# Seed Cryopreservation of Seven Spanish Native Pine Species

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(Received 31st March 1998)

**Summary**

Progressive forest decline requires the development of conservation strategies to avoid the loss of genetic diversity. Among the *ex situ* methods for the preservation of forest tree germplasm, conventional seed banking is considered the most efficient method for the majority of species. However, in some forest trees the rapid decrease of seed viability requires new storage methods. Cryopreservation (storage at ultralow temperatures, < –130 °C) offers great promise for long-term storage of forest tree seeds. In this paper the effect of storage in liquid nitrogen (–196 °C) on seven Spanish pines was evaluated. For most of these species, no significant differences were found in seed germination before and after cryopreservation. Only in *P. pinea* did cryopreservation negatively affect seed viability.

*Key words:* Cryopreservation, *Pinus,* seeds, gene conservation.

*FDC:* 232.315; 232.318; 181.524/.525; 174.7 *Pinus*; (460).

## Introduction

Deforestation by human activities is presently one of the main global environmental problems (MCNEELY et al., 1995). The irreparable loss of genetic diversity in many tree species may result in severe ecological and economic consequences (LEDIG, 1988; AHUJA, 1994). Thus, since the 70's, integrated strategies of *in situ* and *ex situ* conservation have been proposed, implemented and widely spread to preserve the remaining diversity. The latter includes botanical gardens, plantations, *in vitro* banks, and seed banks (MILLAR, 1993). Seed banking is considered the most efficient and economic method for the conservation of genetic resources (GÓMEZ-CAMPO, 1985; HAWKES, 1990; CHIN, 1994). The dessicated seeds of many species can be stored for long periods at temperatures below 0 °C without a loss of viability. These type of seeds are defined as true-orthodox, a category that includes most of the species of the tree genera of temperate areas, such as *Abies, Alnus, Betula, Fraxinus, Larix, Picea, Pinus,* etc. (BONNER, 1990).

Traditional banking of pine seeds has proven to be an efficient method for short and medium term conservation periods. *Pinus ponderosa* seeds stored at 0 °C for 7 years do not show a loss of viability (ALLEN, 1957). The same behaviour was found in *P. elliotii, P. patula, P. radiata* and *P. taeda* after 6 years of storage at –16 °C (DONALD and JACOBS, 1990). However, longer periods seem to affect seed vigour and viability. BARNETT (1969) reported a 32% decrease in the germination of *P. echinata* seeds after 10 years of storage at 1.1 °C and 6% moisture content. Recently, DONALD and JACOBS (1990) reported that storage periods of over 15 years in *P. elliotii, P. patula, P. radiata* and *P. taeda* resulted in an increase in abnormal germination. After 50 years, the germination rate fell to approximately 25% in *P. echinata* and 66% in *P. elliotii* seeds with a notable loss of vigour, which could be correlated with chromosomal aberrations (BARNET and VOZZO, 1985).

The above comments clearly denote that seed viability and seedling vigour are only maintained for short periods in comparison to the span-life cycle of pines. Thus, the use of traditional methods of seed storage is clearly limited and not recommended because the potential storage period is shorter than the natural interval between germination and seed production for the next generation (BONNER, 1990). An alternative method to minimize the current problems associated with traditional seed banking of tree seeds is cryopreservation (AHUJA, 1986, 1991; PITA et al., 1997), which is based on storing seeds at ultra-low temperatures (< –130 °C) (PRITCHARD, 1995; BAJAJ, 1995). At these temperatures metabolism is virtually stopped and consequently the deterioration process is greatly reduced (STANWOOD, 1985). Under these conditions, the storage period is theoretically indefinite and the management problems of traditional seed banking, such as periodic regeneration, viability controls, risk of loss by disease and environmental problems, are reduced or even eliminated (EBERHART et al., 1991).

Cryogenic storage has been successfully carried out in many orthodox seed species (STANWOOD, 1985), including different pine species, such as: *P. sylvestris* (AHUJA, 1986; PITA et al., 1997), *P. echinata* (ENGSTROM, 1966), *P. ponderosa* (STANWOOD and BASS, 1978), *P. nigra* (PITA et al., 1997). However, in some species with orthodox seeds authors have pointed out problems after cryopreservation such as, cotyledon detachment (STANWOOD, 1985; PRITCHARD et al., 1988), abnormal germination (STANWOOD, 1980; HARRISON and CARPENTER, 1977) or seed death by internal freezing injury (VERTUCCI, 1989). In these cases, the role of seed characteristics (seed size, seed moisture content and/or chemical composition) have been studied.

These facts suggest the need for evaluating the effect of cryopreservation on each species before proposing this conservation method. Thus, the objective of this study was to examine the effect of cryopreservation on the seven Spanish pines (*P. canariensis, P. halepensis, P. nigra, P. pinaster, P. pinea, P. sylvestris, P. uncinata*) with very different biogeographical and ecological features and different seed characteristics (AMARAL-FRANCO, 1986).

## Material and Methods

### *Plant material*

Seeds of *P. canariensis, P. halepensis, P. nigra, P. pinaster, P. pinea, P. sylvestris, P. uncinata,* collected in 1996, were obtained from the Institute for Nature Conservation (ICONA), Ministry of Agriculture. Seeds were extracted after submitting the cones to a treatment of 55 °C to 65 °C during 2 hours and subsequently stored at 6 °C in darkness.

### *Seed moisture content*

Seed moisture content was determined in all species using the oven method with samples being held at 103 °C for 17 hours as recommended by the International Seed Testing Association (ISTA, 1996). Moisture contents are expressed as percentage of fresh weight.

### *Seed storage in liquid nitrogen*

Seeds were wrapped in aluminium foil and directly immersed in liquid nitrogen (–196 °C, seed cooling rate of approximately 200 °C $min^{-1}$) for 4 days. The storage of seed accesions in liquid nitrogen took place in L'Air liquid GT-55 cryotanks until germination tests were done. The warming procedure

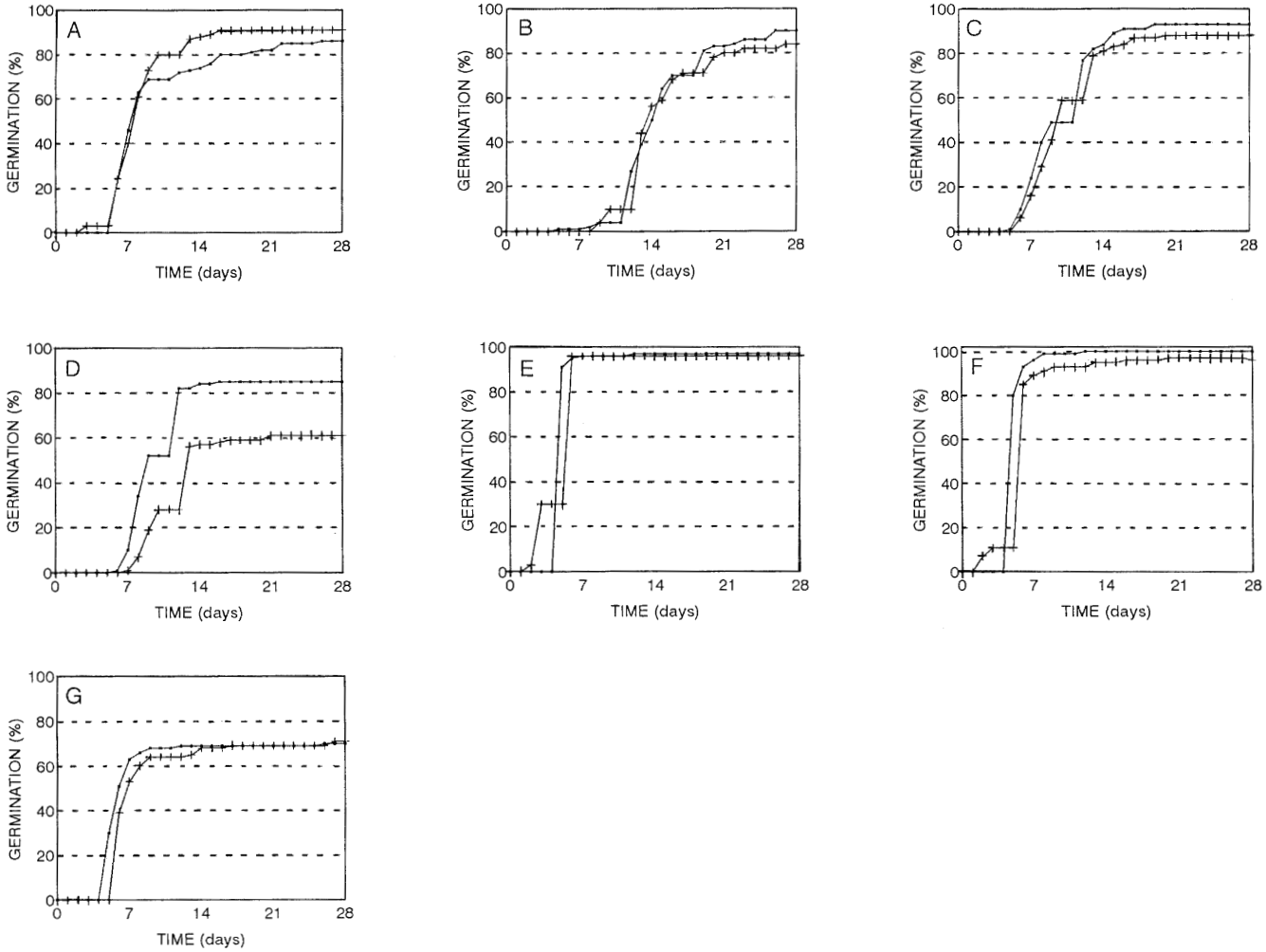


*Figure 1.* – Effect of cryopreservation on seed germination of seven Spanish pines incubated during 28 days at 25 °C/15 °C light/darkness, with 16 h/8 h light/darkness regime. A: *Pinus canariensis.* B: *P. halepensis.* C: *P. pinaster.* D: *P. pinea.* E: *P. nigra.* F: *P. sylvestris.* G: *P. uncinata.* Control (–■–). Inmersion in liquid nitrogen, 4 days (–|–).

consisted of letting the samples warm under ambient laboratory air temperature and humidity (20 °C to 25 °C/40%) to equilibrium (24 h).

*Seed germination tests*

For germination, four lots of 25 seeds were placed in Petri dishes (9 cm diameter) on top of two filter paper disks moistened with distilled water. The seeds were incubated at 25 °C/15 °C day/night, with 16 h/8 h light/darkness 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 28 days of seed incubation were calculated.

*Statistical analysis*

A one-way ANOVA was performed to test the effect of cryopreservation on final germination percentage. Germination percentages were previously submitted to the arcsine transformation and comparison of means was carried out using the SCHEFFÉ test ($p < 0.01$).

## Results and Discussion

Seed germination of the seven Spanish pines after cryopreservation is shown in *figure 1.* For most of these species, no significant differences were found in final seed germination percentages and T50 (*Table 1*) before and after cryopreservation. Only in *P. pinea* did cryopreservation negatively affect seed viability. Thus, the final germination percentage fell to 24% and T50 increased by four days.

Several factors have been postulated in order to explain the adverse effect of cryopreservation on germination, such as seed moisture content, physical damage and/or chemical composition (EBERHART et al., 1991). STANWOOD (1985) pointed out that moisture contents ranging between 10% to 30% could lead to a decrease in seed survival after cryopreservation due to the formation of intracellular ice during the freezing-thawing process (VERTUCCI, 1989). *P. pinea* seeds are close to the lower limit with a moisture content of 9.82%. However, this isolated factor does not seem to explain the loss of viability as *P. canariensis* and *P. pinaster* seeds, which were not affected by cryopreservation, which had similar moisture contents, (9.57% and 9.15% respectively *Table 1*). As indicated by PRITCHARD (1995) the most suitable seed moisture content for cryopreservation varies with each species. In some cases, dessication before immersion in liquid nitrogen has been proposed to avoid freezing damage (IRIONDO et al., 1992; GONZÁLEZ-BENITO et al., 1994). Recently, we have shown that germination percentages of *Pinus nigra* and *P. sylvestris* seeds dessicated with silica-gel (60 days) did not significantly differ from non-dessicated seeds after cryopreservation (PITA et al., 1997), although the initial

*Table 1.* – Final germination percentage (G%) of the seven Spanish pines before and after cryopreservation in liquid nitrogen (LN2). SD (standard deviation). T50 (days to reach 50% germination). HC (moisture content). L (seed length). W (seed weight).

| | Control | | LN2 (4 days) | | | | |
|---|---|---|---|---|---|---|---|
| ESPECIE | G%±SD | T50 | G%±SD | T50 | HC (%) | L (cm) | W (g) |
| *P. canariensis* | 86±2,7 | 7 | 91±0,5 | 7 | 9,57 | 1,11±0,13 | 0,12±0,03 |
| *P. halepensis* | 90±0,6 | 14 | 84±2,0 | 14 | 7,63 | 0,51±0,05 | 0,02±0,01 |
| *P. pinaster* | 94±0,6 | 11 | 88±2,2 | 10 | 9,15 | 0,68±0,10 | 0,07±0,02 |
| ***P. pinea* *** | **85±1,5** | **9** | **61±3,3** | **13** | 9,82 | 1,68±0,14 | 0,70±0,12 |
| *P. nigra* | 97±1,5 | 5 | 96±1,2 | 6 | 7,28 | 0,53±0,70 | 0,02±0,01 |
| *P. uncinata* | 70±1,5 | 6 | 71±1,9 | 7 | 7,26 | 0,37±0,05 | 0,01±0,00 |
| *P. sylvestris* | 100±0,0 | 5 | 97±1,0 | 6 | 7,71 | 0,38±0,04 | 0,01±0,00 |

*) Only in *P. pinea* significant differences were found in final germination percentages before and after cryopreservation.

moisture content was already very low (6% to 7%) probably as a consequence of the seed extraction procedure.

Physical damage due to the cooling/warming process may cause abnormal germination, detachment of cotyledons or production of seedlings with abnormalities (STANWOOD, 1985; PRITCHARD, 1995). These alterations, in some cases, could be due to a large seed size. *Phaseolus vulgaris* seeds split in half when rewarmed, however viability was not affected (STANWOOD, 1985). *P. pinea* seeds presented the largest and heaviest of all the Spanish pines (*Table 1*), but in our assays, no physical alterations were detected in the germinated seeds. Furthermore, there is no linear relationship between size and the presence of physical damage, as there is for small seeds (*Linum usitatissimum* or *Raphanus sativus*) which show notable physical damage after cryopreservation (STANWOOD, 1985).

The third suggested factor is the chemical composition of the seed, mainly oil seed content. It is hypothesized that the interaction between the lipid and water during freezing is responsible of the formation of ice crystals that were large enough to cause lethal damage (VERTUCCI, 1989). This author who worked with *Pisum sativum, Helianthus annuus* and *Glycine max,* reported sensitivity to cryopreservation in the two species with high lipid contents. Pine seeds are rich in oil (31% to 68% by weight) (WOLFF and BAYARD, 1995) mainly fatty acids (IMBS and PHAM, 1996), and in *Pinus pinea* the fatty acid content is close to a 50% (CARVALHO, 1996). This chemical composition might explain the effect of cryopreservation in this species, although no loss of viability has been found in other species with high lipid contents such as *Datura ferox, Datura stramonium, Onopordum nervosum* and *Halimium atriplicifolium* after cryopreservation (IRIONDO et al., 1992).

An integrated strategy must be used for conserving plant genetic diversity (FALK, 1990). Traditional seed banking (storage at low temperatures and seed moisture content) may be considered the most efficient method for the majority of species (GÓMEZ-CAMPO, 1985; HAWKES, 1990; CHIN, 1994). Nevertheless, for tree seeds, the results obtained in this study indicate that it can only be carried out for a limited period without a considerable loss of viability and vigour (BARNET and VOZZO, 1985; DONALD and JACOBS, 1990). When this period does not exceed the natural interval between germination and seed production for the next generation, other alternatives should be considered (BONNER, 1990). Cryopreservation may be an interesting alternative (AHUJA, 1986; JÖRGENSEN, 1990; RAO and RILEY, 1994; PITA et al., 1997), as the preservation period is not limited and costs are significantly reduced (STANWOOD and BASS, 1981).

Our results demonstrate that cryopreservation can be used for most Spanish pines. However in *Pinus pinea* further studies are necessary to determine the causes of the adverse effect of cryopreservation. A set of factors (seed moisture, seed size, chemical composition, etc.) must be considered. Nevertheless, seed oil content may be the main factor involved in the freezing damage. In this case, the cryopreservation protocol could be modified using a cooling rate under 200 °C $min^{-1}$, minimizing the damage in seeds with high lipid contents (VERTUCCI, 1989). In any case, a more complex cryopreservation protocol incurring higher costs may make this method unadvisable for the preservation of *P. pinea* seeds.

As suggested by AHUJA (1989), cryopreservation is a promising method for germplasm conservation of tree species. Seeds have been submitted to cryopreservation and no significant decreases in viability have been detected in *Abies alba, Fagus sylvatica, Larix decidua, Picea abies, Populus* spp. (AHUJA, 1986), *Ulmus pumila* (ENGSTROM, 1966), *Abies concolor* (STANWOOD and BASS, 1978), and even in some pines, such as *Pinus lambertiana, P. ponderosa* (STANWOOD and BASS, 1981), *P. sylvestris* (AHUJA, 1986; PITA et al., 1997), *P. echinata* (ENGSTROM, 1966), *P. ponderosa* (STANWOOD and BASS, 1978) and *P. nigra* (PITA et al., 1997).

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# A Strategy for the Third Breeding Cycle of Loblolly Pine in the Southeastern U.S.

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(Received 9th June 1998)

**Abstract**

A strategy for the North Carolina State University – Industry Cooperative Tree Improvement Program's third-cycle breeding for loblolly pine (*Pinus taeda* L.) was developed to provide genetic gain in the short-term as well as to maintain genetic diversity so that long-term genetic gains will also be possible. Our strategy will be to manage a hierarchy of three populations, each at a different level of intensity. The mainline population will consist of about 160 selections that are available to each cooperator in a given geographic region (i.e. recruitment population). These populations will be managed as subdivided breeding populations (40 sublines of 4 trees each) primarily to provide for long-term genetic gain and diversity. The most intensively selected and managed hierarchy will be the elite populations. A highly selected group of trees (approximately 40 selections) will be managed to provide short-term genetic gain for each member's program. A third hierarchy will be the genetic diversity archives managed to preserve and breed genotypes with extreme breeding values for individual traits (not necessarily for all traits combined) as an insurance population for environmental or selection criteria changes in future generations.

The improved efficiency of this breeding strategy along with the reduction in population sizes compared to the current program, will result in a substantial reduction in effort by individual cooperators. The increase in selection intensity from reducing the population sizes and the increased rate of breeding made possible by mating fewer trees will substantially increase gains in subsequent generations. While the most intensive effort will be devoted to those populations providing immediate genetic and financial gain, the long-term well-being of the genetic resource will be maintained by judicious management of all three hierarchies.

*Key words:* Elite breeding populations, genetic gain, mating designs, *Pinus taeda* L., testing designs.

*FDC:* 165.3; 165.41; 165.6; 232.13; 174.7 *Pinus taeda;* (756).

## Introduction

As the North Carolina State University – Industry Cooperative Tree Improvement Program progressed toward the third cycle of breeding, an efficient, cost-effective breeding strategy was developed to ensure both short- and long-term benefits for

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