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# Cryopreservation of dormant *orthodox* seeds of European hornbeam (*Carpinus betulus*)

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## Summary

The sensitivity of dormant seeds of *Carpinus betulus* of Polish provenances to extreme desiccation and/or the ultra-low temperature of liquid nitrogen, LN (-196°C), was investigated. Germination and seedling emergence tests in this study did not reveal any critical seed water content (WC) for deeply desiccated seeds (nuts desiccated to 0.02 g $g^{1}$ (1.6% of fresh weight)). The seeds were tolerant to freezing (24 hours) in LN within a range of WC of 0.03–0.20 g $g^{-1}$ (3.2–16.5% of fresh weight). Two years storage in LN, after desiccation to a safe WC, did not decrease germination after thawing, in comparison to two years storage at -3°C. Storage of dormant seeds in LN showed that when seeds were stored after their dormancy was broken, germination after storage was lower because of the need for reducing seed WC to 0.08–0.11 g $g^{-1}$ (7.7–9.8% of fresh weight) after stratification, i.e. before storage. The results show the possibilities of long-term cryopreservation of European hornbeam seeds in forest gene banks.

## Introduction

*Carpinus betulus* L. (European or common hornbeam) is a hardwood species important to the economy and to ecology (Suszka *et al*., 1996). Mature seeds of this species tolerate desiccation, so they are classified as *orthodox* (Roberts, 1973; Suszka *et al*., 1996). The longevity of orthodox seeds depends on maintenance of a relatively low, constant water content (WC) throughout the storage period (Zasada and Densmore, 1977). European hornbeam seeds tolerate drying to nut WC 8-10% (0.09–0.11 g $g^{-1}$), at which they can be stored in tightly closed containers at 3°C for 14 months, and at -3°C for 6-10 years (Suszka *et al*., 1996). The seeds are characterized by physiological dormancy (endogenous dormancy of the embryo), which during maturation can be supplemented by lignification of the pericarp (Suszka, 1993).

Conventional storage of orthodox seeds in standard conditions for long periods can sometimes lead to a reduction in their germination. Similar decreases in viability have been observed in orthodox seeds of some plant species stored at -18°C for over 50 years in the National Center for Genetic Resources Preservation (Walters *et al*., 2004; Walters *et al*., 2005).

Long-term storage of orthodox seeds in gene banks can involve their cryopreservation in liquid nitrogen (LN) (Pence, 1991; Stanwood, 1985; Touchell and Dixon, 1993; Wang *et al.*, 1993). Walters *et al.*, (2004) extrapolated that seeds stored in nitrogen vapour would survive for 500 years, while those stored in LN would survive 3400 years. The beneficial influence of storage in nitrogen vapour on seed longevity was slightly less if they had previously been stored at 5°C.

Orthodox seeds cooled to extremely low temperatures, even at a low WC, are exposed to severe physical stress, so the reaction to the LN temperature should be determined for each species separately. If the seed structure has the potential to shrink or stretch during cooling or heating stress, then its physical structure will not be damaged. This rationale applies to most seeds that tolerate dehydration and the temperature of LN. In seeds of some species, the temperature of LN causes physical injuries that arise in the thawing stage (-196° to -150°C) (Stanwood, 1985).

There are few reports on the storage of European hornbeam seeds in LN. Kuhn *et al*. (1995) attempted cryopreservation of hornbeam seeds dried to WC 10% (0.11 g $g^{-1}$). After 48 hours storage in LN, seed viability (TTC – tetrazolium test) was not decreased, as compared to non-frozen seeds. As we have found for seeds frozen in LN in our earlier experiments (Chmielarz, unpublished data), results of seed viability after thawing, determined in a TTC test, differ from results of germination and emergence obtained in germination and emergence tests. It was proved for highly hydrated seeds of *Acer pseudoplatanus* and *Fagus sylvatica* stored in LN, that after thawing from LN 80-90% of such seeds were viable (TTC test) but none from such seeds germinated and emerged in laboratory tests (Chmielarz, unpublished data).

The aim of this study was to assess the sensitivity of European hornbeam seeds of Polish provenances to deep dehydration and the very low temperature of liquid nitrogen (-196°C). The research addressed determination of the safe, critical WC of dried seeds (sensitivity to deep dehydration) and determination of the range of safe WC of seeds frozen in LN (freezing for 24 hours). Another question addressed was the influence on seed germination and seedling emergence after thawing of two years of storage in LN (-196°C), with the use of dormant seeds and of seeds whose dormancy was broken (after stratification).

## Materials and methods

*Plant material*

Seeds of European hornbeam (*C. betulus* L.) from Godzieszów (near Jelenia Góra, SW Poland) and Kórnik (near Poznań, W Poland) were collected directly from the tree when ripe (wings turned yellow), in October 2000 and 2001. The seeds were dried in room temperature (20-23°C) for one week to WC 0.11 g $g^{-1}$ (9.8% of fresh weight, fw), placed in tightly closed containers and preliminarily stored at 3°C.

In the following we use the words “seed” or “seeds” for European hornbeam for the true seed with the pericarp attached (true seed + pericarp = nut), which is the germination unit for this species.

*Water content of seeds*

Depending on the planned WC, seeds with an initial WC of 0.11 g $g^{-1}$ (9.8% of fw) were dried over silica gel or moistened in a tightly closed vessel, and the seed sample weight was checked. The required weight of seeds, corresponding to the measured WC, was calculated using a previously described formula (Suszka, 1975).

To reach a higher seed WC, the seeds were sprayed several times with water to a specified weight and left in tightly closed containers (conditioning) for 5-7 days at 3°C to even out the WC of the whole seed. Seed WC levels lower than 0.11 g $g^{-1}$ (9.8% of fw) were reached by slow seed desiccation above activated silica gel. The duration of drying seeds to WC < 0.01 g $g^{-1}$ (1% of fw) took one to several days. The seeds were placed on blotting paper (in a layer with a thickness not exceeding double the seed size) in a box (25 cm long, 15 cm wide, 9 cm high) filled with activated silica gel (200 g). Differences between individual seed WC levels were around 0.01 g $g^{-1}$, within a WC range of 0.02–0.46 g $g^{-1}$ ± 0.01 g $g^{-1}$ (1.6–31.3% of fw) ± 1-2%.

The WC of seeds was assessed in 3 samples of 10 seeds each by drying them at 103 ± 2°C for 17 hours, until seeds had reached constant dry weight. Water contents were expressed on a dry weight basis (as a g $H_2O$/g dry mass, g $g^{-1}$). Moisture content on the basis of fresh weight is indicated in brackets.

*Drying of seeds after stratification*

Drying of stratified seeds, before storage for one or two years in LN or at -3°C, was conducted at room temperature at 20-23°C for 1-2 days to the previously (24 h storage) experimentally determined WC level within the desired safe range of WC. The safe range was determined on the basis of tolerance to the temperature of LN for 24 hours by seeds with 16 levels of WC (range 0.02–0.46 g $g^{-1}$ ± 0.01 g $g^{-1}$ (1.6–31.3% of fw ± 1-2%), provenance Kórnik).

*Cryopreservation*

*Cryostorage for 24 h*

Before cryostorage the seeds were placed in CryoFlex tubes (polyethylene, elastic cylinders ca. 1 cm in diameter, 5-15 cm long, sealed at both ends). Tubes were plunged directly into LN and were stored in a liquid nitrogen phase throughout the study period.

*Cryostorage for 2 years*

Seeds were stored and frozen in LN in the same way as seeds stored for 24 h. Control seeds for one and two years storage were stored in a similar way at -3°C ± 1.0°C. To assess the effect of storage for up to two years before and after dormancy breaking on seed germination capacity, the seeds were stored at the temperatures mentioned above before or after stratification.

*Thawing of samples after storage*

After cryostorage samples were thawed at 40°C in a water bath for 15 minutes. Samples stored at -3°C were thawed at room temperature as it is commonly applied for such storage.

*Stratification*

The substrate for seed stratification was a moist (Gordon and Rowe, 1982) mixture (v/v, 1:1) of quartz sand fraction < 1 mm with sieved peat of pH 3.5-4.5. Seeds mixed with the substrate at a ratio of 1:3 were placed in plastic containers (200 ml), and they were closed with a lid with several holes, which enabled gas exchange with ambient air and simultaneously protected the substrate with seeds against excessive drying. The condition of seeds and the substrate was checked every week during warm stratification at 20°C, and every 2-3 weeks during cold stratification at 3°C. When the first seedlings appeared the intervals between controls were reduced to one week. During the periodical checks, the water level of the stratification substrate was checked, and water was added by spraying, if necessary. In addition, the condition of seeds was observed (fungal infections, insect larvae), and the first germinated seeds were counted and removed (i.e., those with radicles 2-3 mm long, which are visible signs of dormancy release) if any appeared. Stratification of the seeds was conducted in a substrate, initially at 20°C for 4 weeks, and then at 3°C for 16 weeks, i.e., until the appearance of the first germination (Suszka *et al*., 1996).

*Germination and seedling emergence tests*

Seed germination tests were performed after stratification in darkness, in a similar substrate and bottles as those used for seed stratification. Optimum thermal conditions for seed germination were ensured by alternating temperatures of 3°/20°C (16 + 8 hours a day) (Suszka *et al*., 1996). Every week, germinated seeds were counted and removed and water in the substrate was replenished.

Seedling emergence tests were also conducted in a mixture of sand with peat, similar to that used for the stratification and germination tests. This time, stratified seeds were sown in plastic boxes, into the substrate at a depth of 1 cm, and covered with a layer of sand. To ensure suitable moisture, the boxes were covered with a transparent lid (allowing penetration of light to emerging seedlings), which was removed when seedlings reached ca. 2-3 cm in height. Seedling emergence tests, like germination tests, were conducted at alternating temperatures (3°/20°C, 16 + 8 hours a day), until seedlings reached ca. 2-3 cm in height. Boxes with seedlings were then moved to light (60 $\mu mol \cdot m^{-2}s^{-1}$ for 16 hours a day), in a chamber with a controlled temperature of 25°C.

*Applied nomenclature concerning seed evaluation and germination tests*

The term ‘cutting test’ in this work was used to denote seed viability evaluation (4 × 50 seeds) performed after collection (initial evaluation) and after the stratification and germination test (final evaluation). The seeds were cut along the longitudinal axis (with a secateur) and across the cotyledons and the embryo axis.

In the group of non-germinated seeds the following groups of seeds were distinguished: healthy, decayed, and empty seeds. The results are presented in %, as a mean of all replications, for each fraction separately. Healthy seeds had fleshy and shiny cotyledons and embryo axes and thus could be capable of further development into normal seedlings. Seeds that decayed partly or completely as a result of primary infection (i.e., originating from this seed) were classified as decayed seeds. Such seeds did not develop into seedlings. Empty seeds were distinguished by their containing less than 50% of seed tissue.

*Statistical analysis*
For statistical analysis of the data, STATISTICA software (StatSoft Polska, 1995-2005) was used. To analyse the significance of differences between means, analysis of variance (ANOVA) was applied, as was the Tukey test for pair-wise comparisons. The Tukey test was performed after arc-sine transformation, always at a significance level of $P < 0.05$. Separate ANOVAs and Tukey tests were performed for germination and emergence. All variables used in the experiments (time of seed storage, the way of seed storage, the seed storage temperature) were fixed. Three-way ANOVA was used for tables: time of storage (1 or 2 years), the way of seed storage (before or after stratification) and storage temperature (-3°C or -196°C). Two-way ANOVA was used for figures 1 and 2: the seed water content (16 levels of seed water content) and the seed treatment (- LN, + LN). One-way ANOVA was used for figure 4A (the seed storage: non-stratified or stratified and for figure 4B (the temperature of seed storage: -3°C or -196°C.

In laboratory tests of germination and emergence, each experimental treatment included 4 replications of 50 seeds each.

## Results

*Desiccation sensitivity of seeds*
European hornbeam seeds maintained high germination percentage and seedling emergence after desiccation to a very low WC 0.02 g $g^{-1}$ (1.6% of fw) (figures 1 and 2).

*Cryostorage for 24 hours*
Seeds stored for 24 h in LN tolerated storage within WC range of 0.03–0.20 g $g^{-1}$ (3.2–16.5% of fw) (the safe range of WC, figure 2) and germination percentage and seedling emergence were not reduced compared to the seeds not treated with LN for 24 h (control) (figures 1 and 2).

The application of warm–cold stratification in a substrate, with a 4-week warm stage at 20°C and a 16-week cold stage at 3°C, resulted in seed germination of 80-90% (figure 2).

No significant differences were observed in the germination curves of seeds with WC 0.10 g $g^{-1}$ (8.8% of fw) untreated (-LN) and treated (+LN) with LN for 24 hours (figure 3). All seeds germinated within four weeks. The seedling emergence curves, like germination curves, did not reveal any substantial differences between LN-treated and LN-untreated seeds (figure 3).

*Cryostorage for 2 years*
For seeds with WC 0.11 g $g^{-1}$ (9.8% of fw) stratified after storage for one and two years at -3°C or at -196°C germination ranged 42-68% and seedling emergence 18-44%. Two-year storage of stratified seeds resulted in a significant decrease in germination percentage (11-17%) and seedling emergence (5-12%) (tables 1 and 2). Mean values show that stored dormant seeds (nS, non-stratified) germinated at 56% and emerged at 26%, while seeds in which the dormancy was broken (S, stratified) before storage germinated at 16% and emerged at 10% (figure 4A).

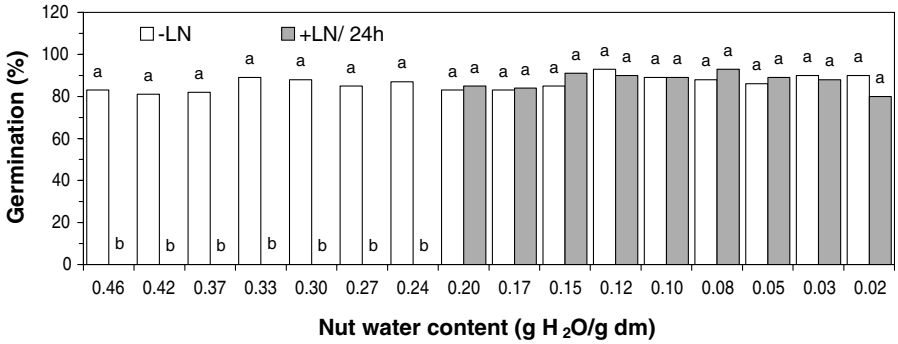


Figure 1. Germination of *Carpinus betulus* seeds (provenance Kórnik), after drying or hydrating to 16 levels of water content 0.02–0.46 g $g^{-1}$ (1.6–31.3% of fresh weight), untreated (-LN) and treated (+LN) for 24 h with liquid nitrogen. Non-germinated seeds were finally classified as dormant (1-6%, for various levels of water content), empty (1-1.5%), and decayed (all the others). The values marked with the same letter are not significantly different at $P < 0.05$, Tukey test.

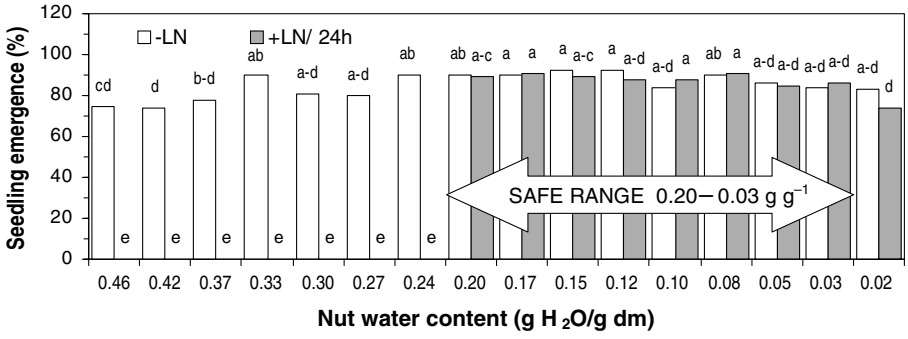


Figure 2. Seedling emergence of *Carpinus betulus* seeds (provenance Kórnik), after drying or hydrating to 16 levels of water content 0.02–0.46 g $g^{-1}$ (1.6–31.3% of fresh weight), untreated (-LN) and treated (+LN) for 24 h with liquid nitrogen. The values marked with the same letter are not significantly different at $P < 0.05$, Tukey test.

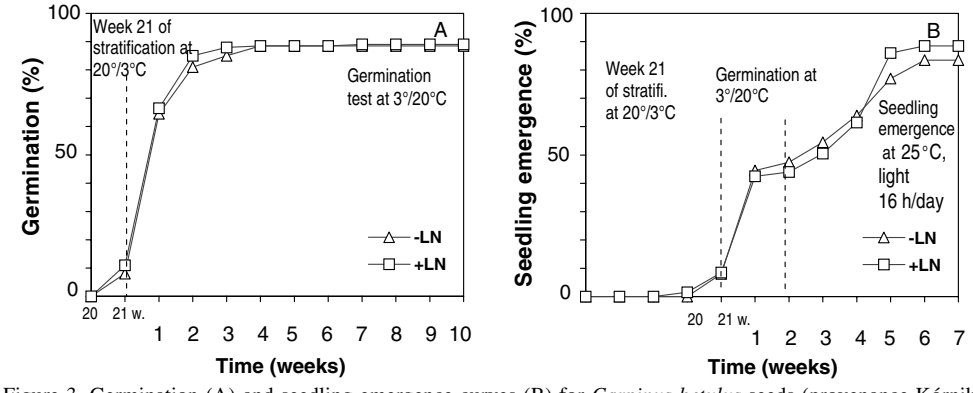


Figure 3. Germination (A) and seedling emergence curves (B) for *Carpinus betulus* seeds (provenance Kórnik) with WC 0.10 g $g^{-1}$ (8.8% of fresh weight) untreated (-LN) and treated (+LN) for 24 h with liquid nitrogen.

Table 1. Germination of *Carpinus betulus* seed (provenance Godzieszów), stored with WC 0.11 g $g^{-1}$ (9.8% of fresh weight) before dormancy release (non-stratified seeds, nS) and WC 0.08 g $g^{-1}$ (7.7% of fresh weight) after dormancy release (stratified seeds, S) for one or two years at -3°C (control) or -196°C. Values followed by the same letter are not significantly different at $P < 0.05$, Tukey test (only for germinated seeds).

| Germination (%) | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Storage → stratification (nS) | | | | | | | | | | | | | Stratification → storage (S) | | | | | | | | | | | |
| Not stored seeds | | | | Storage time | Seeds stored at | | | | | | | | Not stored seeds | | | | Not stored seeds. Desiccated after stratification** | | | | Storage time | Seeds stored at | | | | | | |
| | | | | | -3°C | | | | -196°C | | | | | | | | | | | | | -3°C | | | | -196°C | | |
| Germinated | Non-germinated* | | | | Germinated | Non-germinated | | | Germinated | Non-germinated | | | Germinated | Non-germinated | | | Germinated | Non-germinated | | | | Germinated | Non-germinated | | | Germinated | Non-germinated | | |
| | do | de | em | | | do | de | em | | do | de | em | | do | de | em | | do | de | em | | | do | de | em | | do | de | em |
| **62** ab | 22 | 8 | 8 | 1 Yr*** | **42** abc | 50 | 6 | 2 | **50** ab | 46 | 4 | 0 | **34** bcd | 55 | 7 | 4 | **16** cd | 69 | 7 | 8 | 1 Yr | **21** cd | 67 | 3 | 9 | **11** d | 66 | 19 | 4 |
| | | | | 2 Yr | **64** a | 29 | 6 | 1 | **68** a | 25 | 5 | 2 | | | | | | | | | 2 Yr | **12** d | 72 | 12 | 4 | **17** cd | 67 | 12 | 4 |

* Non-germinated seeds: dormant (do), decayed (de), empty (em)
** Stratified seeds desiccated before storage to WC 0.08 g $g^{-1}$ (7.7% of fresh weight)
*** Yr - years

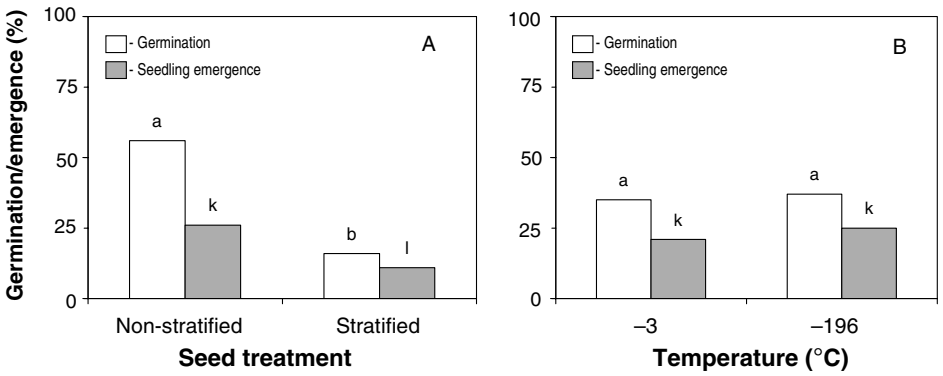


Figure 4. Germination and seedling emergence of non-stratified (nS) and stratified (S) *Carpinus betulus* seeds (provenance Godzieszów) stored for 1 or 2 years at -3°C (A) or -196°C (B). Values are means of results obtained after 1 and 2 years of storage. The values marked with the same letter are not significantly different at $P < 0.05$, Tukey test (2 separate ANOVAs and 4 Tukey tests).

Table 2. Seedling emergence of *Carpinus betulus* seed (provenance Godzieszów), stored with WC 0.11 g $g^{-1}$ (9.8% of fresh weight) before dormancy release (Non-stratified seeds, nS) and WC 0.08 g $g^{-1}$ (7.7% of fw) after dormancy release (stratified seeds, S) for 1 or 2 years at -3°C (control) or -196°C. The values marked with the same letter are not significantly different at $P < 0.05$, Tukey test.

| Seedling emergence (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Storage → stratification (nS) | | | | Stratification → storage (S) | | | | |
| Not stored seeds | Storage time | Seeds stored at | | Not stored seeds | Not stored seeds. Desiccated after stratification* | Storage time | Seeds stored at | |
| | | -3°C | -196°C | | | | -3°C | -196°C |
| 53 a | 1 Yr** | 44 ab | 41 ab | 28 abc | 24 bcd | 1 Yr | 12 cd | 5 d |
| | 2 Yr | 18 bcd | 28 abc | | | 2 Yr | 9 cd | 14 cd |

* Stratified seeds desiccated before storage to WC 0.08 g $g^{-1}$ (7.7% of fresh weight)
** Yr - years

After one year of storage of stratified seeds at -196°C, the seed germination was 11% comparing to 50% of germination for non-stratified seeds stored for the same period (table 1). Similarly, seedling emergence of stratified seeds stored in LN for one year was lower than for seeds that were stored as dormant and stratified after the storage (table 2). The proportion of non-germinated fresh (still dormant) seeds was higher in the seeds stored in LN after stratification (after one year of storage 67%, after two years 72%), as compared to seeds stored in LN without stratification, where the proportion of non-germinated healthy seeds was smaller and reached 50% and 29%, respectively (table 1).

No significant differences in germination and seedling emergence were observed between seeds stored for up to 2 years at -3°C and -196°C (figure 4B).

## Discussion

In this work possibilities of cryopreservation of European hornbeam seed as well as the effect of seed stratification after (Suszka *et al*., 1996; Tylkowski, 1995; Tylkowski, 1998) or before seed storage (rarely used) were studied. Seeds of European hornbeam desiccated to the WC 0.07-0.10 g $g^{-1}$ (6-9% of fw), like other deciduous forest tree species: *Tilia cordata* (Chmielarz, 2002), *Populus deltoides* (Pence, 1996) and coniferous species: *Pinus canariensis*, *P. halepensis*, *P. nigra*, *P. pinaster*, *P. uncinata* (Pita *et al.*, 1998), *P. sylvestris* (Chmielarz, 1998a; Pita *et al.*, 1998) and *Picea abies* (Chmielarz, 1998b) can be safely stored in LN. Although some orthodox seeds of forest tree species like *Prunus avium* (Chmielarz, 2009) and *Pinus pinea* (Pita *et al.*, 1998) despite of a relatively low level of WC in cryopreserved seed do not fully tolerate the temperature of LN.

Storing of stratified seed provides a material ready for sowing immediately after storage (Tylkowski, 1995). However, for storage of seed after stratification it is necessary to lower the high WC of seeds kept during stratification, to ca. 10% (0.11 g $g^{-1}$), at which seeds are usually stored. Such a reduction of seed WC can decrease seed germinability. Studies conducted by Bonner (1984) and Tylkowski (1995) showed that seed desiccation after stratification, conducted at room temperature, can contribute to a reduction of germination in some species subjected to this procedure. Nevertheless, Piotto (1997) indicated that seed desiccation in narrow-leafed ash (*Fraxinus angustifolia* Vahl.), even at room temperature, to WC 9.5% (0.10 g $g^{-1}$), did not significantly decrease the germination of seeds stored for 12 months at -3°C in tightly closed containers. Desiccation of stratified European hornbeam seeds at room temperature also did not decrease the germination of this species in a study of Suszka (1975). In the case of apple seeds, desiccation after stratification at a low temperature (5°C) and a high temperature (20°C) did not decrease the germination of seeds (Kamiński, 1974).

In our study, desiccation of European hornbeam seeds at 23°C, when they were at the final stage of cold stratification did not cause a significant decrease in their germination, but storage of such seeds decreased their germination in comparison with seeds stored before stratification (table 1).

Reduced germination percentage was observed in mature nondormant seeds of the herb *Amaranthus caudatus*, as a result of seed incubation at 45°C (seeds germinated easily at 25-35°C) due to secondary dormancy, which was partly caused by the seed coat (Kępczyński, 2002). In seeds of Japanese barberry (*Berberis thunbergii*), secondary dormancy of seeds was also initiated after a 5-day warm treatment at 25°C (Larsen and Eriksen, 2004). Tylkowski (2006) found that European hornbeam seeds were very sensitive to dehydration (to about 10%) after stratification. This sensitivity was reflected in a strong reduction of their germination and seedling emergence, as compared to un-dried seeds, and the reduction increased when their WC after desiccation decreased. Seedling emergence was stimulated by only a slight desiccation of seeds (to 16-22%) after stratification (Tylkowski, 2006). The current study also shows that the deeper the desiccation of hornbeam seeds to 0.08 and 0.11 g $g^{-1}$ (6.9% and 9.8% of fw), the lower the germination (20% and 50%, respectively). After the germination test, from 67% to 50% of seeds were healthy but non-germinated. The healthy seeds that did not germinate

probably developed secondary dormancy arising from seed desiccation after stratification at 23°C. However, it is unclear if the secondary dormancy of European hornbeam seeds was caused by the high temperature that for a short time affected the gradually dehydrated seeds, or only by the decrease in the WC of seeds after stratification (Suszka, 1967). Also insufficient stratification and primary dormancy in seeds could cause the high percentage of non-germinated healthy (fresh) seeds; 55% after stratification (seed not desiccated after stratification and not stored, table 1). Other experiments are needed for the general conclusion, examining more seedlots.

Secondary dormancy is most often induced in conditions that contrast with those breaking primary dormancy (Suszka, 1967), i.e., usually by a high temperature (Corbineau *et al*., 1993). Thus, if breaking of primary dormancy requires the application of long-term cold stratification, then drying of seeds at a high temperature can induce secondary dormancy, as in wild cherry seeds, where interruption of the cold stage by short-term warm stratification induced secondary dormancy (Suszka, 1975). Seeds of the Australian herb *Arctotheca calendula*, whose primary dormancy was broken at temperatures > 30°C, developed secondary dormancy at low temperatures when water potential in seed tissues was low, equal to -1.5 MPa (Ellery, 2002). Secondary dormancy may be endogenous, as in the case of the African plant *Striga hermonthica* (Gbehounou *et al.*, 2000). In spite of the similar reaction of seeds that develop primary and secondary dormancy (i.e., inhibition of germination in both cases), the two types of dormancy under the influence of temperature are regulated by different physiological processes, as observed in dormant seeds of remote sedge *Carex remota* (Brandel, 2005). In the current study, secondary dormancy was caused by external factors, probably by the high temperature of seed desiccation at the end of stratification. Jones *et al.* (1998) showed that secondary dormancy may also be induced during storage of desiccated seeds and not exclusively as a result of seed dehydration during the drying procedure. The research presented here did not reveal any deepening of secondary dormancy in European hornbeam seeds as a result of seed storage after desiccation and stratification.

The results of this study indicate that the temperature and time of storage of dormant European hornbeam seeds, within the safe range of WC for 1-2 years, undoubtedly less strongly affected their germination and emergence than did the time of stratification. If seeds were stored in LN after stratification, then it was necessary to dry seeds to a safe WC (storage in LN), but this approach was not favourable for seeds, as it could induce secondary dormancy. Lower germination was also observed in the case of similarly treated *Prunus avium* seeds stored after stratification (Chmielarz, 2009).

In conclusion, the limits of the safe range of seed WC for storage in LN were determined for the first time in European hornbeam: minimum 0.03 g $g^{-1}$ (3.2% of fw), maximum 0.20 g $g^{-1}$ (16.5% of fw). Within this range of WC, seeds tolerated the temperature of LN. This study also confirmed that properly desiccated seeds can be safely stored in LN for 2 years. Our results show how to optimize the storage conditions for long-term preservation of European hornbeam seeds in gene banks.

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