Acta Biologica Hungarica 61(2), pp. 224–233 (2010)
DOI: 10.1556/ABiol.61.2010.2.10

CRYOPRESERVATION OF THE NON-DORMANT ORTHODOX SEEDS OF *ULMUS GLABRA*

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(Received: May 20, 2009; accepted: June 25, 2009)

In order to evaluate the feasibility of cryopreservation of Wych elm (*Ulmus glabra* Huds.) seeds, we evaluated the seeds sensitivity to extreme desiccation and/or the ultra-low temperature of liquid nitrogen (LN; –196 °C). We also determined the critical water content (WC) of desiccated seeds and the high-moisture freezing limit of seeds desiccated or moistened to various WCs and frozen for 24 h or up to two years in LN. Germination tests revealed no critical WC for seeds to 0.03 g H_2O g^{-1} dry mass, g g^{-1}. Seeds tolerated freezing in LN within safe ranges of WC 0.03–0.21 g g^{-1} (nuts). Seeds desiccated to the safe WC and stored in LN for two years had similar germination as seeds stored at –3 °C for two years. Therefore, long-term cryopreservation of *U. glabra* seeds in gene banks is feasible.

Keywords: Ulmus glabra – desiccation – liquid nitrogen – seed storage – water content

INTRODUCTION

Wych elm (*U. glabra* Huds. = *U. montana* With. = *U. scabra* Mill.) is native to most European countries. Elms are endangered species in Europe because of Dutch elm disease (DED) pandemics, which caused dramatic mortality in elm populations [8]. Therefore long-term *ex situ* conservation management should be ensured in selected populations. European elms (*Ulmus* spp.) genetic resources conservation strategies are developed through the European Forest Genetic Resources Programme (EUFORGEN) to protect genetic diversity of elms in Europe [8].

According to the recommendations of the Food and Agriculture Organization/ International Plant Genetics Resources Institute [9], *orthodox* seeds of trees should be stored at –18 °C after desiccation to 3–7% WC (0.03 – 0.08 g g^{-1}). *Ulmus glabra* seeds tolerate storage in tightly closed containers after desiccation [28]. Buszewicz and Holmes [6] found that seeds of this species can be dried for several days at room temperature to a moisture content (mc) of 9.4% (0.11 g g^{-1}) and maintain very high viability. Tylkowski [27] stored seeds of *U. glabra* dried to a mc of 10% (0.11 g g^{-1}) at –3 °C for two years and recorded high germination and seedling emergence.

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American elm seeds (*Ulmus americana*) had a germination rate of 33% after deep dehydration to 3% mc (0.03 g g^{-1}) and storage at room temperature for four years [1].

Maintaining high seed viability during long-term storage is facilitated by freezing in liquid nitrogen (LN; 29), and seed WC is one of the most important factors for successful cryopreservation. The high-moisture freezing limit for storage in LN (HMFL) is the seed WC threshold above which seed viability is reduced during storage in LN [20, 21].

Wych elm seeds dried to a mc of 10% (0.11 g g^{-1}) and stored in LN for 48 hours had reduced viability (by up to 20%) after thawing [14]. Elm seed (*U. carpinifolia*) with the mc ranges from 3–19% (0.03 – 0.23 g g^{-1}) stored at –75 °C did not differ significantly in longevity from seed kept at –13 °C [26]. When seeds were stored at this temperature with mc of 20 – 21% (0.25 – 0.27 g g^{-1}) their germination decreased to 0% [26]. In an effort to preserve the genetic resources of European elm species (*U. minor*, *U. laevis*, *U. glabra*) and their hybrids, dormant buds were cryopreserved in LN. After thawing, *in vitro* bud growth was restored in most of the 26 clones. Plantlets developed from the thawed explants and they continued to grow properly in the nursery [12].

The main hypothesis of these investigations was that seeds of Wych elm can be stored in LN without loss of viability. We assessed the sensitivity of seeds from Wych elm trees of Polish provenances to deep dehydration and the very low temperature of LN (–196 °C). Accordingly, we determined the safe and critical WC of severely dried seeds (sensitivity to deep dehydration) and determined the range of safe WC of seeds frozen in LN (for 24 hours). We also evaluated the influence of two-year storage in LN on seed germination and seedling emergence after thawing.

MATERIALS AND METHODS

Plant material

Seeds from two populations Kołobrzeg 1 (initial germination rate = 79%) and Kołobrzeg 2 (initial germination rate = 63%) from NW Poland were used. Seeds were collected when fully mature in the year 2000 and were dried to a WC of 0.10 – 0.15 g g^{-1}.

The word "seed/seeds" refers to the true seed with the pericarp (true seed + pericarp = nut), which is the germination unit for elm.

Adjusting seed water content

Before the experiments, seeds were dried or moistened to various WC levels. Depending on the target WC, seeds were dried over silica gel or moistened in a

tightly closed vessel and the sample weight was controlled. The required weight of seeds corresponding to the measured WC was calculated as follows [24]:

$$X = \frac{M \times (100 - Wp)}{100 - Wd},$$

where
X = final weight of seeds required to attain the target WC
M = fresh weight of moistened seeds
Wp = initial WC of seeds (before moistening)
Wd = target WC of seeds

To reach a higher seed WC (by moisturization), seeds were sprayed several times with water to a specified weight, and next left in tightly closed containers (conditioning) for 3 days at 3 °C, to even out the WC of the sample. Seed WC levels lower than 0.11 g g^{-1} were reached by slow desiccation of nuts above dry silica gel. Seed desiccation usually lasted one or several days. The seeds were placed in a box, on blotting paper (in a layer whose thickness did not exceed double seeds height).

Seeds were dried to a WC range of 0.03–0.25 g g^{-1}. Differences between individual seed WC levels exceeded 0.02 g g^{-1} in most cases. The WC of seeds (3 replications of 50 seeds each), was determined every time by drying them at 103 ± 2 °C for 17 hours. Seed water contents were expressed on a dry weight basis [as a g H_2O g^{-1} dry mass (dm); g g^{-1}].

Assessment of the safe range of seed WC (storage for 24 h)

To determine the safe range of seed WC for freezing in LN, seeds were dried or moistened to about a dozen levels of WC, placed in vials (*Nunc* 1.8 ml), and directly plunged into LN, where they were stored for 24 h (Kołobrzeg 1 provenance). In a similar way, control seeds were stored at –3 °C.

Seed storage for one-two years

Seeds (in nuts), tightly closed in vials, were placed in a 100-liter container (Cryoson, Germany). The vials were frozen by direct plunging into LN. The samples were stored in LN throughout the specified period. Control seeds were stored in a similar way at –3 °C. The level of LN in the freezer with the samples was controlled by the level control and alarm unit (Cryoson, NR2A-10), which signalled the minimum and maximum level of LN. Liquid nitrogen in the freezer with the stored material was automatically replenished.

After freezing in LN, the vials containing seeds were thawed in a water bath at 40 °C for 5 min. The seeds stored at –3 °C were thawed at room temperature.

Germination and seedling emergence tests

Germination tests were conducted in a Jacobsen germinator. The temperature was maintained at 23 °C for 22 hours and 27 °C for 2 hours per day and light was delivered in a 12-hour cycle (irradiance 22 μmol m^{-2} s^{-1} at the level of blotting paper) with illumination occurring during the period with the highest temperature every day. Germination tests lasted 14 days.

Seedling emergence tests were performed for seeds stored for 1 and 2 years. They were conducted in plastic boxes filled with a mixture of sand with peat (v/v, 1 : 1). From the time of sowing till the appearance of shoots, they were covered with a transparent lid, to ensure proper humidity and light. Seeds were not covered with sand. Optimum seedling emergence was ensured by a constant temperature of 20 °C, with illumination for 16 hours a day (irradiance 60 μmol m^{-2} s^{-1}). Seedling emergence tests lasted 14 days.

In addition to the germination and seedling emergence tests (after 14 days), we also conducted tests to determine the germination vigor and seedling emergence after 7 days (emergence vigor). Germination vigor was defined as the percentage of seeds that germinated by day 7 of the germination if the entire germination process lasted 14 days (storage for 24 h, provenance Kołobrzeg – 1) or as the percentage of seeds that germinated by day 4 of the germination test if the germination process lasted 8 days (storage for 1 and 2 years, provenance Kołobrzeg – 2). Emergence vigor after 7 days was defined as the percentage of seedlings with developed cotyledons recorded after half of the time necessary for complete shoot appearance (14 days).

Statistical analysis

For statistical analysis of the data, STATISTICA software was used [23]. To analyse the significance of differences between means, analysis of variance (ANOVA) was applied, and the Tukey test for pair-wise comparisons. The Tukey test was performed after arc-sin transformation, always at a significance level of $P < 0.05$. Separate ANOVAs and Tukey tests were performed for germination rates, germination vigor, and emergence vigor.

Significance of differences between individual values of germination, germination energy or seedling emergence, and mean values of those parameters, was marked with small letters of the alphabet (the values marked with the same letter are not significantly different at $P < 0.05$). If a table or figure presented results of several tests, they were marked with groups of letters from the beginning, middle and end of the alphabet, e.g. *a, b, c...* for the first test; *k, l, m* for the second test, and *s, t, u* for the third test.

In laboratory tests of germination, germination vigor, seedling emergence, emergence vigor each variant included four replications of 50 seeds.

RESULTS

Seeds of Wych elm tolerated drying to WC 0.03 g g^{-1} (the lowest tested WC level). Germination of such seeds were about 70%, i.e. similar to those for seeds dried to the safe WC 0.10 g g^{-1} (Fig. 1).

The safe range of seeds WC preserved in LN was 0.03 – 0.21% (Figs 1 and 2). This was indicated by both germination and germination vigor of seeds after freezing in LN. Germination of such seeds after freezing was about 75%, i.e. similar to that of seeds that had not been frozen in LN. Freezing of seeds with a higher WC caused a decrease in germination to 16% (Fig. 2).

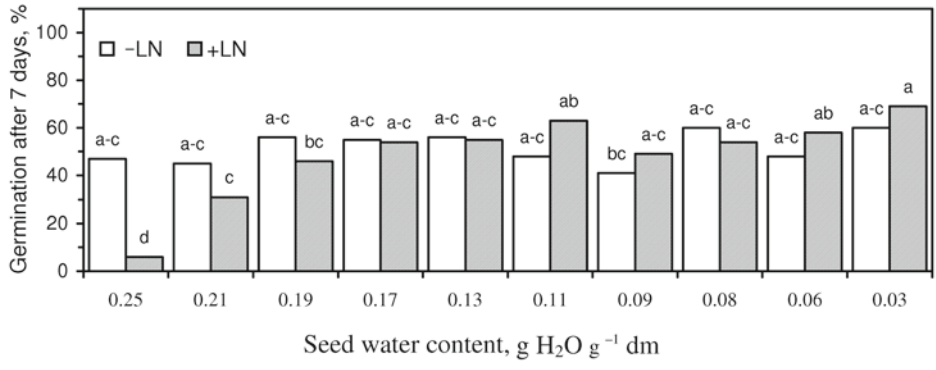

Fig. 1. Ulmus glabra. Germination after 7 days (vigor) for seeds dried or moisturized to 10 levels of water content (0.03 – 0.25 g g^{-1}), untreated (–LN) and treated (+LN) with liquid nitrogen, the values marked with the same letter are not significantly different at $P < 0.05$, Tukey test, provenance Kołobrzeg 1

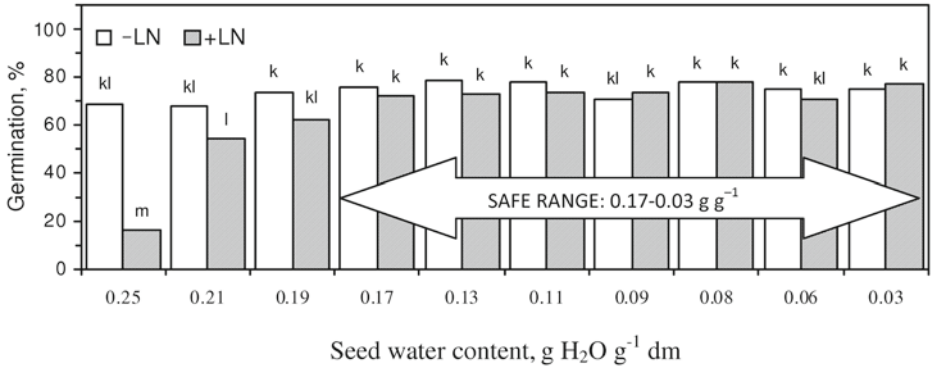

Fig. 2. Ulmus glabra. Germination (after 14 days) for seeds dried or moisturized to 10 levels of water content (0.03 – 0.25 g g^{-1}), untreated (–LN) and treated (+LN) with liquid nitrogen, the values marked with the same letter are not significantly different at $P < 0.05$, Tukey test, provenance Kołobrzeg 1

Wych elm seeds frozen in LN after drying to WC 0.08 g g^{-1} germinated with the same vigor as seeds that had not been frozen in LN. The final percentage of seeds germinated after 14 days of the germination test (Fig. 3) was similar for LN-treated and control seeds.

Storage for one–two years

No significant differences in seed germination after 1 and 2 years of storage were observed between seeds stored at –3 °C and –196 °C. This applies to germination and seedling emergence after 7 and 14 days (Table 1).

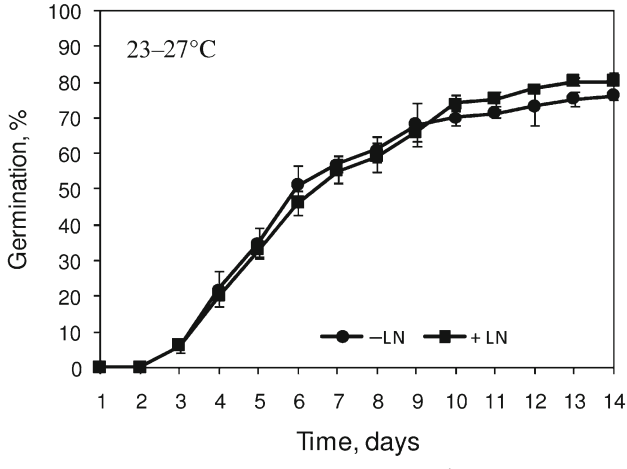

Fig. 3. Ulmus glabra. Germination curve for seeds (WC 0.08 g g^{-1}) untreated (–LN) and treated (+LN) with liquid nitrogen for 24 hours, provenance Kołobrzeg 1

Table 1
Germination and seedling emergence after storing nuts with a water content of 0.09 g H_2O g^{-1} dry mass at –3 °C or –196 °C for 1 or 2 years

	Germination percentage				Seedling emergence percentage			
	After 4 days		After 14 days		After 7 days		After 14 days	
Storage time	–3 °C	–196 °C	–3 °C	–196 °C	–3 °C	–196 °C	–3 °C	–196 °C
1 year	27a	32a	47l	50l	11p	9p	47w	47w
2 years	16b	25ab	84k	87k	24p	40o	98u	96u

Values marked with the same superscript letter do not differ significantly from one another at $P < 0.05$, Tukey test, provenance Kołobrzeg 2.

Initial germination before storage = 79%.

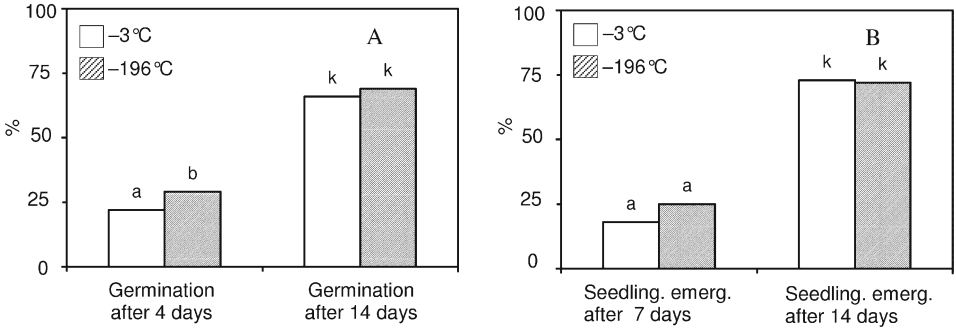

Fig. 4. Ulmus glabra. Effect of storage of seeds (WC 0.09 g g^{-1}) for 2 years at –3 °C or –196 °C on germination (after 14 days), germination vigor (after 4 days) – A and seedling emergence (after 14 days) and emergence vigor (after 7 days) – B. Values are means of results obtained after 1 and 2 years of storage. The values marked with the same letter are not significantly different at $P < 0.05$, Tukey test, provenance Kołobrzeg 2

Germination, germination vigor, seedling emergence and emergence vigor tests show that only germination vigor (mean for 1 and 2 years of storage, Fig. 4) depends on the temperature of seed storage: 22% at –3 °C and 29% at –196 °C. Any statistical differences were observed for other tests for seeds stored at –3 °C or –196 °C.

DISCUSSION

Wych elm seeds are non-dormant [25]. Cold stratification of dried seeds stored at 5 °C for two to three months increases the germination [17]. Cicek and Tilki [7] showed that two-year storage of Wych elm seeds at 4 °C increased the light requirement for optimum germination. After such storage, a greater percentage of seeds germinated if they were exposed to light for eight hours as compared to seeds kept in darkness at cycling temperatures of 30°/20 °C for 8/16 hours per day. More seeds germinated under cycling temperatures (30 °C/20 °C for 8/16 hours per day) than under a constant temperature of 20 °C. In addition, Tylkowski [27] found that seedling emergence for seeds sown onto the surface of the medium and in light was higher than for seeds placed in the substrate at a depth of 1 cm. In our study, seeds were sown onto the surface of the medium in light, which ensured a high seedling emergence.

This is the first report on the complete WC safe ranges for seeds of Wych elm exposed to the temperature of LN. The WC safe range for seeds of Wych elm was determined on the bases of germination and germination vigor, which are indicators of seed viability [11] or a loss of seed viability [2]. This study allowed us to detect a decrease in seed viability manifested by delayed or absent germination [2]. We found that the germination vigor of Wych elm seeds stored at –196 °C was higher than that of seeds stored at –3 °C; however, we detected no significant differences in germina-

tion for seeds stored at the different temperatures (–3 °C and –196 °C). The literature contains data about a positive effect of the temperature of LN on germination and germination vigor of *orthodox* seeds [19]. The faster and more vigorous germination of Wych elm seeds exposed to the temperature of LN could have resulted from microlesions of the seed coat caused by LN freezing and thawing. In contrast to Kuhn and co-workers [14], we did not observe that Wych elm seeds were intolerant to the temperature of LN within the safe WC range. Perhaps in Kuhn and co-workers [14], only seeds with a low initial viability were used, which could have affected their tolerance to freezing damage at –196 °C. Our data showing the tolerance of elm seeds to freezing at –196 °C open the door to successful cryopreservation of the genetic resources of this species in gene banks. Due to the widespread decline of elm trees in Europe due to Dutch elm disease [3, 4, 8, 10, 18] species cryopreservation is increasingly important. Similar to our results for elm seeds, Stanwood and Bass [22] did not detect any decrease in viability of American elm (*U. americana*) seeds subjected to cryopreservation. In a study on phase transitions in cell membranes of deeply dehydrated seeds of Siberian elm (*U. pumila*), membrane microviscosity was measured by diphenylhexatriene fluorescence anisotropy [30]. Deeply dehydrated seeds were characterized by mostly liquid crystalline membrane structures as compared to similarly dried seeds stored at –20 °C [30], which could indicate that deep dehydration does not eliminate the physiological activity of seeds to the same extent as deep dehydration plus low temperature. Results from our study support this hypothesis.

Despite deep dehydration (WC < 0.05 g g^{-1}), the cell membranes of o*rthodox* seeds maintain adequate physical stability [30] and membrane fluidity. Seeds of the species analyzed in this study were dehydrated to a WC of 0.02 – 0.03 g g^{-1}. According to Wang and co-workers [30], in thusly dehydrated seeds of *U. pumila*, carbohydrate molecules (mostly sucrose and stachyose) help to maintain the fluidity of cell membranes. In deeply dehydrated *orthodox* seeds, carbohydrate molecules (disaccharides and oligosaccharides) are located in the intercellular spaces and their presence protects membranes against damage during phase transitions from liquid crystalline into gel [15]. An additional factor protecting cell membranes against damage is the transition of the carbohydrate solutions located in intercellular spaces into an 'amorphous glass' [5]. This process is one of the major elements that enables cell survival during deep dehydration [5, 13]. In a study by Macherel and co-workers [16], mitochondria of seeds tolerant to severe desiccation were characterized by the accumulation of specific proteins (*heat-shock proteins*) and a special composition of phospholipids that protected mitochondria against effects of extreme temperature. Similar defense mechanisms probably protected the seeds analyzed in our study, which largely tolerated deep dehydration.

In conclusion sensitivity of Wych elm seeds to deep dehydration and ultra-low temperature were investigated. Results showed that Wych elm seeds tolerate very deep desiccation in nuts under silica gel, confirming that these are *orthodox* species. We identified, for the first time, a safe range of water content for seeds frozen 24 h in liquid nitrogen (–196 °C, LN), which allows seeds to survive such a storage.

Seeds of Wych elm were successfully stored for one and two years in liquid nitrogen, desiccated to the safe range of water content. Therefore, long-term cryopreservation of *U. glabra* seeds in gene banks is also possible using cryopreservation techniques.

ACKNOWLEDGEMENTS

We thank Mrs. Elżbieta Drzewiecka-Pieniężna and Anna Całka for providing technical assistance during the experiments in Kórnik. Partial financial support from the State Committee for Scientific Research, Poland, grant no. 5 P06H 057 18, is also gratefully acknowledged.

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