeladas em nitrogênio líquido (+NL), a -263 ºC.min.$^{-1}$ e após 72 h foi descongeladas à temperatura ambiente de 25 ± 2 ºC, a 5 °C.min.$^{-1}$. A avaliação da germinabilidade das sementes antes (-NL) e após congelamento (+NL) foi conduzida a 25 °C, substrato rolo de papel, fotoperíodo 16 h luz/8 h escuro e contagens de plântulas normais por 38 dias. Os percentuais germinativos foram de 88% (T_0-NL), 98% (T_0+NL), 61% (T_1-NL), 95% (T_1+NL), 78% (T_2- NL) e de 89% (T_2+NL). Os tempos médios para a formação de plântulas foram de 23 (T_0-NL), 22 (T_1-NL e T_2-NL) e de 30 dias para as sementes expostas ao NL. O congelamento rápido, o descongelamento lento e os três teores de água testados foram adequados para a criopreservação de sementes de *Pyrostegia venusta*.
Palavras-chave: cipó de São João, dessecação, germinação

Introduction

Lianescent, herbaceous, shrub and arboreal species of the Bignoniaceae family are widely distributed throughout South America (Ortolani 2007; Camarinha *et al.* 2015). Among the lianescent species of this botanical family, *Pyrostegia venusta* (Ker Gawl.) Miers, orange trumpet vine, is a Brazilian native plant found in distinct vegetation formations in all regions of Brazil (Hortenci *et al.* 2018). Although *P. venusta* is generally cited as an invasive plant and weed in pastures, the species is a popular ornamental, cultivated for landscaping due to its lush inflorescences that hold striking orange tubular flowers (Oliveira & Wendling 2013, Online Compendium Gerson Luiz Lopes 2018, Plantamed 2018). In Brazilian folk medicine, aerial parts and flowers of the plant are used in infusions or decoctions and administered orally as a general tonic, as well as for the treatment of diarrhea, vitiligo, cough and common infectious diseases of the respiratory system such as bronchitis, flu and cold (Ferreira *et al.* 2000, Mostafa *et al.* 2013). Pharmacological studies, analyzing hidroethylic extracts of flowers, aerial parts and roots successfully isolated biologically active compounds oleanolic acid, acacetin 7-O-β-glucopyranoside and β-sitosterol, with anti-inflammatory, antitumor,

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antioxidant, antimicrobial and anti-HIV, antiallergic and immunomodulatory properties (Veloso *et al.* 2010, Mostafa *et al.* 2013, Figueiredo *et al.* 2014, Moreira *et al.* 2015). *P. venusta* is an alternative source for *in vitro* production of these bioactive phenolic compounds (Braga *et al.* 2015).

Due to its multiple uses, conservation of *P. venusta* germplasm is considered a priority in order to ensure availability of the species' genetic variability to meet demands of current and future research programs in pharmacology, bio products production and genetic improvement. Conservation of seeds in gene banks in cold rooms is the conventional approach for plant germplasm conservation. However, one of the physiological characteristics of seeds of species of the Bignoniaceae family is that they are short-lived under storage (Aguiar 2010, Tresena *et al.* 2010, Martins *et al.* 2014). This is generally attributed to the instability of lipid compounds present in the bilobed cotyledons of the seeds (Gabrielli 1988, Renó *et al.* 2007). In addition, during storage, shorter seed longevity can be associated with oxidative stress of macromolecules such as nucleic acid, proteins and lipids (Sano *et al.* 2016). In the particular case of *P. venusta*, studies showed that seeds stored at room temperature, at 10 ° C and 4 ° C maintained viability for short periods of time (Gabrielli 1988, Rossatto & Kolb 2010). However, there are no studies on these seed's viability after storage at subzero temperatures such as -20 °C, in a conventional gene bank, or at -196 °C, in a cryobank. Considering that seeds with lipid reserves, such as those of *P. venusta*, present viability loss when stored in cold rooms at -20 °C, the most promising approach to ensure long-term conservation of this species germplasm is cryopreservation in liquid nitrogen, (Michalak *et al.* 2013). Cryopreservation includes a range of techniques for freezing and storing biological material in liquid nitrogen, at -196 °C (Panis 2019). These techniques are currently the most effective approach for long-term conservation of plant germplasm, because cryopreservation requires minimal storage space, labor and maintenance, all the while preserving the integrity and preventing aging of stored samples for unlimited periods (Panis 2019). Cryopreservation techniques are now used in an increasing number of gene banks and research facilities around the world (Kalaiselvi *et al.* 2017, Panis 2019).

Plant cells contain high amounts of water and are not inherently freeze-tolerant, so they are extremely susceptible to freezing injury. This is a critical point in the development of a cryopreservation protocol, because plant structures with high levels of cellular water will be injured during cryopreservation due to crystallization of the water present in the cell into ice upon exposure to freezing temperatures (Mazur 1984). Therefore, plant cells dehydration prior to exposure to liquid nitrogen is essential to protect them from damage caused by intracellular ice formation.

The objective of this work was to establish a procedure for cryopreservation of *Pyrostegia venusta* seeds in liquid nitrogen, at -196 ° C.

Materials and methods

Mature semi-open *Pyrostegia venusta* septicidal capsules were harvested in September of 2019 (three months after flowering) from 15 plants growing at Embrapa Genetic Resources and Biotechnology experimental field, located in Brasília, Federal District-DF (05°43'53"S and 47°53'53"W). The experiments were conducted at the Seed Laboratory and the Plant Cryobiology Laboratory of this institution. The seeds, extracted manually from the capsules, were cleaned, selected, homogenized and divided into three samples of 240 seeds. The samples were desiccated with blue indicator silica gel (4g silica/1g seed) for 0 h (T_0), 7 h (T_1) and 24 h (T_2), at a constant temperature of 25 °C, in a Percival germinator. Seed moisture contents (mc) after each desiccation period were determined by the gravity-convection method (Brazil, 2009) Four repetitions of ten seeds were used for the determination of the mc, and the results were expressed as average percentages of seed fresh weight.

Following desiccation, each sample (200 seeds) were divided into two subsamples (100 seeds each), one for freezing in LN and another to be used as control. Seeds were packed in aluminized bag, sealed with parafilm and identified with the respective desiccation period. For freezing in LN, the packets were immersed directly into liquid nitrogen (LN), at -196 °C, at a cooling rate -263 °C.min.$^{-1}$ (Salomão 2002). After 72 hours, the material was removed from the LN and kept on the laboratory bench to thaw slowly at room temperature (25 ± 2 °C), at a cooling rate of approximately 5 °C.min^{-1} (Salomão 2002). Control samples (-LN) were maintained in the laboratory bench, at room temperature (25 ± 2 °C), for 72 hrs. After 72 hrs, desiccated (- LN) and desiccated and frozen (+ LN) seeds were immersed in a commercial neutral detergent solution at 2% (v/v) concentration for 5 min, followed by rinsing under running tap water until complete removal of the product. After surface disinfection, germination tests were conducted with four replications of 25 seeds per treatment, on paper roll substrate. This standard germination substrate was prepared by distributing seeds evenly onto two sheets of Germitest® paper moistened with distilled water (volume equivalent to 2.5 times the mass of the dry paper), covering seeds with another sheet of moist paper and then making a roll of the paper sheets containing the seeds. Paper rolls were placed into plastic bags, incubated in a germination chamber set at constant temperature of 25 °C, photoperiod 16 hrs light provided by eight 40 w fluorescent bulbs and 8 hrs dark, with daily counts of germinated seeds for 38 days. Final results were expressed as percentage of germinated seeds that formed normal seedlings, that is, those presenting the first pair of expanded eophils and the main root.

Mean days for seedling formation were calculated according to Santana & Ranal 2004:

$$M_{days} = N_1G_1 + N_2G_2 + ... + N_nG_n / G_1 + G_2 + ... + G_n$$

In which:

$N_1, N_2, ..., N_n$ = number of days counted from sowing to observation day.

$G_1, G_{2...}, G_n$ = number of seeds germinated on observation day.

The experimental design was completely randomized with factorial arrangement of three mc of non-frozen (- LN) and frozen (+ LN) seeds. The germination and M_{days} data were subjected to analysis of variance (ANOVA), at $P < 0.05$, to test for the effects of mc levels and of freezing in liquid nitrogen on the germinability of the seeds. The means of germination percentages were compared by Bonferroni's Multiple Comparison Test, at $P < 0.05$. All data analysis were performed using the statistical program GraphPad Prism (@ 2017 Graph Pad Software Inc.).

Results and Discussion

Pyrostegia venusta seeds had an initial mc of 6.5%, which corresponded to the desiccation time T_0. This initial value of mc can be attributed to water loss during maturation drying, a natural phase of seed development, but also to the fact that the capsules were already semi dehiscent by the time they were harvested, which allowed the loss of seed moisture under field conditions. After desiccation on silica gel for seven hrs (T_1) seeds reached 4.4% mc, and at the end of 24 hrs of desiccation, seeds reached mc of 3.8% (T_2). The germination percentages of unfrozen (-LN) seeds were 88% (T_0), 61% (T_1) and 78% (T_2) (Figure 1). Seeds of T_1 had lower germination percentage value (61%) and it differed statistically ($P < 0.05$) from the other germination percentages, 88% (T_0) and 78% (T_2). However, it was observed that T_1 had the largest number of empty seeds, which probably contributed to this reduction in germination percentage. Reduction of mc from 6.5% (T_0) to 4.4% (T_1) and 3.8% (T_2) apparently did not have a deleterious effect on seed viability, as evidenced by a low percentage of abnormal seedling formation. Shankaralingappa *et al.* (2019) reported that seeds of papaya cultivars Akra Prabhath and Akra Surya desiccated to 4.1% and 4.2%, respectively, maintained initial viability and vigor, while seeds of the cultivar Sunrise solo showed reduced germination after drying to 3.6% mc, indicating that reduction in mc below 4% was harmful for seeds of this species. Zhang *et al.* (2019) concluded that desiccation to extreme low moisture contents stabilized longevity of *Calocedrus macrolepis* seeds stored at room temperature for 5 years, since germination percentages and vigor of seeds desiccated to 2.1% and 3.0% mc were higher than the values obtained for non-dehydrated control seeds (mc = 7.5%). However, for some species, the effect of extreme reduction in seed mc below a critical moisture level is unpredictable and might result in mechanical structure changes and deleterious effects on germination and viability (Ballesteros & Walters, 2011; Colville & Pritchard, 2019). The mc of the seeds had no significant effect ($P < 0.05$) on germination values after freezing in LN. However, freezing in LN at -196 °C had a significant enhancing effect ($P > 0.05$) on the germination performance of *P. venusta* seeds (Figure 1, Table 1). After exposure to LN, germination percentages were, 98% (T_0), 95% (T_1), 89% (T_2), values higher than those obtained with desiccated unfrozen seeds (- LN). Seeds of some species develop cracks in the seed coat during immersion in liquid nitrogen, a phenomenon that promote seed germination as they serve as openings for water entry, triguering germination processes (Salomão 2002). Liquid nitrogen exposure has been used in many cases as a dormancy breaking treatment for species with impermeable seed coats (Wu *et al.* 2017).

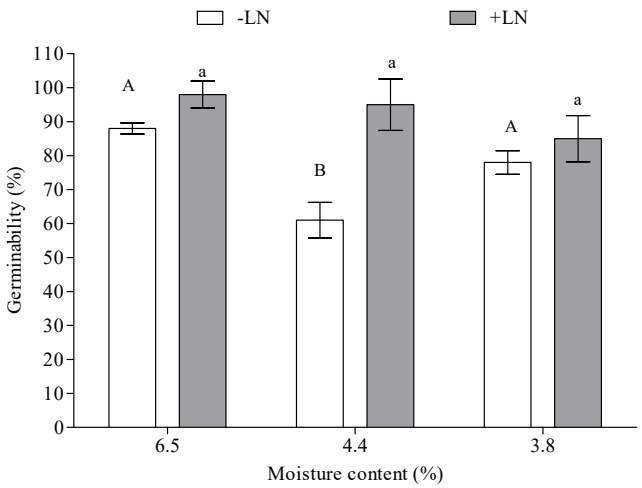

Figure 1. Germination percentages of *Pyrostegia venusta* (Ker Gawl.) Miers seeds with different moisture contents before (-LN) and after (+LN) liquid nitrogen exposure. The same capital letter (-LN) and the same small letter (+LN) are not significantly different for ANOVA at $P < 0.05$ by Bonferroni's Multiple Comparison Test. Vertical error bars represent SE of means of four replications ($P < 0.05$).

Pyrostegia venusta seeds can withstand both desiccation and LN exposure. However, one of the intrinsic characteristics of seeds that can have a detrimental effect on survival after LN freezing is mc. Because of this, the first step in establishing a cryopreservation protocols is to adjust seed mc to values that prevent freezing damage. Such damage may become cumulative in subsequent steps of the protocol, *i.e.* thawing and imbibition during seed germination (Pereira *et al.* 2014, Salomão *et al.* 2015). One of the approaches to promote cellular dehydration is exposure of plant structures, such as seeds, to silica gel, which will cause controlled loss of water by evaporation (Santos & Salomão 2017). As shown in Table 1, the variable mc did not interfere in the germination process of the seeds of *P. venusta*. Unfrozen (- LN) and frozen (+ LN) seeds dehydrated on silica gel reaching different mc, 6.5% (T_0), 4.4% (T_1) and 3.8% (T_2) maintained high germinability.

Despite of the lower germination percentage (61%) for seeds with 4.4% mc compared to the other germination values attained by seeds with lower or higher mc, this germination rate is similar to those observed in a study with *P. venusta* seeds treated with germination promoters KNO_3 (2%) or GA_3 (150 $mg.L^{-1}$), when germination percentages were 62% and 63.3%, respectively (Scalon *et al.* 2008, Santos 2016).

Post-seminal phases of seedling development were followed for up to 38 days. Seeds at all three mc germinated promptly and seedling morphology showed normal development with formation of aerial parts and root systems (Figure 2). For control seeds, M_{days} for normal seedling formation, showing the first pair of expanded eophiles and the main root, were 23 days (T_0-LN) and 22 days (T_1-LN and T_2-LN). For frozen seeds (+ LN), the M_{days} were 30 days for the three mc tested (Table 2).

Freezing and thawing rates, as well as seed mc, are decisive factors for the maintenance of functional and structural integrity of cryopreserved seeds. If inadequate, these factors result in biochemical and biophysical damage that compromise seed survival. The difference between M_{days} values for unfrozen *P. venusta* seeds (- LN) and frozen seeds (+ LN) was presumably due to cell destabilization due to freezing or thawing process. In this case, longer soaking time was required to activate the cellular repair mechanisms, as well as, for the mobility of nutrients responsible for embryonic axis growth. Results obtained suggest that for cryopreserved seeds longer soaking time contributed not only to the repair of damage, but also to improve the performance of subsequent phases of the germination process, since germination percentages were 98% (T_0+LN), 95% (T_1+LN), 89% (T_2+LN), without abnormal seedling development (Figure 2). Another factor that probably contributed to these results may have been that LN pressure exerted on the embryo or seed structure (integument, endocarp and others) can induce disruption or a network of cracks, make them less rigid and improve imbibition. Such effects of LN are beneficial as they allow greater water absorption by seeds, improving germination performance (Jordan *et al.* 1982, Jitsopakula *et al.* 2012). However, in seeds of some species there may be greater leakage of solutes through these cracks or by the malleability of tissues. This results in, among other events, slower seedling development, as longer time is required for structural repair, especially of the membranes of the tonoplast and plasmalema (Khanna *et al.* 2014). A similar pattern was observed in a study with *Tabebuia aurea* (Silva Manso) Benth and Hook F. *ex* S. Moore in which seeds frozen in LN required longer soaking time and absorption of bigger volume of water for seedling formation to occur than unfrozen seeds or seeds frozen at -20 °C (Salomão & Fujichima 2002).

Pyrostegia venusta seeds can be stored for short periods, with reported storage longevity of five months at room temperature, six months at 10 ° C, one year when packed in silica gel and more than two years at 4 °C (Gabrielli 1988, Rossatto & Kolb 2010, Santos 2016). The loss of germinability, viability and vigor in lipid seeds after storage at low or subzero temperatures may possibly be due to hydrolytic and oxidative rancidity of fatty acids

Table 1. Effect of moisture content (mc) and liquid Nitrogen (LN) exposure on seed germination of *Pyrostegia venusta* (Ker Gawl.) Miers. ANOVA obtained from variance analysis, $P < 0.05$ level of significance.

Variables	Sum-of-squares	Degree of freedom	F	P value	% of total variation
Liquid nitrogen (LN)	88.17	1	529.0	0.0019	99.25
Moisture content (mc)	0.3333	2	1.000	0.5000	0.38
Residual	0.3333	2			

Table 2. Mean days for seedling formation from seeds of *Pyrostegia venusta* (Ker Gawl.) Miers with different moisture contents before (-LN) and after (+LN) liquid nitrogen exposure.

Desiccation Duration (hrs)	Moisture content (%)	Mean days for seedling formation (M_{days})	
		-LN	+LN
0 (T_0)	6.5	23 B	30 A
7 (T_1)	4.4	22 B	30 A
24 (T_2)	3.8	22 B	30 A

The same capital letter in the column is not significantly different at $P < 0.05$ by variance analysis (ANOVA).

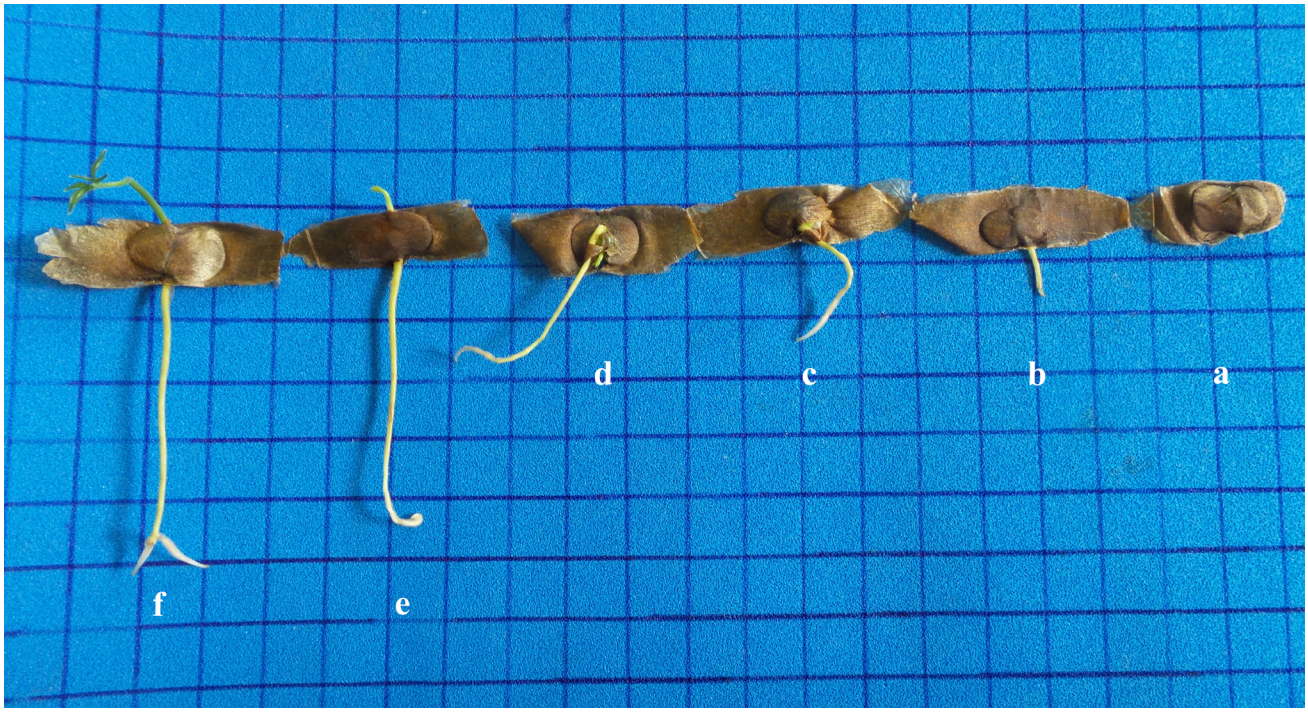

Figure 2. *Pyrostegia venusta* (Ker Gawl.). Miers post-seminal phases of seedling development. a. radicle protrusion. b-d. radicle development. e. epicotyl emergence. f. first pair of expanded eophils and the main root (scale: square side on the background corresponds to 1 cm).

(Chmielarz 2009, Balešević-Tubic *et al.* 2010). Currently, the recommendations to maintain viability of seeds with short life span and low storability are the conservation using cryogenic methods, at -196 °C (Normah *et al.* 2019, Hay & Probert 2013). The successful cryopreservation of seeds of several tropical species has been attributed to the adjustment of mc according to the specificity of each species, the use of methods that allow rapid freezing by direct immersion in LN and slow thawing at room temperature (Santos *et al.* 2013, Salomão & Santos 2018). During rapid freezing, there is uniformity of subcellular water cooling and the formation of small intra and extracellular ice crystals, preventing damage by cell dehydration and rupture of cell membranes (González-Arnao *et al.* 2014). Fast thawing may cause ice crystal formation, biophysical and biochemical changes in lipids and proteins that can result in critical or lethal damage to seeds while slow thawing maintains seed morphophysiological integrity (González-Arnao *et al.* 2014). Some examples of tropical species that achieved high viability or germination percentages after cryopreservation in LN are *Epidendrum quitensium* Rchb.f., *E. andressonii* Hágsater and Dodson, *Passiflora edulis* Sims, *P. mucronata* Lam., *P. suberous* L., *Encholirium magalhaesii* L. B. Sm. *E. spectabile* Martius *ex* Schultes f., *E. subsecundum* (Baker) Mez, *Magonia pubescens* A. St. Hil., *Ormosia flava* (Ducke) Rudd. *Peltogyne confertiflora* (Mart. *ex* Hayne) Benth. and *Sparattanthelium tupiniquinorum* Mart. (Cerna *et al.* 2018, Araújo *et al.* 2016, Ferrari *et al.* 2016, Salomão *et al.* 2015, Tarré *et al.* 2007).

The results obtained with *P. venusta* seeds after exposure to LN are compatible with those observed in seeds of other species of the Bignoniaceae family. Liquid nitrogen had a stimulatory effect on germinability for seeds of six species with mc ranging from 4.2% to 8.5% and germination percentages before and after exposure to LN were 68% - 84% (*Anemopaegma arvense* (Vell.) Stellfeld *ex* de Souza) 88% - 93% (*Handroanthus serratifolius* (Vahl) S. Grose), 78 - 89% (*Jacaranda* cuspidifolium Mart.), 78% - 89% (*Tabebuia aurea* (Silva Manso) Benth and Hook. F. *ex* S. Moore), 20% - 62% (*Cybistax antisyphilitica* (Mart.) Mart.) [Salomão 2002, Salomão *et al.* 2018] and 57% - 76% (*Handroanthus impetiginosus* (Mart. ex DC.) Mattos) [Martins *et al.* 2009]. For seeds of *Handroanthus chrysotrichus* (Mart. *ex* DC.) Mattos (Tresena *et al.* 2010), *Jacaranda decurrens* Cham. (Salomão 2002) and *Tabebuia pentaphylla* Helmsl (Aguiar *et al.* 2010), with mc of 4.0%, 5.2% and 7.1%, respectively, there was no significant loss of germinability after freezing. The germinative percentages before and after freezing in LN were 87% - 82% (*H. chrysotrichus*), 89% - 89% (*J. decurrens*) and 89% - 88% (*T. pentaphylla*).

Rapid freezing (-263 °C.min^{-1}) followed by slow thawing (5 °C.min^{-1}) increased germinative percentages of *P. venusta* seeds. Germination rates for seeds with 6.5% mc (T_0) was 88% before (- LN) and 98% after (+ LN) cryopreservation.

Seeds with 4.4 mc (T_1), presented 61% (- LN) and 95% (+ LN). For seeds dehydrated until they reached a mc of 3.8% (T_2), the germinative percentages were 78% (- LN) and 89% (+ LN) (Figure 1).

Conclusions

The behavior of *Pyrostegia venusta* seeds after exposure to LN corroborates the adoption of cryopreservation for long-term conservation of this plant. For successful cryopreservation with high recovery rates it is recommended to desiccate seeds to 4.4% and 6.5% water content, submerge dry seeds rapidly in LN (estimated cooling rate of -263 °C.min^{-1}) and to thaw them slowly at room temperature (25 ± 2 °C), at a cooling rate of 5 °C min^{-1}.

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