Revista Mexicana de Ciencias Forestales Vol. 13 (69)
Enero – Febrero (2022)

DOI: https://doi.org/10.29298/rmcf.v13i69.1198

Article

Crioconservación de semillas de *Cedrela odorata* L.: germinación y establecimiento temprano en vivero

Seed cryopreservation of *Cedrela odorata* L.: germination and early nursery establishment

Mario Valerio Velasco-García[1], Dalia Grisel Hernández-Arroyo[1], Liliana Muñoz-Gutiérrez[1], Carlos Román Castillo-Martínez[1], Miguel Ángel Vallejo-Reyna[1], Florencia García-Campusano[1]*.

Resumen
La crioconservación de semillas representa una alternativa para la conservación a largo plazo de germoplasma forestal, sobre todo de especies tropicales intermedias o recalcitrantes, cuya viabilidad decae rápidamente bajo condiciones estándares de almacenamiento. Por ello, se evaluó en semillas de cedro rojo el impacto del congelamiento rápido en nitrógeno líquido (N_2L) sobre la germinación *sensu stricto* y desarrollo temprano de la plántula. Para este fin, semillas deshidratadas o sin deshidratar se sometieron a pretratamientos de encapsulación/deshidratación en presencia de agentes osmoprotectores (LS, PVS2 y PVS3), previo a su congelación en N_2L. Después de la descongelación a temperatura ambiente, se compararon la capacidad y velocidad de germinación de las semillas de los distintos tratamientos, así como el establecimiento, supervivencia y crecimiento de las plántulas después de cuatro meses en vivero. Los resultados muestran que las semillas de cedro rojo tienen la capacidad de sobrevivir el congelamiento rápido, aunque esto tuvo en general un efecto perjudicial durante las etapas de germinación y emergencia temprana, el cual fue menos severo en semillas sin encapsular. No obstante, los pretratamientos favorecieron la supervivencia de la plántula en vivero; el sistema radicular tuvo mayores afectaciones que la parte aérea en todos los tratamientos de congelación, lo cual incidió en una alta relación PSA/PSR. Se concluye que las semillas de *Cedrela odorata* sometidas al congelamiento rápido tienen el potencial de sobrevivir, germinar y producir plántulas para trasplante en vivero, aunque es necesario afinar el protocolo para optimizar la respuesta.
Palabras clave: Cedro rojo, conservación *ex situ,* germinación, germoplasma, nitrógeno líquido, osmoprotector.

Abstract
Seed cryopreservation represents an alternative for the long-term conservation of forest germplasm, especially of intermediate or recalcitrant tropical species, whose viability declines rapidly under standard storage conditions. The impact of rapid freezing of Spanish cedar seeds in liquid nitrogen (L_2N), on *sensu stricto* germination, and on the early stages of seedling development was evaluated. To this end, both dehydrated and non-dehydrated seeds were subjected to encapsulation/desiccation pre-treatments in the presence of various anti-vitrifying agents (LS, PSV2, and PSV3), prior to freezing. After thawing at room temperature, germination speed and capacity were compared, as were plantlet establishment, growth and survival after transplanting to the nursery. Results show that Spanish cedar seeds have the potential to survive fast freezing, although, in general, this had a damaging effect during the germination and early stages of plantlet establishment, being less severe in seeds that had not been encapsulated. However, the pretreatments favored plantlet survival in the nursery. The roots were the most affected organ, which led to a high shoot root dry weight ratio (S:R). It is concluded that *Cedrela odorata* seeds subject to rapid freezing have the potential to survive, germinate and produce plantlets that can be transferred to nurseries, although further fine tuning of the protocol is required to optimize response.
Key words: Red cedar, *ex situ* conservation, germination, germplasm, liquid nitrogen, osmoprotectant.
Fecha de recepción/Reception date: 18 de agosto de 2021
Fecha de aceptación/Acceptance date: 15 de noviembre de 2021

1Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, Cenid-Conservación y Mejoramiento de Ecosistemas Forestales. México.
*Autor para correspondencia; correo-e: garcia.florencia@inifap.gob.mx

Introduction

Cedrela odorata L. is the second most commercially important precious wood species in the tropical and subtropical regions of Mexico and Latin America (Pennington and Sarukhán, 2005); therefore, it is considered a priority species for reforestation and commercial forest plantation programs. However, due to the destruction of its habitat by deforestation and excessive use, it was included in Appendix III of the 2013 Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in order to regulate its trade and prevent its unsustainable exploitation; is also part of the Red List of the International Union for Conservation of Nature (IUCN) as a vulnerable species (Mark and Rivers, 2017); in Mexico, it is listed in the NOM-059-SEMARNAT-2019 norm as a species subject to special protection (Semarnat, 2019). It is essential to establish strategies for seed management and conservation in order to preserve this genetic resource in germplasm banks, as well as to meet the growing demand for seeds for plantation and reforestation programs.

Based on their storage characteristics, *C. odorata* seeds are classified as intermediate, as they are relatively tolerant to dehydration, although sensitive to chilling (Andrés *et al*., 2011). However, their viability declines a few months after collection, which limits the possibility of mid-to-long term maintenance under *ex situ* conditions (García and Abdelnour, 2013) and reinforces the need to seek alternatives for their preservation.

Cryopreservation, or storage of cells, tissues or organisms at ultra-low temperatures, in liquid nitrogen (N_2L, below -150 °C), has been successfully implemented for the conservation of agricultural (Hor *et al*., 2005; Acosta *et al*., 2020), ornamental, (Hirano *et al*., 2009) and forest species (Pita *et al*., 1998; Michalak *et al*., 2015). In addition, it represents an option for endangered, as well as tropical species with intermediate or recalcitrant seeds, particularly those that can withstand partial dehydration (Hor *et al*., 2005), such as Spanish cedar. The

technique is intended to halt metabolic processes and cell division, and, thereby, to prevent deterioration (Engelmann and Dussert, 2013).

However, the freezing process has consequences as it can induce crystal formation and osmotic shock, which is lethal to the tissue; therefore, success depends on the proper management of cellular moisture content (Dussert *et al*., 2001; Hor *et al*., 2005). Because the water and macromolecule content differs in seeds of different species, standardized protocols are required to limit damage during freezing and subsequent thawing. On the other hand, it is necessary to evaluate the effectiveness of the process, both to preserve the viability and germinability of the seeds, as well as their potential to produce good quality plants.

Research on tissue cryopreservation in the *Cedrela* genus is scarce, but promising. Studies on *C. fissilis* Vell. (Da Costa *et al*., 2003) show that seeds tolerate freezing with N_2L and recover to produce vigorous seedlings, as long as the moisture content remains between 6 and 7 %. Similarly, in *C. odorata*, vitrification and freezing of apices and seeds, as well as subsequent recovery under *in vitro* conditions promotes high survival and efficient germination, even after several months of freezing (García and Abdelnour, 2013). However, the impact of different seed management strategies prior to N_2L treatment on germination, survival, rooting and overall quality of the seedlings obtained is still unknown. Therefore, the objectives of this work were: 1) to determine the efficiency of different cryopreservation strategies on freezing tolerance in seeds of *C. odorata*, and 2) to test the rooting ability, survival and growth of seedlings 16 weeks after transplanting to the nursery.

Materials and Methods

Plant material

A composite mixture of *C. odorata* seeds collected in 2018 from the states of *Chiapas, Tabasco,* and *Veracruz,* Mexico, was used. From this mixture, a working sample of 1 100 seeds was obtained without physical damage or evidence of

contamination by pests or diseases. The moisture content (MC) of the lot was 8 % and was determined prior to the establishment of the experiments, according to ISTA guidelines (2016).

Seed pretreatment and deep freezing

The effect of freezing in N_2L was compared in intact seeds that did not receive any handling (naked - T0), with seeds subjected to different pretreatments (Table 1), in which the effect of dehydration (T1) and encapsulation/dehydration (T2 and T3) was tested; as well as the additional application of different osmoprotectants, alone (T4, T5 and T6) or in combination (T7 and T8). Five replications of 20 seeds were used in each treatment, for a total of 100 seeds. Untreated and unfrozen seed was used as a germination control (TP).

Table 1. Cryopreservation treatments for seeds of *Cedrela odorata* L.

Treatment	Preconditioning					Exposure in N_2L
	Dehydratation (%)	Encapsulation	Vitrification			
			LS	PVS2	PVS3	
TP	No	No	No	No	No	No
T0	No	No	No	No	No	Yes
T1	5	No	No	No	No	Yes
T2	No	Yes	No	No	No	Yes
T3	5	Yes	No	No	No	Yes
T4	5	Yes	Yes	No	No	Yes
T5	5	Yes	No	Yes	No	Yes
T6	5	Yes	No	No	Yes	Yes
T7	5	Yes	Yes	Yes	No	Yes
T8	5	Yes	Yes	No	Yes	Yes

For treatments that included encapsulation/dehydration, the *C. odorata* seeds were placed in a 2 % sodium alginate solution (Sigma); then, they were individually

transferred to a 0.1 M calcium chloride polymerization solution (Sigma) during 15 minutes. Finally, they were placed in a desiccation chamber with activated silica gel until reaching constant weight (5.13 % CH in the seed without encapsulation and 5.24 % in the encapsulated seed, after 2.5 hours of dehydration). This procedure was repeated with the rest of the treatments.

In the osmoprotectant treatments, the encapsulated/dehydrated seeds were immersed for 10 minutes in LS (Loading solution: Murashige and Skoog (1962) medium, 2.0 M glycerol and 0.4 M sucrose) (Nishizawa *et al*., 1993); then left for 20 min, in PVS2 (glycerol 30 %, ethylene glycol 15 %, dimethyl sulfoxide 15 %, sucrose 0.4 M) (Sakai *et al*., 1990), or in PVS3 (glycerol 50 %, sucrose 1.5 M) (Nishizawa *et al*., 1993).

The seeds of the different treatments were immersed in N_2L for 20 minutes, and then thawed at room temperature.

Germination and seedling emergence

After applying the different treatments, the seeds were placed in germination boxes (Seedburo™) on filter paper moistened with sterile double distilled water. Incubation was carried out in a Memmert HPP750 climatic chamber (Memmert GmbH + Co.Kg, Germany), at 28 °C (day/night) and room relative humidity. The seeds were checked daily for 22 days and were considered as germinated when the root protruded from the testa by a length of approximately 3 mm. Germination capacity (*GC*) was evaluated as the percentage of germination at the end of the test; germination energy (*GE*) was evaluated as the number of days in which 50 % germination was achieved (higher values indicated lower *GE*) (Juárez-Agis *et al*., 2006); the germination value (*GV*) was estimated obtained using the Czabator equation (1962):

$$GV = ADG \times PV$$

Where: *ADG* (average daily germination) resulted from dividing the final percentage of germinated seeds by the number of days of testing (Viveros-Viveros *et al*., 2015); and the peak value (*PV*) corresponds to the cumulative percentage at the inflection point of the germination curve (González-Zertuche and Orozco-Segovia, 1996).

Once germinated, the seeds were placed on moist, sterilized substrate (35 % of peat moss, 35 % of perlite, and 30 % of vermiculite) under greenhouse conditions. The design was completely randomized in accordance with the ten treatments and four replications. After 15 days, the percentage of seeds with main root growth, generation of secondary roots, hypocotyl growth, and the appearance of true leaves were counted.

Survival, growth and biomass of seedlings

15 rooted seedlings were randomly selected from each of the treatments and transplanted into 250 mL tubes with sterilized substrate (35 % of peat moss, 35 % of perlite, and 30 % of vermiculite) and 4.2 g L^{-1} of Multicote® for growth in the nursery during 16 weeks. A randomized complete block experimental design was applied, with 10 treatments, five replications (blocks) and three plants per experimental unit. Survival (%), hypocotyl height (mm), total height (mm), basal diameter (root collar) (mm), crown diameter (mm), main root length (cm), with a Truper® digital vernier caliper. Root fresh weight (*RFW*) (g), leaf fresh weight (LFW) (g), aerial dry weight (ADW) (g) and root dry weight (*RWW*) (g) were also evaluated, which were determined with an analytical scale (OHANUS, model Galaxy ® 200) after dehydration of the plants in an electric oven (Riossa, OHF-125 model) at 96 °C, until constant weight was obtained; the total dry weight (*TWD*) (g) and the S:R ratio (g) were also estimated (Ruano, 2003).

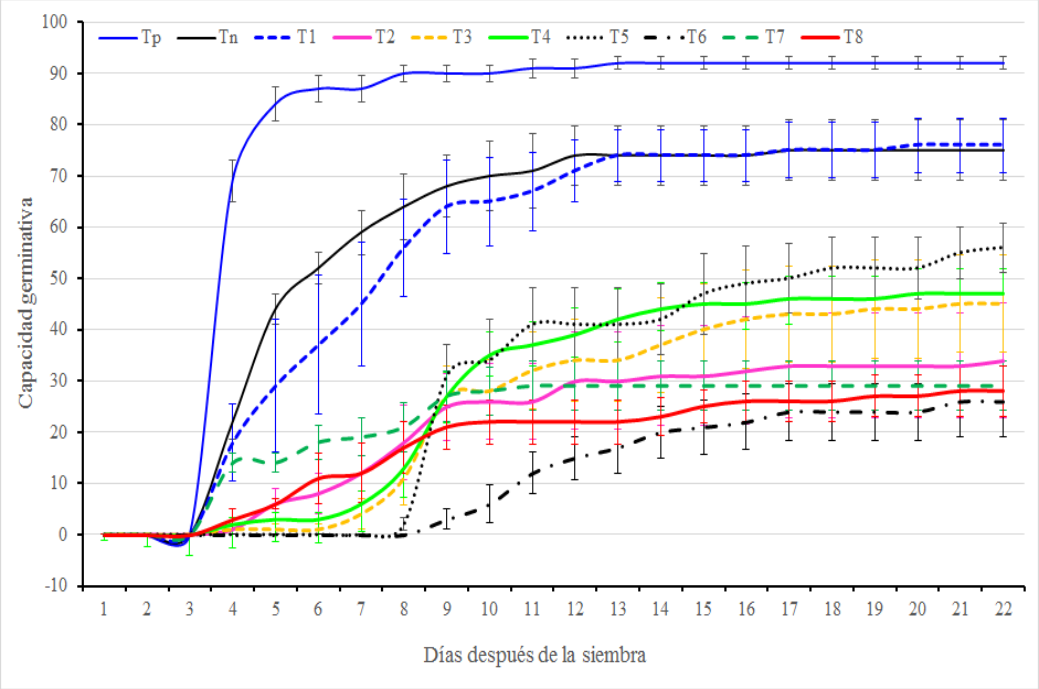
Data analysis

The assumptions of normality and homogeneity of variances of the data for all variables (germination, rooting, survival, and growth) were verified with the Shapiro-Wilk and Levene tests, respectively. Only the percentage of established seedlings met the assumptions ($p \geq 0.0893$), for which a Tukey's analysis of variance and comparison of means was performed ($p \leq 0.05$). The rest of the variables did not meet the assumptions ($p \leq 0.0433$); therefore, they were analyzed using nonparametric tests of variance and multiple comparisons of ranks (Kruskal and Wallis, 1952; Conover, 2012). For survival, growth variables and S:R ratio ($p \leq 0.0335$), analyses of variance, and RT-3 nonparametric mean comparisons were performed (Conover, 2012), while for root length data ($p \geq 0.2479$) a parametric analysis of variance and Tukey's mean comparison was performed. Finally, the differences between treatments were determined by considering the joint effect of germination and growth variables (survival, growth and S:R ratio); two separate principal component analyses were performed, and the comparison of means was obtained for principal component one (CP1). All analyses were carried out using the SAS V. 9.3.1 statistical analysis software (http://support.sas.com/software/93/).

Results

Effect of deep freezing on germination

Seed freezing in N_2L reduced the final germination percentage (*GC*) between 15 and 60 % with respect to the unfrozen control (TP), depending on the treatment. The best results for this stage were obtained for seeds that were frozen without encapsulation (naked), without dehydration (T0) or dehydrated (T1), which reached similar values among themselves and were comparable to the control for final germination (*GC*), germination vigor (*GV*) and germination energy (*GE*) (Figure 1, Table 2). Seed encapsulation (T2 and T3) caused a significant decrease in all germination parameters evaluated ($p < 0.0001$).



Días después de la siembra = Days after sowing; *Capacidad germinativa* = Germination capacity.

Each point represents the average percentage (%) of germinated seeds from five replications and the vertical bars represent the standard error.

Figure 1. Germination curve of *Cedrela odorata* L. seeds subjected to different pretreatments and to freezing in liquid nitrogen.

Table 2. Germination parameters. Average values and comparison of means are shown for the ten cryopreservation treatments of seeds of *Cedrela odorata* L.

Treatment	Germination capacity (*GC*) (%)	Germination value (*GV*)	Germination energy (*GE*)	Principal Component One	Emergence of seedlings (%)
TP	92.00 a	200.59 a	4.00 a	2.527 a	76.84 a
T0	74.00 ab	67.42 a	5.00 ab	1.683 ab	38.86 b
T1	75.00 ab	62.12 a	6.40 bc	1.400 b	34.93 b
T3	34.00 de	7.45 bcd	8.00 c	-0.881 cd	30.00 b
T4	44.00 cd	10.56 bc	8.40 d	-0.589 cd	33.33 b
T7	47.00 cd	11.54 b	9.20 d	-0.443 cd	60.56 ab
T5	55.00 bc	15.50 b	9.80 de	-0.390 cd	46.00 ab
T8	26.00 e	3.17 d	11.80 e	-2.060 e	42.87 b
T2	29.00 e	12.19 bc	4.60 a	-0.134 c	50.03 ab
T6	27.00 e	6.01 cd	7.80 c	-1.113 de	33.33 b

Values with different letters in the column are statistically different Tukey ($p \leq 0.05$).

The addition of the osmoprotective agents LS, PVS2, and PVS3 to the encapsulated/dehydrated material, alone (T4, T5 and T6) or in combination (T7 and T8) did not significantly reverse the detrimental effect of encapsulation; only the PVS2-only treatment (T5) significantly increased GC, although the onset of germination was late, which caused GE and GV values to remain low. In general, the lowest values for all parameters were obtained when PVS3 was incorporated (T6 and T8). An important feature at this stage was that freezing, in any of the treatments, significantly increased the variance of germination capacity (TP = 15.8, rest of treatments = 76.5 - 309.2).

In the Principal Component Analysis, principal component one (PC1) of the germination parameters explained 76.82 % of the variance. *GC* and *GV* contributed similar values (0.608 and 0.655, respectively) to explain the variability, while *GE* had a lower contribution (-0.449). CP1 of germination parameters showed statistical differences ($p < 0.0001$) among treatments, with values ranging from -2.03 (T8) to 3.56 (TP). CP1 showed three groups of treatments: the first group consisted of treatments T0, T1 and the control treatment (TP); the second group consisted of T2, T3, T4, T5 and T7; while T6 and T8 formed the third group (Table 2).

Effect of deep-freezing and seed pretreatment on seedling emergence

The ability of germinated seeds to form complete seedlings suitable for transplanting was statistically different between cryopreservation treatments ($p < 0.049$). In contrast to what was observed during *sensu stricto* germination, the freezing of uncapsulated seeds (T0 and T1) negatively affected subsequent development, since the number of emerged seedlings was significantly reduced.

Encapsulation allowed a partial recovery of the response when used in combination with dehydration (T2), or depending on whether the osmoprotective agent PVS2 was applied alone (T7) or in combination with LS (T5); this achieved statistically similar results to the unfrozen control (TP) (Table 2).

Survival, growth and biomass of seedlings in greenhouses

The seedling survival after 16 weeks of greenhouse growth, as well as the shoot/root dry weight ratio (*S:R*) and all the growth variables exhibited statistical differences ($p < 0.05$) between cryopreservation treatments, with the exception of the basal (root collar) diameter ($p = 0.2094$) (Table 3).

Table 3. Survival and growth variables of seedlings under cryopreservation treatments for *Cedrela odorata* L.

Treatment	Survival (%)	Hypocotyl height (mm)	Basal diameter (mm)	Total height (mm)	Crown diameter (mm)	Root length (mm)	S:R Ratio	Principal component 1
TP	73.33 b	36.31 a	3.80 a	113.81 abc	169.29 a	11.32 a	3.83 c	1.139 a
T0	46.67 b	38.14 a	5.08 a	103.49 abc	156.22 a	8.47 bc	2.84 ab	0.768 ab
T1	66.67 ab	39.79 a	4.94 a	116.04 a	139.64 abcd	10.11 ab	4.25 c	0.714 ab
T3	80.00 ab	28.99 b	3.67 a	85.83 c	113.65 d	6.79 c	3.06 abc	-0.987 c
T4	86.67 ab	28.54 b	4.05 a	112.50 a	132.85 abcd	8.42 bc	2.24 a	0.008 abc
T7	100.00 a	24.53 b	3.63 a	91.52 bc	120.27 bcd	7.39 c	3.40 bc	-1.060 c
T5	100.00 a	27.55 b	4.83 a	99.93 abc	133.80 abcd	8.06 bc	3.71 c	-0.208 abc
T8	91.67 ab	27.00 b	3.56 a	85.20 c	116.37 cd	8.42 bc	3.56 bc	-0.853 bc
T2	80.00 ab	29.34 b	3.96 a	111.78 ab	150.54 abc	9.75 ab	3.96 bc	0.406 abc
T6	72.73 ab	28.51 b	4.48 a	126.69 a	154.21 ab	9.30 bc	5.04 c	0.739 ab

Mean values and comparison of means (different letters in the same column indicate significant differences, *p* less than or equal to 0.05).

Seedlings from uncapsulated seeds (T0 and T1) had similar survival to unfrozen seeds (TP), as well as for practically all growth variables, except for root length and S:R ratio in which seedlings from dry seeds (T0) had significantly lower values than seedlings from unfrozen seeds. The above indicates a positive effect of seed dehydration at this stage, and in particular on root quality, which may have impacted survival. As in the establishment stage, encapsulation, in the absence (T2 and T3) or in the presence (T4 to T8) of osmoprotective agents, promoted survival, particularly in treatments including PVS2 (T5 and T7), which reached 100 % and surpassed even unfrozen seeds (TP). However, the hypocotyl length of seedlings from encapsulated seeds was significantly shorter than that obtained for uncapsulated seeds.

The CP1 of survival, growth variables, and *S:R* ratio accounted for 41.82 % of the variance. Basal diameter, root length, total height and crown diameter had the greatest contribution to the total variance (0.4103 to 0.5267), followed by hypocotyl height (0.2830); on the other hand, the survival and S:R ratio registered lower contributions to the total variance (0.007 and 0.0374). CP1 showed statistical differences between treatments (p = 0.0111), with values ranging from -1.060 (T7) to 1.139 (TP). In general, CP1 determined three groups of treatments, the first group was made up of treatments (TP, T0, T1, T6) with high values; the second, with intermediate values (T2, T4, T5), and the third (T3, T7, T8), with low values (Table 3).

Discussion

The implementation of cryopreservation of *C. odorata* seeds offers an alternative for its long-term management in germplasm banks. Coupling deep freezing with

osmoprotective pretreatments is a widely used strategy to maintain seed viability during freeze/thawing. However, the impact of these management practices, as a whole, on the development of the future plant must be explored in its different stages —from germination to establishment to subsequent growth— in order to assess their feasibility as a conservation strategy.

Under the conditions proposed in the documented trials, it was determined that the effect of the different pretreatments varied at different stages of development. Between 20 and 70 % of the germplasm was found to be lost during *sensu stricto* germination and the early stages of seedling emergence, depending on the pretreatment; therefore, these represent the stages that are most sensitive to freezing. In particular, it was observed that the best germination response was obtained in the treatments of dehydration and freezing of naked seeds, with or without dehydration (T0 and T1), with respect to encapsulated/dehydrated seeds, without (T2 and T3) or with osmoprotective solutions (T4 to T8). The incorporation of an encapsulation/dehydration and vitrification step with osmoprotective agents prior to freezing was expected to maintain the germination capacity and overall viability of the seeds. However, this was not the case, and these manipulations had no impact (dehydration) or even a detrimental impact (encapsulation/dehydration/vitrification), since the final percentage, as well as the speed and energy of germination, decreased. These strategies are widely used because they favor cell water control by limiting the formation of ice crystals that can damage membranes during freezing and thawing (Gantait *et al*., 2017).

For example, glycerol behaves as an antifreeze that replaces intracellular water, making it very useful as a cryoprotectant; however, it is highly toxic, and, therefore, its use must be carefully adjusted (Kim *et al*., 2009). The seeds of *C. odorata* may be sensitive to the chemical toxicity of glycerol, which is why its use

was counterproductive. In addition, the PVS2 and PVS3 formulations, although highly recommended (Sakai *et al*., 1990; Nishizawa *et al*., 1993), have high sucrose concentrations, especially PVS3 (treatments T6 and T8), which can generate an osmotic shock.

This reflects the limitations of the tested treatments, which must be addressed in order to succeed in using deep-freezing as a tool for the long-term preservation of *C. odorata* seeds. However, the fact that the germination response was maintained suggests that, despite its cited sensitivity to cold, it is possible to adapt a cryopreservation strategy for its seeds, which contrasts with what has been observed for the seeds of other tropical tree species such as *Pithecellobium saman* (Jacq.) Benth. and *Gliridicidia sepium* (Jacq.) Kunth ex Walp (Abdelnour *et al*., 2007).

One aspect to consider is that seed viability and subsequent seedling development were possibly affected by the thawing at room temperature used in the present study, since work with the same taxon (García and Abdelnour, 2013) and for *C. fissilis* (Da Costa *et al*., 2003), indicated that seeds stored directly in N_2L maintain 100 % of their germination capacity, when thawing occurs at 40 ºC. While room temperature thawing is widely used, short incubation in heat may be favorable for certain species, especially those with high lipid content, as it appears to promote thawing of triacylglycerols before they interact with water during imbibition, which limits intracellular structural damage (Volk *et al*., 2006).

In contrast to what was observed during *sensu stricto* germination, in the initial establishment of the seedling, and during its subsequent development, a positive effect was observed in plants originating from seeds treated by encapsulation/dehydration, in particular with the use of PVS2 as an osmoprotectant (T5 and T7). This contrasts with plants whose seed received no treatment (T0 and T1), or was encapsulated without osmoprotectants (T2 and T3), or in seeds treated with PVS3, in which less than 50 % continued their development, indicating that components such as ethylene glycol or DMSO,

present in PVS2, may exert a protective effect (Kim *et al.*, 2009) and, therefore, should be optimized for the species.

Finally, the negative effect of freezing with N_2L decreased with the progress of seedling development, which probably indicates a gradual recovery from the stress thus provoked, as has been observed in sorghum, bean, corn, and tomato (Cejas *et al*., 2012; Arguedas *et al*., 2018; Acosta *et al*., 2020). In particular, seed management by means of some pretreatment (T2 to T8) favored seedling survival once transplanted in the nursery. This may be a reflection of the selection pressure exerted by the treatments in previous stages, which results in only the most vigorous seeds being able to produce seedlings with the potential to establish (see, for example, T6). However, seedlings grown from encapsulated/dehydrated seeds treated with PVS2 (T5 and T7) reached higher values even than the unfrozen control (TP), suggesting that the combination of compounds present in this solution effectively provides a benefit that is expressed after germination. When analyzing the growth variables of the aerial part (shoot) of the seedling, the treatments with dry (T0) and dehydrated (T1) seeds were similar to the unfrozen control (TP), which contrasts with the treatments with PVS2 (T5 and T7) that presented maximum survival but slow growth. Although height is related to the photosynthetic capacity of the seedlings and transpiration surface, as well as to a greater likelihood of competing for resources, an adequate root system is required to support the growth of the seedlings (Rodríguez, 2008; Prieto and Sáenz, 2011).

Root development was affected in all freezing treatments —more than the development of the shoot— and influenced the high values of the *S:R* ratio observed, whose optimum value is expected to be within the 1.5 and 2.5 range (Rodríguez, 2008). The smaller shoot size observed in T5 and T7 generated a more balanced *S:R* ratio; this could benefit the growth of the seedlings in restrictive sites, since it has been demonstrated that plants with lower height and

a smaller diameter maintain a better water status, with a moderate water consumption in situations of water deficiency (Leiva and Fernández, 1998).

Given the results of this study, an ultrafreezing protocol for Spanish cedar seeds will require further exploration of thawing alternatives, germination conditions, and the incorporation of PVS2 in order to enhance the survival and vigor of the different developmental stages.

Conclusions

Seeds of *C. odorata* subjected to flash freezing have the potential to survive, germinate and produce vigorous seedlings for nursery transplanting. The critical phases for the success of a cryopreservation protocol are *sensu stricto* germination and early seedling emergence. The evaluation of different pretreatments of encapsulation, dehydration, as well as the use of osmoprotectants showed that freezing seeds directly, without the application of any pretreatment, is the most adequate protocol to maintain germination capacity, although it is necessary to optimize the thawing conditions in order to obtain a better yield. The use of the osmoprotective solution PVS2 has a beneficial effect on early emergence and survival of seedlings in the nursery. This suggests a route to follow for optimizing a protocol that combines these factors and provides a viable alternative for the long-term conservation of *C. odorata* seeds.

Acknowledgments

The authors thank the individuals and institutions that donated the seeds. The results are part of the project "Transcriptomic analysis of germination in *Cedrela odorata* L. for the identification of molecular markers for seed conservation", number 941634436, financed by the INIFAP Fiscal Fund.

Conflict of interests

Florencia García Campusano, Mario Valerio Velasco-García and Liliana Muñoz-Gutiérrez declare that they did not participate in any editorial activity related to this document.

Contribution by author

Mario Valerio Velasco-García and Liliana Muñoz-Gutiérrez: design of nursery experiments, nursery data collection, data analysis, and drafting of the manuscript; Dalia Grisel Hernández Arroyo: execution of cryopreservation experiments and data collection; Carlos Castillo Martínez: design and execution of cryopreservation experiments; Miguel Ángel Vallejo Reyna: design of experiments, discussion of results, review and editing of the manuscript; Florencia García Campusano: interpretation of the results and drafting of the manuscript. All authors gave their approval to the final version.

References

Abdelnour E., A., G. Rojas y U. Alfaro. 2007. Estudios preliminares para la crioconservación de especies forestales arbóreas. Revista Tecnología en Marcha 20(1):98-103. https://revistas.tec.ac.cr/index.php/tec_marcha/article/view/95. (31 de agosto de 2021).

Acosta, Y., L. Pérez, C. Linares, L. Hernández, D. Escalante, A. Pérez, B. E. Zevallos, L. Yabour, M. E. Martínez-Montero, I. Cejas, D. Fontes, Sershenand and J.C. Lorenzo. 2020. Effects of *Teramnus labialis* (L.f.) Spreng seed cryopreservation on subsequent seed and seedling growth and biochemistry. Acta Physiologiae Plantarum 42:7. Doi:https://doi.org/10.1007/s11738-020-3012-9.

Andrés, P., C. Salgadoand and J. M. Espelta. 2011. Optimizing nursery and plantation methods to grow *Cedrela odorata* seedlings in tropical dry agroecosystems. Agroforestry Systems 83:225–234. Doi:https://doi.org/10.1007/s10457-011-9404-5.

Arguedas, M., A. Villalobos, D. Gómez, L. Hernández, B. Zevallos, I. Cejas, L. Yabor, M.E. Martínez-Montero and J. C. Lorenzo. 2018. Field performance of cryopreserved seed-derived maize plants. CryoLetters 39(6):366-370. http://www.cryoletters.org/Abstracts/vol_39_6_2018.htm#366 (31 de agosto de 2021).

Czabator, F. J. 1962. Germination Value: An index combining speed and completeness of pine seed germination. Forest Science. 8(4):386-396. Doi:https://doi.org/10.1093/forestscience/8.4.386.

Cejas, I., K. Vives, T. Laudat, J. González-Olmedo, F. Engelmann, M. E. Martínez-Montero and J. C. Lorenzo. 2012. Effects of cryopreservation of *Phaseolus vulgaris* L. seeds on early stages of germination. Plant Cell Reports. 31:2065-2073. Doi:https://doi.org/10.1007/s00299-012-1317-x.

Conover, W. J. 2012. The rank transformation-an easy and intuitive way to connect many nonparametric methods to their parametric counterparts for seamless teaching introductory statistics courses: The rank transformation. Wiley Interdisciplinary Reviews. Computational Statistics. 4(5):432–438. Doi:https://doi.org/10.1002/wics.1216.

Da Costa N., E., E. E. Benson, A. C. Oltramari, P. S. Araújo, J. R. Moser and A. M. Viana. 2003. *In vitro* conservation of *Cedrela fissilis* Vellozo (Meliaceae): a native tree of the Brazillian Atlantic Forest. Biodiversity & Conservation 12:837–48. Doi:https://doi.org/10.1023/A:1022492226341.

Dussert, S., N. Chabrillange, G. Rocquelin, F. Engelmann, M. Lopez and S. Hamon. 2001. Tolerance of coffee (*Coffea* spp.) seeds to ultralow temperature exposure in relation to calorimetric properties of tissue water, lipid composition and cooling procedure. Physiologia Plantarum 112:495–504. Doi:https://doi.org/10.1034/j.1399-3054.2001.1120406.x.

Engelmann, F. and S. Dussert. 2013. Cryopreservation. *In*: Normah, M., H. Chin and B. Reed (eds.). Conservation of Tropical Plant Species. Springer, New York, NY. USA. pp.107-119. Doi:https://doi.org/10.1007/978-1-4614-3776-5_6.

Gantait, S., U. R. Sinniah, G. Shukla and N. C. Sahu. 2017. Cryoconservation Methods for Extended Storage of Plant Genetic Resources. *In*: Ansari, A. A., S. S. Gill, Z. K. Abbas and M. Naeem (eds.). Plant biodiversity: monitoring, assessment and conservation. CABI. pp. 458–464. Doi:http://dx.doi.org/10.1079/9781780646947.0458.

García R., T. y A. Abdelnour E. 2013. Crioconservación de ápices y semillas de cedro (*Cedrela odorata* L.) mediante las técnicas de vitrificación y deshidratación. Agronomía Costarricense 37(1):113-126. Doi:https://doi.org/10.15517/rac.v37i1.10717.

González-Zertuche, L. y A. Orozco-Segovia. 1996. Métodos de análisis de datos en la germinación de semillas, un ejemplo: *Manfreda brachystachya*. Boletín de la

Sociedad Botánica de México 58:15-30. https://www.botanicalsciences.com.mx/index.php/botanicalSciences/article/view/1484 (12 de julio de 2021).

Hirano, T., T. Godo, K. Miyoshi, K. Ishikawa, M. Ishikawa y M. Mii. 2009. Cryopreservation and low-temperature storage of seeds of *Phaius tankervilleae*. Plant Biotechnology Reports 3:103–109. Doi:https://doi.org/10.1007/s11816-008-0080-5.

Hor, Y. L., Y. J. Kim, A. Ugap, N. Chabrillange, U. R. Sinniah, F. Engelmann and S. Dussert. 2005. Optimal hydration status for cryopreservation of intermediate oily seeds: Citrus as a case study. Annals of Botany 95(7):1153-1161. Doi:https://doi.org/10.1093/aob/mci126.

International Seed Testing Association (ISTA). 2016. Reglas internacionales para el análisis de las semillas. https://vri.umayor.cl/images/ISTA_Rules_2016_Spanish.pdf (31 de agosto de 2021).

Juárez-Agis, A., J. López-Upton, J. J. Vargas-Hernández y C. Sáenz-Romero. 2006. Variación geográfica en la germinación y crecimiento inicial de plántulas de *Pseudotsuga menziesii* de México. Agrociencia 40(6): 783-792. https://agrociencia-colpos.mx/index.php/agrociencia/article/view/509 (31 de agosto de 2021).

Kim, H. H., Y. G. Lee, D. J. Shin, H. C. Ko, J. G. Gwag, E. G. Cho and F. Engelmann. 2009. Development of alternative plant vitrification solutions in droplet-vitrification procedures. CryoLetters 30(5):320–334. http://www.cryoletters.org/Abstracts/vol_30_5_2009.htm#320 (31 de agosto de 2021).

Kruskal, W. H. and W. A. Wallis. 1952. Use of ranks in one-criterion variance analysis. Journal of the American Statistical Association 47(260):583-621. Doi:https://doi.org/10.2307/2280779.

Leiva, M. J. and R. Fernández A. 1998. Variability in seedling water status during drought within a *Quercus ilex* subsp. *ballota* population, and its relation to seedling morphology. Forest Ecology and Management 111(2-3):147-156. Doi:https://doi.org/10.1016/S0378-1127(98)00320-X.

Mark, J. and M. C. Rivers 2017. *Cedrela odorata*. The IUCN Red List of Threatened Species 2017: e.T32292A68080590. https://dx.doi.org/10.2305/IUCN.UK.2017-3.RLTS.T32292A68080590.en (6 de agosto de 2021).

Michalak, M., B. P. Plitta, T. Tylkowski, P. Chmielarz and J. Suszka. 2015. Desiccation tolerance and cryopreservation of seeds of black poplar (*Populus nigra* L.), a disappearing tree species in Europe. European Journal of Forest Research 134(1):53-60. Doi: https://doi.org/10.1007/s10342-014-0832-4.

Murashige, T. and F. Skoog. 1962. A revised medium for the rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum 15:473–497. Doi:https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Nishizawa S, A. Sakai, Y. Amano and T. Matsuzawa. 1993 Cryopreservation of asparagus (*Asparagus officinalis* L. Osb.) embryogenic suspensión cells and subsequent plant regeneration by vitrification. Plant Science 91(1):67–73. Doi:https://doi.org/10.1016/0168-9452(93)90189-7.

Pennington, T. D. y J. Sarukhán. 2005. Árboles Tropicales de México. Manual para la identificación de las principales especies. UNAM/FCE. México D.F., México. 523 p.

Pita, J. M., V. Sanz and A. Escudero. 1998. Seed cryopreservation of seven Spanish native pine species. Silvae Genetica 47(4):220-223. https://www.thuenen.de/media/institute/fg/PDF/Silvae_Genetica/1998/Vol._47_Heft_4/47_4_220.pdf (31 de agosto de 2021).

Prieto P., J. A. y J. T. Sáenz. 2011. Indicadores de la calidad de planta en viveros de la sierra madre occidental. Libro Técnico Núm. 3. Campo Experimental Valle del Guadiana. Centro de Investigación Regional Norte Centro. INIFAP. Durango, Dgo. México. 212 p.

Rodríguez T., D. A. 2008. Indicadores de calidad de planta forestal. Mundi-Prensa. Madrid, España. 156 p.

Ruano M., J. R. 2003. Viveros forestales. Manual de cultivo y proyectos. Mundi-Prensa. Madrid, España. 281 p.

Sakai, A., S. Kobayashi and I. Oiyama. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. brasiliensis Tanaka) by vitrification. Plant Cell Reports 9(1):30-33. Doi:https://doi.org/10.1007/BF00232130.

Secretaria de Medio Ambiente y Recursos Naturales (Semarnat). 2019. Modificación del Anexo Normativo III, Lista de especies en riesgo de la Norma Oficial Mexicana NOM-059-SEMARNAT-2010, Protección ambiental-Especies nativas de México de flora y fauna silvestres-Categorías de riesgo y especificaciones para su inclusión, exclusión o cambio-Lista de especies en riesgo. 30 de diciembre de 2010. DOF. Ciudad de México, México. 108 p.

Viveros V., H., J. D. Hernández P., M. V. Velasco García, R. Robles S., C. Ruiz M., C., A. Aparicio R., M. D. J. Martínez H., J. Hernández V. y M. L. Hernández H. 2015. Análisis de semilla, tratamientos pregerminativos de Enterolobium cyclocarpum (Jacq.) Griseb. y su crecimiento inicial. Revista mexicana de ciencias forestales, 6(30): 52–65. Doi:https://doi.org/10.29298/rmcf.v6i30.207.

Volk, G. M., J. Crane, A. M. Caspersen, L. M. Hill, C. Gardner and C. Walters. 2006. Massive cellular disruption occurs during early imbibition of *Cuphea* seeds containing crystallized triacylglycerols. Planta 224(6):1415-1426. Doi:https://doi.org/10.1007/s00425-006-0310-4.