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SEED VIABILITY OF LESS SPREAD INTRODUCED CONIFER PLANTS AFTER STORAGE IN LIQUID NITROGEN

Tolerance of seeds of plants from the family Cupressaceae (Cypress) Chamaecyparis lawsoniana (Lawson's cypress), Cryptomeria japonica (Japanese cypress), Platycladus orientalis (Oriental broadleaf) and Pinaceae (Pine) Larix kaempferi (Kaempfer's larch), Pseudotsuga menziesii (Menzies' pseudotsuga), Tsuga canadensis (Canadian hemlock), which according to the International Classification are less spread plants, to storage conditions in liquid nitrogen were investigated for the first time. The results of scientific research into the preservation of seeds in liquid nitrogen, as a source of genetic information, confirmed that the investigated seeds of plants introduced in Western Ukraine retained their viability after storage. Soil germination of cryopreserved seeds increased compared to the control variant on 0.7% for C. japonica, 7.4% for L. kaempferi or decreased in T. canadensis by 2%. The growth and development of the control seedlings do not differ from seedlings obtained from cryopreserved seeds.

Key words: *seeds, liquid nitrogen, cryopreservation, viability, conifers, gene pool, Cupressaceae, Pinaceae.*

Changes in the key parameters of climate negatively affect plant growth, development, and seed formation. Modern conservation concepts require shifting the focus toward preserving plant diversity *ex situ*, outside the influence of biotic and abiotic stress factors. An advanced and adequate method for the longterm preservation of plants as biological resources—allowing *ex situ* conservation of research material while maintaining its viability and genetic characteristics — is cryopreservation, *i.e.*, storage of biological objects in liquid nitrogen (–196 °C) [21, 24, 26].

Studies on the effects of low temperature storage on seed viability were initiated in the 19th century by the Swedish botanist A. P. de Candolle and the French biologist P. Becquerel [6].

Fundamental research on the possibility of storing both agricultural crop seeds [23, 15, 19, 22] and wild flora species [10, 9, 17] in liquid nitrogen, as well as various isolated seed structures and other plant organs [11, 16, 7, 29], began abroad and in Ukraine in the 20th century.

Seeds represent the predominant form of plant preservation [2, 3, 26, 27], as they contain the full spectrum of genetic hereditary material [4, 7, 12, 13, 22]. Seeds of introduced plant species are of particular interest, especially those whose natural populations are threatened with extinction. When introduced into new climatic conditions, such species develop adaptive geographic variability, which becomes fixed in their seeds. The formation of fully developed seeds in such plants — especially under altered environmental conditions compared to their natural habitats — allows their preservation as a seed material [1], the main suppliers of which are plants cultivated in botanical garden collections [5], which play a fundamental role in conserving plant diversity *ex situ* [25, 28].

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The cryopreservation has been tested on seeds of a wide range of plant groups [2, 3, 26, 27]. Longterm experimental data confirm the effectiveness of this method as an alternative for prolonged storage of research material without loss of its genetic characteristics [4, 7, 12, 13, 22]. Protocols optimized for each specific object ensure the preservation of both genetic and morphogenetic traits [18]. The key parameter of any storage protocol is the assessment of seed viability for each species after cryopreservation [20].

The objects of this study are coniferous plants, which occupy approximately 31% of the world's forested areas and are among the most significant carbon sinks. Although the ecological evolution of conifers has resulted in broad adaptive plasticity, many species remain vulnerable to rising ambient temperatures and reduced precipitation. The species investigated in this work, introduced into the Botanical Garden of the Ivan Franko National University of Lviv, have adapted to new photo and thermoperiodic conditions and produce viable seeds.

MATERIALS AND METHODS

Plant material. Seeds of coniferous species from the family *Cupressaceae — Chamaecyparis lawsoniana* (Lawson's cypress), *Cryptomeria japonica* (Japanese cedar), *Platycladus orientalis* (Oriental thuja) — and from the family *Pinaceae — Larix kaempferi* (Kaempfer's larch), *Pseudotsuga menziesii* (Douglas fir), and *Tsuga canadensis* (Canadian hemlock) — were collected in 2022. According to the Plant of the World Online (POWO) database, these species are classified as Near Threatened (NT), except for *L. kaempferi* and *P. menziesii*, which are listed as Least Concern (LC). Information on the natural distribution ranges and dates of first description was taken from POWO, while the introduction period in Ukraine was taken from Kohno (Table 1) [14].

The studied species originate from the temperate biome (POWO) in their natural habitats and are currently cultivated within the same geographical region.

Seed Storage in Liquid Nitrogen. Seeds of each species — 100 seeds per replicate for *L. kaempferi*, *Ch. lawsoniana*, *C. japonica*, *T. canadensis*, and *P. menziesii*, and 30 seeds per replicate for *P. orientalis* — were placed in paper envelopes and stored for one month in Dewar vessels filled with liquid nitrogen at the Center for Nanotechnologies and Low Temperature Research of the Ivan Franko National University of Lviv. Three replicates were used for each species.

Control seeds were stored under laboratory conditions at room temperature.

Rewarming. After storage in liquid nitrogen, the seeds were kept under laboratory conditions at a temperature of 23—25 °C for three days.

Table 1. **Characteristics of the introduced species**

Species name	Natural distribution range	Year of first species description	Year of introduction in Ukraine	Sites of cultivation of introduced species in Ukraine
Chamaecyparis lawsoniana	From southwestern Oregon to northern California	1864	1811	Botanical gardens and arboreta
Cryptomeria japonica	Japan	1838	second half of the 19th century	Botanical gardens and arboreta, especially in the western regions of ukraine
Larix kaempferi	Japan	1856	late 19th century	Botanical gardens
Platycladus orientalis	From the Russian Far East to east central China, and Korea	1949	1809	Ornamental species
Pseudotsuga menziesii	From Alaska to Mexico	1950	1827	Botanical gardens and arboreta
Tsuga canadensis	From eastern Canada to northern, central, and eastern United States	1855	1860	Botanical gardens and arboreta

Fig. 1. *Cryptomeria japonica* (Japanese cedar): *1* — seedlings from control seeds; *2* — seedlings from cryopreserved seeds

Fig. 2. *Platycladus orientalis* (Oriental thuja): *1* — seedlings from control seeds; *2* — seedlings from cryopreserved seeds

Fig. 3. *Larix kaempferi* (Kaempfer's larch): *1* — seedlings from control seeds; *2* — seedlings from cryopreserved seeds

Table 2. **The influence of liquid nitrogen temperature (–196 °C) on soil germination of seeds of introduced conifer species, %**

Species name	Control	Treatment (–196 °C)
Chamaecyparis lawsoniana (A. Murray bis) Parl.	2.7	3.7
Cryptomeria japonica (Thunb. ex L.f. D. Don)	16.3	17.0
Larix kaempferi (Lamb.) Carrière	27.3	34.7
Platycladus orientalis (L.) Franco	36.7	40.0
Pseudotsuga menziesii (Mirb.) Franco	1.7	3.0
Tsuga canadensis (L.) Carrière	4.7	2.7

Fig. 4. Five-month-old seedlings of *Platycladus orientalis* (Oriental thuja): *1* — seedlings from control seeds; *2* — seedlings from cryopreserved seeds

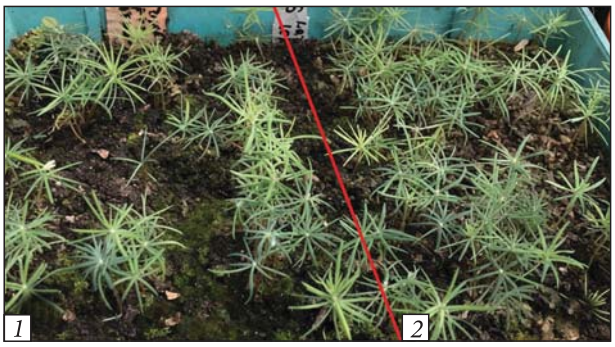

Fig. 5. Five-month-old seedlings of *Larix kaempferi* (Kaempfer's larch): *1* — seedlings from control seeds; *2* — seedlings from cryopreserved seeds

Fig. 6. Two-year-old seedlings of *Platycladus orientalis* (Oriental thuja): *1* — seedlings from control seeds; *2* — seedlings from cryopreserved seeds

Seed Viability. Seeds were sown into germination trays filled with soil and cultivated in a greenhouse. Final germination was determined by calculating the mean value of three replicates. The time of cotyledon emergence was recorded, and the growth and development of seedlings were monitored. At the beginning of summer, containers with seedlings were moved from the greenhouse to open ground, and during the winter period they were covered with conifer branches.

RESULTS AND DISCUSSION

For the first time, the effect of liquid nitrogen temperature (−196 °C) on the viability of freshly collected seeds of *Chamaecyparis lawsoniana*, *Cryptomeria japonica*, *Larix kaempferi*, *Platycladus orientalis*, *Pseudotsuga menziesii*, and *Tsuga canadensis*, introduced in Western Ukraine (Precarpathia), has been investigated. The obtained results indicate that the studied seeds are tolerant to liquid nitrogen temperatures. Seeds retained their viability after cryogenic storage. The influence of storage conditions on seed germination was species-specific (Table 2).

Seeds of *L. kaempferi*, *P. menziesii*, and *P. orientalis* germinated simultaneously in both the control and cryopreserved variants three weeks after sowing, while *Ch. lawsoniana* germinated after four weeks. *C. japonica* and *T. canadensis* belong to the group of species with prolonged germination; their seedlings appeared after six weeks.

Completion of germination (control and cryopreserved seeds) occurred as follows: *P. orientalis* — five weeks; *Ch. lawsoniana* — six weeks. Storage in liquid nitrogen accelerated final germination in *L. kaempferi*, which reached completion in five weeks compared to seven weeks in the control. In *C. japonica*, the germination period in the cryopreserved variant lasted up to seven weeks, whereas the control completed germination in five. For *T. canadensis*, final germination of cryopreserved seeds occurred after two months, while control seeds required three months.

The results of the analysis of liquid nitrogen temperature effects on seed viability show that the final percentage of germinated seeds after cryopreservation was higher than in the control for *Chamaecyparis lawsoniana*, *Cryptomeria japonica*, *Larix kaempferi*, *Platycladus orientalis*, and *Pseudotsuga menziesii*. Storage in liquid nitrogen increased the germination of *P. menziesii* seeds compared with the control (Table 2), although according to published data [2], the germination of cryopreserved seeds of this species was 55% compared with 61% in the control. In the present study, the germination of *T. canadensis* seeds in the control was higher than after cryopreservation. However, in earlier experiments conducted in 2017 with seeds of this species collected in the Botanical Garden, germination in the control was lower than in cryopreserved seeds [1].

Moreover, according to the published data, soil germination of *T. canadensis* seeds varies widely—from 30—55% [14] to as low as 3—5% [8]. Coniferous species are characterized by substantial intraspecific variability, which may explain the discrepancies in experimental data for some species.

The number of the first leaves of the seedling, *i. e.*, the cotyledons, is a species-specific trait. Seedlings of the studied species in both the control and cryopreserved variants exhibited the same number of cotyledons. *Pseudotsuga menziesii* seedlings had 5—7 triangular cotyledons, *C. japonica* had 3 cotyledons, *L. kaempferi* had 6, and *P. orientalis* had two linear-lanceolate cotyledons (Figs 1—3).

Analysis of the development of seedlings obtained from seeds stored in liquid nitrogen revealed no morphological differences compared with seedlings from the control variant (Figs 4, 5). The shoots developed leaves known as scales (*P. orien-talis*, *Ch. lawsoniana*) or needles in *C. japonica*, *L. kaempferi*, and *P. menziesii*. One-year-old seedlings overwintered successfully in open ground. Coniferous species differ in the rate of aboveground growth. *P. menziesii*, *L. kaempferi*, and *P. orientalis* belong to fastgrowing species.

On average, two-year-old seedlings reached the following heights: 25 mm in *T. canadensis*, 70 mm in *C. japonica*, 90 mm in *P. menziesii*, and 110 mm in *L. kaempferi*. In *P. orientalis*, spiral branch arrangement began to form in the second year, and seedlings reached an average height of 18.5 cm in both the control and cryopreserved groups. The growth and development of twoyearold seedlings derived from the seeds stored in liquid nitrogen did not differ morphologically from the control seedlings for any of the studied species (Fig. 6).

CONCLUSIONS

Cryogenic storage of seeds of introduced coniferous species from the Botanical Garden collection (*Chamaecyparis lawsoniana*, *Cryptomeria japonica*, *Larix kaempferi*, *Platycladus orientalis*, *Pseudotsuga menziesii*, *Tsuga canadensis*) was carried out for the first time.

The results of the study confirm the feasibility of cryopreserving seeds formed under the new climatic conditions of Western Ukraine. The experimental seeds did not require any special pretreatment before immersion in liquid nitrogen, and the

storage and rewarming protocols proved effective. Seeds retained their viability and morphogenetic potential after cryopreservation. The growth and development of seedlings derived from cryopreserved seeds did not differ from those of the control.

Based on the obtained experimental results, which are consistent with previous findings for herbaceous species from the Botanical Garden collection — where viability, germination, as well as genetic and biochemical characteristics were preserved after exposure to liquid nitrogen [1] — it can be concluded that cryopreservation is an effective method. Cryopreservation can be recommended as an alternative approach for preserving plant genetic information, complementing traditional cultivation in open ground.

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ЖИТТЄЗДАТНІСТЬ НАСІННЯ МАЛОПОШИРЕНИХ ІНТРОДУКОВАНИХ ШПИЛЬКОВИХ РОСЛИН ПІСЛЯ ЗБЕРІГАННЯ В РІДКОМУ АЗОТІ

Вперше досліджено толерантність насіння рослин родини Cupressaceae (кипарисові) — *Chamaecyparis lawsoniana* (кипарисовик Лавсона), *Cryptomeria japonica* (криптомерія японська), *Platycladus orientalis* (широкогілочник східний) та родини Pinaceae (соснові) — *Larix kaempferi* (модрина Кемпфера), *Pseudotsuga menziesii* (псевдотсуга Мензиса), *Tsuga canadensis* (тсуга канадська) до умов зберігання в рідкому азоті (–196°C). Згідно з міжнародною класифікацією, ці види перебувають під загрозою зникнення. Дослідження збереження насіння в рідкому азоті як джерела генетичної інформації показали, що насіння рослин, інтродукованих на Заході Україні, зберегло життєздатність після консервування в рідкому азоті. Встановлено, що ґрунтова схожість кріоконсервованого насіння порівняно з контролем підвищувалася на 0,7 % для *C. japonica*, на 7.4 % для *L. kaempferi* та знижувалася на 2,0 % у *T. canadensis*. Ріст і розвиток сіянців контрольного та дослідного варіантів не відрізнялися.

***Ключові слова*:** насіння, рідкий азот, кріозберігання, життєздатність, шпилькові, генофонд, *Cupressaceae, Pinaceae.*