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Cryopreservation of *Pinus sylvestris* seeds for three years*

Abstract

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Scots pine (*Pinus sylvestris*) seeds from three provenances, desiccated to 5% (±0.1-1.0%) of moisture content (fresh weight basis), were stored for three years at –196°C (liquid nitrogen) and –3°C (control). The germination capacity of seeds frozen and thawed from LN2 was about 90% (88-98%). After thawing of seeds there were no significant differences between germination capacity of seeds stored at –196°C and at –3°C. Period of storage (1 hour, 6 months, 12, 24 and 36 months) did not differentiate the germination capacity of seeds for any of the different provenances.

Additional key words: Scots pine, seeds storage, liquid nitrogen.

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INTRODUCTION

Among several *ex situ* methods of long-term conservation of genetic resources of threatened stands considered here is storage of seeds at a very low temperature. Such a possibility is provided by liquid nitrogen at a temperature of –196° (LN2). Since 1992 in the Institite of Dendrology in Kórnik a research work on cryopreservation of genetic resources of recalcitrant seeds has been conducted (Chmielarz 1997a). Cryopreservation of a highly desiccated (c. 10%) orthodox seeds in LN2 and their high resistance to such storage is presented in many publications (Stanwood 1985; Stanwood & Bass 1978, 1981; Stanwood & Ross 1979). But up to now it has not been sufficiently shown how storage of Scots pine seeds in liquid nitrogen affects germinability. Only some authors have briefly mentioned such storage, working on a very low number of seed samples (Ahuja 1986; Jörgensen 1990b).

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The aim of this paper is to investigate the possibility of storage of Scots pine seeds at the ultra low temperature of liquid nitrogen. Results of germination capacities of several thousands of seeds of *Pinus sylvestris* from three Polish provenances, stored several years in liquid nitrogen, is presented.

MATERIALS AND METHODS

Seeds were collected in 1992 from three provenances of north-west part of Poland: Miastko, Kalisz Pomorski and Świdwin. Seeds of each provenance were mixed from many trees. To estimate percent of full seeds, four replicates of 50 seeds were used. Seeds were cut with a scalpel and then the number of full seeds was counted. Seeds taken for the experiment were 98-100% full.

The moisture content (M.c.) of seeds was determined before and after freezing them in LN2. The M.c. is always presented in relation to fresh weight of seeds using a Thermo Control Infrared Dryer (120°C/20 min, ±0.1-1.0% – these measurements were calibrated with the help of the traditional oven method until weight became constant) for 100 seeds in three replicates. Before storage seeds were partially desiccated under silica gel at 20°C to a M. c. of 5%.

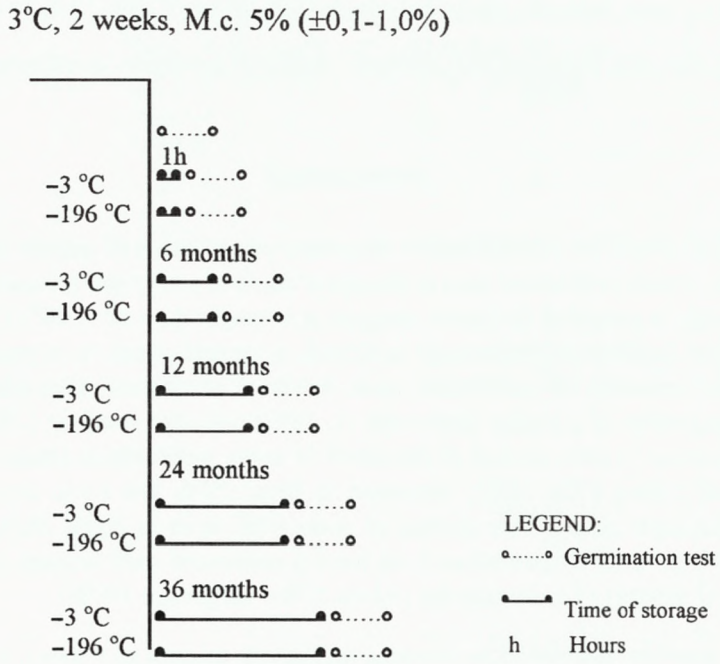

Fig. 1. *Pinus sylvestris* L. Scheme of storing seeds, desiccated to 5% of M.c. (±0,1-1,0%), at temperatures –3° and –196°C for three years

Germination of seeds was determined on Jacobsen apparatus at a temperature of 25°C in a 16/8 h light/darkness photoperiod, with a Photosynthetic photon flux density (PPFD), at the position where seeds were situated (under a bell jar) 20.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (LF 40 W, daylight, Polam, 380-680 nm). Seeds with an arched radicle at least 3 mm long were recorded as germinated. Germinating seeds with a radicle more than 5 mm long were taken off the Jacobsen apparatus and transferred to moist perlite, where they were grown four weeks (light and temperature as on the Jacobsen apparatus).

In each freezing variant seven replicates were used, each containing 40 seeds. Seeds were packed into plastic vials (1.8 ml, Nunc). The vials were welded tightly inside cryoflexes to prevent their explosion during thawing. Samples were frozen from a temperature of 20°C directly into liquid nitrogen and then stored (Fig. 1.), all the time immersed in LN2 (not in its vapour). The level of liquid nitrogen in a Dewars container WSN-100 (capacity of 100 litres) *Cryoson*, was controlled during storage automatically by NR2A-10 *Cryoson* (Liquid nitrogen level control and alarm unit). Loss of liquid nitrogen during storage from the storage container was supplemented every week from the NT-50/R container (capacity of 50 litres) also by the NR2A-10 *Cryoson* unit. Samples were thawed in a 40°C water bath for 15 minutes.

Seed samples stored at –3°C were packed and thawed in the same way.

Results of germination experiments, transformed from percentages to arcsine, have been subjected to variance analysis.

RESULTS

The germination capacity of seeds frozen and thawed from LN2 was about 90% (88-99%). The germination data for Scots pine seeds stored in liquid nitrogen for one hour, six months, 12 months, 24 and 36 months shows that there were no significant differences in germinabilities, maintained on the level of about 90%.

Storage at a temperature of –3°C was considered as the control to the storage at the temperature of liquid nitrogen –196°C. Several-year storage of desiccated seeds of Scots pine at –3°C is considered safe and does not reduce their germinability. Germination percent of seeds thawed from –3° after each period of storage (one hour, six months, 12, 24 and 36 months) was equal to the germination percent of thawed seeds which were being stored at –196°C.

Germination capacity of thawed seeds stayed on the same level for the three studied provenances – Miastko, Kalisz Pomorski, Świdwin as it was before treating them with LN2.

Seeds after storage in LN2 do not show a lower energy of germination. After two and three years of storage the energy of germination in the majority of germination tests was even higher, compared with seeds stored at –3°C (Fig. 2).

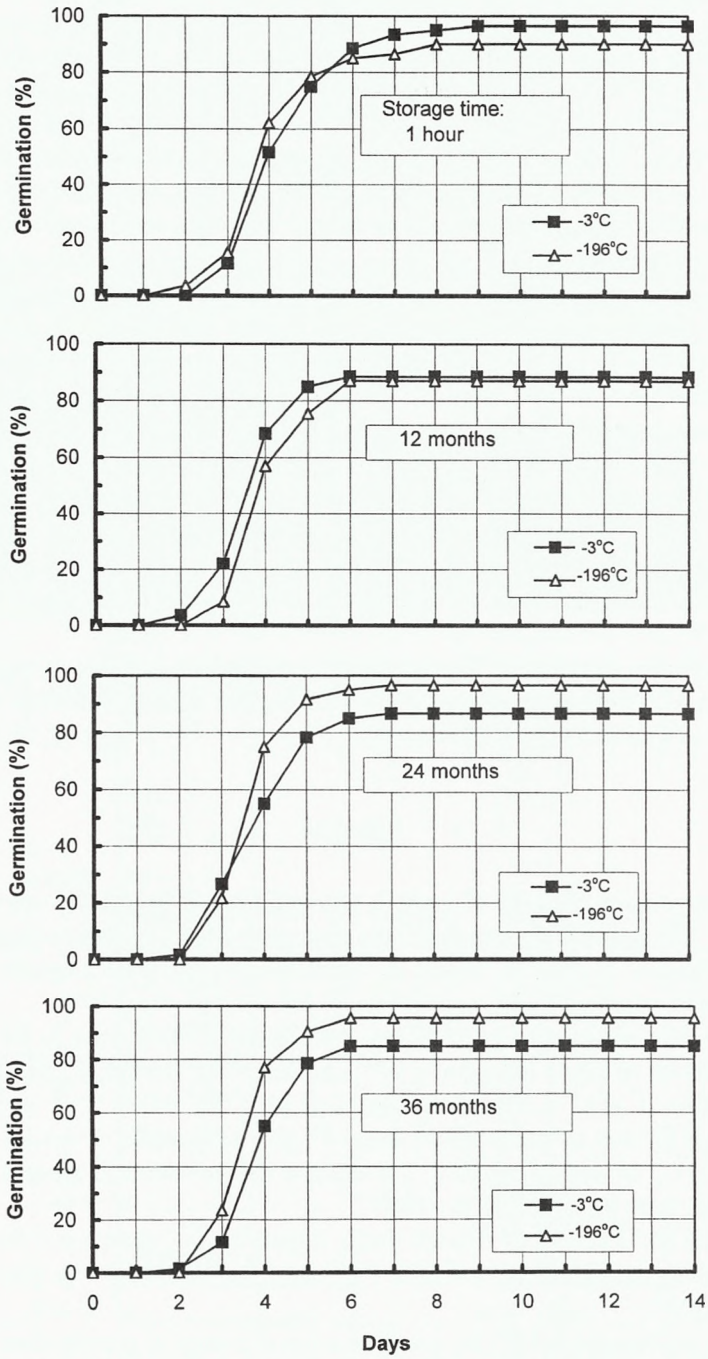

Fig. 2. *Pinus sylvestris* L. Course and attained germination capacity of seeds stored at –3° and –196°C (moisture content 5%, ± 0,1–1,0%), for one hour, 12 months, 24 months and 36 months (*Świdwin provenance*)

DISCUSSION

Seeds of three provenances of Scots pine have been desiccated to a M. c. of 5%, frozen in liquid nitrogen and thawed after up to three-year storage. Germination capacity of seeds after such storage was 88-98% and it did not differ from the germination capacity of non-stored seeds and of those stored for 3 years at a temperature of –3°C. After a short – one hour – storage and after a long half-year, one-year, two or three-year period of storage in LN2, the germination capacity was equally high. Therefore for the further cryopreservation study of sensitivity of partially desiccated orthodox seeds to LN2, a brief (1 hour) storage in LN2 is sufficient. Germination capacity, after one-hour and one-year storage of seeds in LN2, taken from five individual trees of one provenance, desiccated to the same level of moisture content (5%), in both cases of the storage also showed a high level of germination of seeds, above 90% (Chmielarz 1997b).

According to the Kartha (1985) classification of seeds with regard to exposure to LN2 temperatures, seeds of Scots pine can be classified in the first category: "desiccation-tolerant, LN2-tolerant".

Ahuja (1986) stored seeds of Scots pine at 0°C and in liquid nitrogen for six days. He has not observed any decrease in germinability of seeds in both variants of the experiment. Jörgensen (1990) confirmed, that seeds of Scots pine frozen directly in LN2 (moisture content of these seeds has not been evaluated) germinated after thawing on the same level or higher compared with control seeds (not frozen in LN2). In our experiments at the beginning of the germination test, a little higher germinability of seeds stored for two and three years in LN2 has been observed as well (Fig. 2). Such earlier germination during a germination test could be explained by the formation of micro-cracks on the surface of seeds frozen in LN2 and in consequence easier and faster imbibition of water throughout the seed coat.

Both mentioned authors have stored seeds of one provenance only for a very short time, only several days. In this work seeds from three different provenances were used and no significant differences were noticed.

All seeds used in the experiment were characterised by a high energy and a high germination capacity (first quality class). A higher germinability of seeds after storage in liquid nitrogen in comparison with germinability of seeds stored at –3°C could perhaps become evident after longer storage time if the quality of seeds were lower. This would require evidence from further studies.

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Kriokonserwacja nasion sosny zwyczajnej przez 3 lata w ciekłym azocie

Streszczenie

Nasiona sosny zwyczajnej *Pinus sylvestris* L. pobrane z wielu drzew trzech proweniencji: Miastko, Kalisz Pomorski, Świdwin, przechowywano przez trzy lata w temperaturze –196° (ciekły azot) oraz w –3°C (kontrola). Nasiona przed zamrożeniem w ciekłym azocie, podsuszono do wilgotności 5% (± 0,1-1,0% w stosunku do świeżej masy). Zdolność kiełkowania nasion przemrożonych w ciekłym azocie wynosiła 88-98%. Po rozmrożeniu nie obserwowano istotnych różnic w zdolności kiełkowania nasion, przechowywanych w temperaturze –196°C i –3°C. Czas przechowywania nasion (1 godzina, 6 miesięcy, 12, 24 i 36 miesięcy) nie różnicował zdolności kiełkowania nasion trzech badanych proweniencji sosny zwyczajnej.