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Cryopreservation: an Alternative Method for the Conservation of Endangered Populations of Two Iberian Pines (*Pinus nigra* ARNOLD and *Pinus sylvestris* L.)

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Summary

Seed conservation in traditional seedbanks is a possibility for preserving genetic resources, but the rapid decrease in the viability of germplasm of forest trees and the problems associated with collection and multiplication, recommend the use of other storage methods. We have studied the effect of cryopreservation on the viability of *Pinus nigra* and *Pinus sylvestris* seeds. The results showed that germination is not significantly different in any of the blocks included in our experimental design (cryopreservation x desiccation). All this suggests that cryopreservation can be an economical and practical method in the conservation of seeds of *Pinus* species.

Key words: Cryopreservation, diversity loss, *Pinus nigra, Pinus sylvestris,* seeds.

FDC: 232.315.2; 232.318; 174.7 *Pinus nigra;* 174.7 *Pinus sylvestris;* (460).

Introduction

The most conspicuous cause of the loss of biodiversity is habitat destruction induced by man's activities (WILSON, 1985; MCNEELY et al., 1995). Forested areas in temperate regions have suffered a severe reduction in size as a result of habitat fragmentation. Thus, isolated and threatened populations can be highly affected by both natural disturbances, such as wildfires, or man-induced changes, such as acid rain (ENEY and PETZOLD, 1987), or urban development. Extreme events can lead to local extinctions and a consequent loss of genetic variability. The smaller the area and the more severe the disturbance, the higher the rate of local loss of populations of tree species. In this sense several Iberian populations of *Pinus sylvestris* on the southern boundary of its distribution, and of *Pinus nigra* subsp. *salzmannii* are clearly threatened due

to low regeneration capacity, interspecific competition and, above all, disturbance (CEBALLOS and RUÍZ DE LA TORRE, 1966; ELENA-ROSELLÓ and SÁNCHEZ-PALOMARES, 1991; NICOLÁS and GANDULLO, 1969). It seems necessary to improve strategies to preserve endangered small populations in order to save the Iberian forest tree genetic resources.

Several conservation methods have been proposed and developed to avoid or minimize these problems. Seedbanks can be considered the main key to conservation because seed storage is the most practical and economical method for conserving germplasm (GÓMEZ-CAMPO, 1985; HAWKES, 1990; CHIN, 1994), as long as it is used in combination with appropriate *in situ* procedures, such as forest reserves (FALK, 1990).

Lost of viability of some pine seeds and abnormal seedling growth have been detected after 10 to 15 years of storage (DONALD and JACOBS, 1990). This implies that stocks in genebanks must be replenished at short intervals from natural populations. The use of this protocol, therefore, is not viable as a consequence of the biological traits of forest trees. Logically, this procedure can become impractical for threatened populations because the reproductive effort can be seriously damaged by the harvest process or even because the population could disappear after some stochastic disturbance (i.e. wildfire). Cryopreservation has been proposed as an efficient alternative tool for the *ex situ* conservation of forest tree seeds because material can be stored for long periods, minimizing the problems associated with the harvest and multiplication of material (STANWOOD, 1985; AHUJA, 1986, 1991, 1994; JÖRGENSEN, 1990; RAO and RILEY, 1994).

Our aim was to test the viability of *Pinus sylvestris* and *Pinus nigra* seeds after submitting them to different cryopreservation protocols, in order to evaluate this method as an alternative to the traditional seedbanking.

Material and Methods

Seeds of *Pinus nigra* ARNOLD ssp. *salzmannii* (DUNAL) FRANCO and *Pinus sylvestris* L. var. *iberica* SVOB., collected in 1995 in the southern Sistema Ibérico (Cuenca, Spain), were obtained from the Institute for Nature Conservation (ICONA), Ministry of Agriculture. Seeds were extracted after submitting the cones for opening at 55 °C to 65 °C during 2 hours and subsequently stored at 6 °C in darkness.

Half of the seeds of each species were desiccated over silica gel for 60 days in hermetically-sealed glass jars. Seed moisture content was determined before and after desiccation at 105 °C for 24 hours.

Seeds were wrapped in aluminium foil and directly inmersed in liquid nitrogen (–196 °C, seed cooling rate of approximately 200 °C min^{-1}) for 1 or 30 days. The warming procedure consisted of letting the samples warm under ambient laboratory temperature and humidity (20 °C to 25 °C/40%) to equilibrium. For germination, 4 x 25 seeds each were placed in Petri dishes (7 cm diameter) on top of 2 filter paper disks moistened with 3 ml distilled water. The seeds were incubated at 25 °C/15 °C day/night, with 16 h/8 h day/night regime and an irradiance of 35 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (OSRAM L 58W/20). Seeds showing radicle emergence were counted every day and removed from the Petri dishes. The number of days needed to reach 50% germination (T50) and final germination percentage after 30 days of seed incubation were calculated. A two-way ANOVA was performed to test the effect of desiccation (2 levels) and time of cryopreservation (3 levels: control, 1 day and 30 days). Germination percentages were previously submitted to the arcsine transformation and comparison of means was carried out using the SCHEFFÉ test ($p < 0.01$) (ZAR, 1984).

Table 1. – Final germination percentage (G%) of *Pinus nigra* and *Pinus sylvestris* seeds after different treatments. Cryopreservation (LN_2, liquid nitrogen), desiccation and desiccation-cryopreservation. SD (standard desviation). T50 (days needed to reach 50% germination).

	Pinus sylvestris		*Pinus nigra*	
	G% ± SD	T50	G% ± SD	T50
CONTROL	90 ± 0,56	8,5	94 ± 1,29	7,5
LN_2 (1 day)	87 ± 0,96	9	90 ± 1,73	7,5
LN_2 (30 days)	90 ± 1,29	9,5	94 ± 0,56	7,5
Desiccation/LN_2 (1 day)	80 ± 0,95	14,5	91 ± 1,26	11,5
Desiccation/LN_2 (30 days)	80 ± 1,15	10,5	89 ± 1,26	9,5
Desiccation	80 ± 0,81	11	82 ± 1,73	7,5

Results

Cryopreservation did not seem to affect the seed germination of *Pinus sylvestris* or *Pinus nigra* (*Table 1*). In *Pinus sylvestris* germination percentages reached high values in all treatments, 80% to 90%, and there were no differences compared to control as determined by ANOVA (dessication-cryopreservation). The treatments of previously dried seeds (moisture content: 5% to 6%), had a tendency to present the lowest germination percentages (80%). These treatments also showed the highest values for T50 (days to reach 50% of germination) 11 days to 14.5 days, likely due to a longest imbibition.

Similar results were obtained with seeds of *Pinus nigra.* Germination percentages for cryopreserved seeds were between 82% to 94%, and there were no differences with the control. The treatments of previously dried seeds had the highest T50 values.

Discussion

In spite of the wide distribution of *Pinus nigra* and *P. sylvestris* in the Iberian Peninsula, some of their natural populations are currently represented by only scattered small stands with very few trees. In the case of *P. sylvestris* the most isolated and reduced populations are located above 2000 m in some of the main ranges of Southern Spain (Sierra de Baza) and in the central portion of the Cantábrica Range on the northern fringe of the Iberian Mediterranean region. The former populations have been named *P. sylvestris* var. *nevadensis* although its taxonomic status is far from clear and form the southern edge of this species in the world *(*AMARAL-FRANCO, 1986). For *P. nigra*, the most threatened populations are located in the oromediterranean belt of the Sierra de Gredos, in central Spain, which are the only ones growing in acid soils in Spain, together with some stands in the southeastern ranges of the Iberian Peninsula (Sierra de María, Sierra de Mágina and Sierra de Baza) and some stands located in Soria in northern Spain (CATALÁN, 1991).

The genetic variability of these populations represents a valuable resource for the future exploitation of these forest trees in heterogeneous environments, thus one of the main foci of forestry management must be the preservation of these species and ecotypes. Furthermore, these genetically isolated populations present problems in their regeneration and in many cases are subjected to severe disturbances, both natural, such as wildfires, and, mainly, man-induced. Usually, the genetic conservation of forestry resources has relied on a

double approach: *in situ* conservation through the establishment of natural reserves and legal protocols, and *ex situ* conservation by traditional germplasm banks, storing seeds in optimal conditions.

Our results show that seed viability of these 2 Iberian pines is not modified by cryopreservation. This fact has previously been reported for other pine trees (STANWOOD, 1985) and even for some European populations of *P. sylvestris* (AHUJA, 1986). As a consequence of the heat treatment procedure to extract the seeds from the cones, the moisture content of seeds was very low (6% to 7%). It has been previously reported that seed moisture content is the most critical factor to cryopreservation, both high and excessively low contents can result in a drastic loss of viability (STANWOOD, 1985). The moisture contents after using a heat treatment to extract seeds seemed high enough to avoid a significant loss of viability, thus the desiccation in silica gel in cryopreservation protocols can be omitted.

Before recommending and generalizing this protocol to the *ex situ* conservation of forest tree germplasm of threatened populations, the possible effect of cryoshock, the delay of embryo growth and seedling vigor, must be evaluated.

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