DENDROBIOLOGY
2002, vol. 47, Supplement: 71–77

Paweł Chmielarz

Sensitivity of *Tilia cordata* seeds to dehydration and temperature of liquid nitrogen

Abstract: The aim of the study was to assess the susceptibility of small-leaved lime (*Tilia cordata* Mill.) seeds to drying and freezing in liquid nitrogen (–196°C). Seed samples were frozen in liquid nitrogen for 24 h at 11 different levels of seed moisture content (m.c.), ranging from 3.1% to 22.8% (fresh weight basis). All samples, including unfrozen control samples, were subjected to scarification with concentrated sulphuric acid (Tylkowski 1998) either before or after freezing. Seed pre-treatment before germination (at 3~15°C/16~8h) involved cold stratification at 3°C without substrate. Seed drying to 3.1% m.c. significantly reduced their germinability (to 63%), as compared to the high germinability (82–88%) of seeds with 5.2–20.9% m.c. Thus seeds of this species can be assigned to the 'suborthodox' category. Such a high germinability (79–87%) was preserved after freezing in liquid nitrogen in samples dried to 9.0–17.4% m.c. if scarification was performed before freezing, and in samples dried to 9.1–16.2% m.c. if scarification was performed after freezing. The highest percentage of seedlings emerged after freezing in liquid nitrogen from seeds dried to 11.1–20.1% m.c. (emergence 65–75%) if scarification was performed before freezing, and from seeds dried to 7.3–17.8% m.c. (emergence 53%–71%) if scarification was performed after freezing.

Additional key words: small-leaved lime, cryopreservation, seeds, drying, scarification, liquid nitrogen

Address: P. Chmielarz, Polish Academy of Sciences, Institute of Dendrology, Parkowa 5, 62-035 Kórnik, Poland, e-mail: pach@rose.man.poznan.pl

Introduction

The unfavourable effect of human activity on forest habitats leads to a reduction in biodiversity in forest ecosystems. One of the methods to preserve the gene resources of tree species is storage of their seeds in gene banks. More and more often the seeds are preserved in liquid nitrogen (LN) at –196°C, and this process is termed cryopreservation. This is usually the only possibility of preserving the gene pool of species whose seeds are sensitive to dehydration, classified as *recalcitrant*. Cryopreservation is also one of the safest methods of long-term storage (Ashwood-Smith and Grant 1977) of seeds resistant to dehydration, classified as *orthodox*. For seeds of both categories it was found that the colder the storage of biological material within the safe moisture range, the longer the potential storage time without reducing seed viability (Roberts 1972, Harrington 1972). For the temperature of –196°C, the potential storage time is virtually unlimited (Ashwood-Smith and Grant 1977).

Not all seeds that are resistant to drying in open air at room temperature (usually to 9–12% of moisture content) tolerate further dehydration with the use of a drying factor. Often the air-dried seeds, initially classified as *orthodox*, loose the ability to germinate after drying to less than 7,5 or 3% of moisture content (m.c.). This is the case in *Fagus sylvatica* L., which is now assigned to the intermediate category between *orthodox* and *recalcitrant* seeds (Chmielarz 2000). A decreased germinability of seeds of this species was observed after drying to about 5% m.c. Already Suszka (1966) observed their reduced germinability

after drying to 5.7% m.c. This means that only after drying to a very low moisture content the sensitivity of seeds to desiccation can be precisely determined, which was the major aim of this study concerning seeds of the small-leaved lime, *Tilia cordata* Mill.

Some *orthodox* and intermediate seeds, which are tolerant to dehydration, are sensitive to freezing in LN, e.g. the wild cherry, *Prunus avium* (L.) L., and hazel, *Corylus avellana* L. (Kartha 1985). So far, the sensitivity of *T. cordata* seeds to strong dehydration and freezing in LN has been assessed only for two levels of moisture content: 10% and 20% fresh weight basis (Tylkowski 1998). Thus the safe moisture range for cryopreservation of seeds of this species is still unknown. To form the basis for long-term storage of *T. cordata* seeds, this study aimed also to determine their sensitivity to freezing in LN in a wide range of seed moisture content. Additionally, we compared the influence of LN treatment and scarification on dormancy breakage in seeds of this tree species.

Material and methods

Plant material

Fruits of *T. cordata* were collected in November 2000 from several trees in Puszczykowo near Poznań (mid-western Poland). Pericarps were removed manually by rubbing between two pieces of rubber (Tylkowski 1998). Next, the seeds were cleaned by winnowing and dried to a safe (Suszka 1994) level of 10.8% m.c. (fresh weight basis). Finally, they were placed in sealed plastic bags (VAC 37, polyamide + polyethylene, PA/PE HB, 100 mm thick). Until the beginning of the experiment they were stored at 3°C for 3 weeks.

Wetting/drying of seeds

To reach 11 levels of moisture content, ranging from 1% to 21%, seeds were dried at room temperature above silica gel or wetted in tightly closed vessels, and seed weight was controlled. The required seed weight (S_2), reflecting the needed moisture content (C_2), was calculated from the formula: $S_2=S_1 \quad (100–C_1)/(100–C_2)$, where C_1 is the current moisture content (in %) and S_1 is the current seed weight (Suszka 1975, symbols modified). Seeds were dried above silica gel in a desiccator for 1–7 days. The lowest moisture variant of 1% could not be reached despite drying for 4 weeks. Higher moisture levels were reached by spraying the seeds with water several times to the required weight, followed by incubation in tightly closed vessels for 7 days at 3°C to ensure an even distribution of moisture within the seed sample. The moisture content of seeds was assessed each time by the oven method (105°C for 17 h) for three replications of 15 seeds each. The planned and actual levels of seed moisture content are presented in Tables 1 and 2.

Freezing in LN (–196°C)

Seeds were placed in plastic vials (Nunc), 50 seeds per vial. Three replications of 50 seeds each were used for each of the planned 11 levels of moisture content. The vials with seeds were immersed in LN for 24 h, and then thawed in a water bath at 41°C for 15 minutes. Seeds were scarified either before or after freezing (Figs 1a and 2a). Finally, the seeds were subjected to stratification and both the germination test and seedling emergence test. Control seeds were not frozen, but only dried to the 11 levels of moisture content, scarified before or after drying/wetting, and later subjected to stratification and the germination test (Figs 1b and 2b).

Scarification and stratification

The dormancy of *Tilia* seeds is called paradormancy by Lang et al. (1987). It is due to the presence of the endosperm and the mechanical barrier formed by the hard seed coat, as isolated embryos are not dormant (Tylkowski pers. comm.). The dormancy of seeds was broken by scarification and stratification.

Scarification consisted in soaking the seeds in concentrated 96% sulphuric acid (v/v proportion of seeds

Table 1. *Tilia cordata* Mill. Planned and actual moisture content (m.c.) of seeds frozen after scarification

Drying/wetting of seeds (m.c. after scarification: 9.9%)			
Dried seeds		Wetted seeds	
planned m.c. (%)	actual m.c. (%)	planned m.c. (%)	actual m.c. (%)
9,0	9.0	11.0	11.1
7,0	7.2	13.0	13.2
5,0	5.2	15.0	15.3
3,0	3.1	17.0	17.4
1,0	3.1*	19.0	20.1
		21.0	20.9

*Despite prolonged drying of seeds (4 weeks) the planned level of 1% was not reached.

Table 2. *Tilia cordata* Mill. Planned and actual moisture content of seeds frozen before scarification

Drying/wetting of seeds (m.c. after inial storage 10.8%)			
Dried seeds		Wetted seeds	
planned m.c. (%)	actual m.c. (%)	planned m.c. (%)	actual m.c. (%)
9.0	9.1	11.0	11.3
7.0	7.3	13.0	13.5
5.0	5.5	15.0	16.2
3.0	3.4	17.0	17.8
1.0	3.4*	19.0	20.1
		21.0	22.8

*Despite prolonged drying of seeds (4 weeks) the planned level of 1% was not reached.

to acid 1:2) for 12 minutes (Tylkowski 1994). During scarification the temperature of acid was controlled, so that it did not exceed 27°C. After scarification the seeds were rinsed in tap water for 10 minutes. Scarification was performed either before or after freezing. If before freezing, seeds were scarified at 10.8% m.c., and later rinsed (5 minutes) and dried for 20 h at room temperature to 9.9% m.c. From this level the seeds were dried or wetted to 11 levels of moisture content as described above, and then subjected to LN treatment (Fig. 1a). If scarification was performed after freezing, seeds were scarified at 11 levels of moisture content (Fig. 2a).

Stratification was conducted at 3°C for 18–21 weeks, without substrate. To replenish the water lost during stratification, the seeds were soaked in water for 1 hour every 7 days (Tylkowski 1998).

Effects of freezing in LN and scarification on seed dormancy breakage, were additionally analysed by comparing germinability and seedling emergence in four variants:
(a) seeds not scarified, frozen in LN (-acid +LN);
(b) seeds not scarified, not frozen in LN (-acid -LN);
(c) seeds scarified, frozen in LN (+acid +LN);
(d) seeds scarified, not frozen in LN (+acid –LN).

Germination tests and seedling emergence tests

A signal for starting germination tests was the appearance of the first germinating seeds (3–5% germinated) during stratification. Germination tests were carried out in 200-ml plastic bottles filled with a mixture of moist sand and acid peat (1:1) (Gordon and Rowe 1982) at 3∼15°C (Tylkowski 1998) for 10 weeks, until the complete cessation of germination. Seedling emergence was observed after sowing seeds in boxes filled with a mixture of sand and peat (3 replications of 30 seeds each). The seeds were covered with a 1-cm layer of sand. The boxes were kept at 3∼15°C (Tylkowski 1998) for 10 weeks. Initially the boxes were covered with transparent lids to maintain a higher humidity level in the substrate. After emergence the lids were removed to enable the seedlings to grow freely. After the emergence test, the cutting test was used for assessing the viability the remaining seeds.

Statistical analysis

Results of germination tests and emergence tests were subjected to an analysis of variance and the Newman-Keuls test. Differences were regarded as significant at p 0.05.

Results

Scarification and drying/wetting

Unfrozen seeds scarified at 10.8% m.c. (Fig. 1b) before drying or wetting to 3.1–20.9% m.c., germinated at the highest level (82–88%) at 5.2–20.9% m.c. (Table 3). Seeds of a m.c. of 3.1% had a significantly lower germinability (63–66%). By contrast, the seeds scarified after drying or wetting to 11 levels of moisture content, ranging from 3.4% to 22.8%, were characterized by the highest germinability (80–89%) only at 7.3–16.2% m.c. (Table 3).

Scarification, drying/wetting and freezing in LN

Analysis of variance showed that the timing of scarification (before or after LN treatment) has a significant effect on seed germinability (Table 4). After scarification and freezing in LN, seeds germinated at the highest level (79–88%) at 9.0–17.4% m.c. The high moisture freezing limit (HMFL) of seeds frozen after scarification differed from that of seeds frozen before scarification. In the latter variant, it amounted to 16.2% (Table 5). The lowering of HMFL from 17.4% to 16.2% was due to the high moisture content during scarification preceding the LN treatment (Tables 3 and 5).

No interaction between freezing and scarification (before or after LN) was found (Table 4). A crucial role was played here by the moisture content of seeds

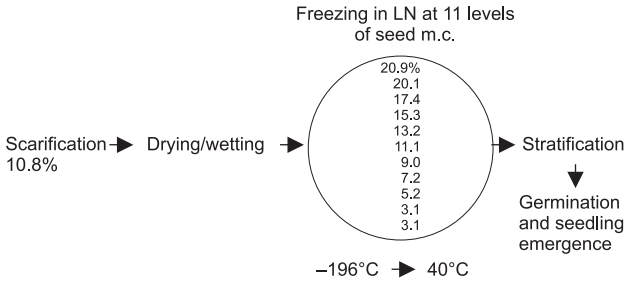

Fig. 1a. *Tilia cordata* Mill. Treatment of seeds scarified at 10.8% of moisture content and frozen in liquid nitrogen (LN) at various levels of moisture content (m.c.)

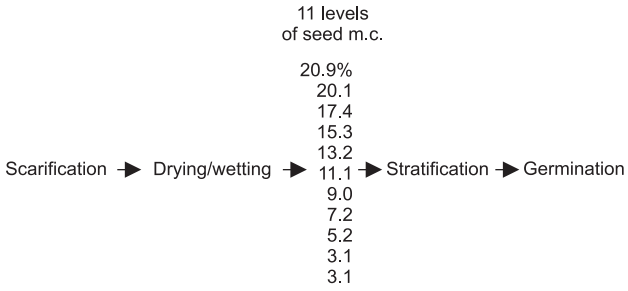

Fig. 1b. *Tilia cordata* Mill. Treatment of unfrozen seeds scarified at 10.8% of moisture content (m.c.)

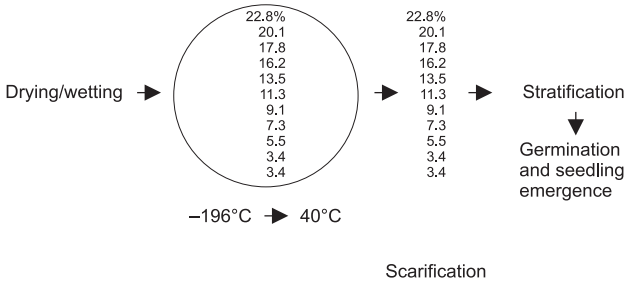

Fig. 2a. *Tilia cordata* Mill. Treatment od seeds frozen in liquid nitrogen and scarified at 11 levels of moisture content (m.c.). Initial moisture content of seeds: 10.8%

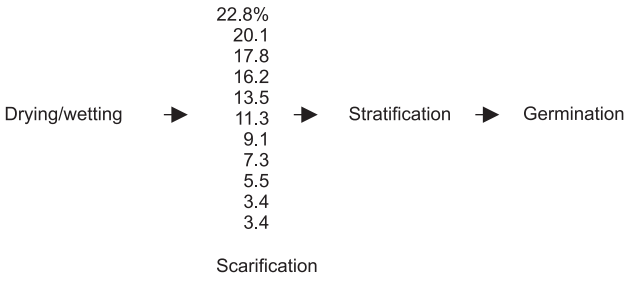

Fig. 2b. *Tilia cordata* Mill. Treatment of unfrozen seeds scarified at 11 levels of moisture content (m.c.). Initial moisture content of seeds: 10.8%

during scarification and freezing, rather than by timing of scarification. Freezing of seeds below their lower limit of safe moisture range, i.e. 3.1% (scarification before LN) or 3.4% (scarification after LN), and above the HMFL, i.e. 20.9% (scarification before LN) or 17.8% (scarification after LN), significantly decreased their germinability (Table 3).

The interaction between seed moisture content and LN treatment was significant (Table 4). Unfrozen seeds were characterized by the highest germinability at 5.2–20.9% m.c., while frozen seeds only at 9.0–17.4% (scarification at 10.8% m.c., Tables 3 and 5).

A strong dehydration of seeds (to 5.2% and 7.2% m.c.) did not affect the germinability of unfrozen seeds but decreased the germinability of frozen seeds (Tables 3 and 5). A similar situation was observed at 7.3% m.c. if seeds were frozen before scarification.

Results of the germination test and seedling emergence are not consistent. The safe moisture range assessed on the basis of seedling emergence is 11.1–20.1% m.c. for seeds frozen after scarification, and 7.3–17.8% m.c. for seeds frozen before scarification (Table 5). A significant decrease in seedling emergence was recorded at the upper limit of the studied moisture range, at 20.9% m.c. when LN was applied after scarification and at 17.8% m.c. and higher levels when LN was applied before scarification. During seedling emergence tests for seeds dried to 3.1% and 5.2% m.c., some abnormal seedlings were observed (marked with asterisks in Table 5). Those seedlings had well-developed roots but their cotyledons were enclosed in the seed coat until the end of observations (10 weeks). After a short period of growth their roots died.

As a result of the conducted experiments we determined the safe range of seed moisture content allowing cryopreservation of *T. cordata* seeds without significant loss of germinability (Tables 3 and 5). The upper and lower limits of this range depend on seed moisture content both during scarification (interaction 1 2, Tables 4 and 6) and during LN treatment (interaction 2 3, Table 4). The limits do not depend on whether stratification is performed before or after freezing (lack of interaction 1 3, Table 4).

Comparison of effects of freezing and scarification on dormancy breakage

The germinability of unscarified seeds was generally the same irrespective of whether the seeds were earlier frozen in LN or not (Table 7). This indicates that freezing in LN does not exert any influence of dormancy breakage in *T. cordata* seeds (Table 7).

Table 3. *Tilia cordata* Mill. Germinability of unfrozen seeds dried/wetted after or before scarification to 11 levels of moisture content (m.c.). In the first variant seeds were scarified at 10.8% m.c., while in the second variant seeds were scarified at 11 levels of m.c.

M.c. of seeds dried/wetted after scarification %	Germinability %	M.c. of seeds dried/wetted before scarification %	Germinability %
20.9	88 ab	22.8	58 prst
20.1	82 abcdefgh	20.1	61 oprs
17.4	83 abcdefg	17.8	76 efghijkl
15.3	85 abcde	16.2	83 abcdefgh
13.2	83 abcdefg	13.5	89 a
11.1	85 abcdef	11.3	85 abcde
9.0	88 ab	9.1	82 abcdefgh
7.2	87 abcd	7.3	80 abcdefghi
5.2	86 abcde	5.5	73 hijklmn
3.1	63 nopr	3.4	67 ijklmnop
3.1	66 klmnop	3.4	67 ijklmnop

Table 4. Analysis of variance for germinability data from Tables 3 and 5. Variables: 1 = timing of scarification (before or after freezing); 2 = moisture content; 3 = freezing in liquid nitrogen

Variable	Degrees of freedom	Mean square	Degrees of freedom for error	Mean square for error	F (Snedecor's coefficient)	Significance
1	1*	798.1553*	88*	6.422386*	124.2771*	0.000000*
2	10*	365.4073*	88*	6.422386*	56.8959*	0.000000*
3	1*	342.9974*	88*	6.422386*	53.4065*	0.000000*
1 2	10*	88.6526*	88*	6.422386*	13.8037*	0.000000*
1 3	1	1.6943	88	6.422386	0.2638	0.608803
2 3	10*	30.4647*	88*	6.422386*	4.7435*	0.000020*
1 2 3	10*	35.6325*	88*	6.422386*	5.5482*	0.000002*

*significant at p 0.05

Table 5. *Tilia cordata* Mill. Germinability and seedling emergence after freezing of seeds in liquid nitrogen (after or before scarification). Before freezing the seeds were dried to various levels of moisture content (m.c.)

M.c. of seeds (%)	Freezing after scarification		M.c. of seeds (%)	Freezing before scarification	
	germinability (%)	seedling emergence (%)		germinability (%)	seedling emergence (%)
20.9	66 klmnop	19	22.8	52 st	35
20.1	78 defghij	75	20.1	51 t	29
17.4	84 abcdefg	75	17.8	65 lmnop	57
15.3	87 abc	75	16.2	79 abcdefghi	67
13.2	79 bcdefghi	67	13.5	76 (?)defghijk	71
11.1	88 ab	65	11.3	85 abcdef	53
9.0	83 abcdefg	45	9.1	81 abcdefghi	61
7.2	78 cdefghi	49	7.3	78 cdefghi	62
5.2	74 ghijklm	2 (+5*)	5.5	75 fghijkl	7 (+16*)
3.1	63 mnopr	35	3.4	54 rst	33
3.1	71 ijklmno	32 (+31*)	3.4	67 ijklmnop	6 (+41*)

Values marked with the same letters (separately for germinability and seedling emergence) are not significantly different at p = 0.05

*percentage of seeds that developed branched roots but never developed any shoots (the seedlings died several weeks after germination)

(?) values for replications for this variant were very different: 64%, 78% and 86%, so the extremely low first value affected the mean of 76% and statistical differences between this and other variants

Table 6. Analysis of variance for data on seedling emergence from Table 5 (seeds frozen in liquid nitrogen). Variables: 1 = timing of scarification (before or after freezing); 2 = moisture content

Variable	Degrees of freedom	Mean square	Degrees of freedom for error	Mean square for error	F (Snedecor's coefficient)	Significance
1	1	0.079	44	26.59759	0.00295	0.956900
2	10*	1791.283*	44*	26.59759*	67.34755*	0.000000*
1 2	10*	221.123*	44*	26.59759*	8.31365*	0.000000*

*significant at p 0,05

Table 7. *Tilia cordata* Mill. Effect of scarification with sulphuric acid (+/– acid) and freezing in liquid nitrogen (+/– LN) on germinability and seedling emergence

Variant	Germinability (%)			Seedling emergence (%)			
	germinating	healthy, not germinating	decayed	emerging	healthy, not emerging	decayed	only germinating (no shoot)
	(%)	(%)	(%)	(%)	(%)	(%)	(%)
– acid* +LN	51	22	27	36	59	0	5
– acid –LN	51	21	28	33	0	67	0
+acid –LN	85	0	15	49	0	48	3
+acid +LN	85	0	15	53	0	47	0

*denotes presence of the factor, '–' denotes absence of the factor

Discussion

This study showed that *T. cordata* seeds are sensitive to strong dehydration above silica gel and to freezing in LN (–196°C) at some levels of seed moisture content. The lowest moisture content reached in our experiment was 3.1%. The planned moisture content of 1% could not be reached. Anyway, dehydration to 5.2% m.c. or below this level resulted in a decrease in both germinability and seedling emergence after freezing in LN, as compared with seeds frozen within the safe moisture range (Table 5). By contrast, unfrozen seeds scarified at 10.8% m.c. and later dried to 5.2% m.c. germinated at the highest level (Table 3).

Generally, the timing of scarification (before or just after freezing in LN) did not have any significant effect on germinability. The most important was the moisture of seeds during scarification and during LN treatment. The germinability of unfrozen seeds and of seeds frozen at 17.8% m.c. was significantly lower than that of seeds germinating at the highest level. However, 16.2% m.c. for seeds frozen before scarification was still a safe moisture level. The question is: what factor decreased the germinability of seeds scarified at 17.8% m.c.? This could be due to a high temperature of acid in the zone adjacent to the exceedingly moist tissues of scarified seeds. It is also possible that some seeds had more permeable seed coats and absorbed more water, and consequently their moisture level was higher than 17.8%.

Seed samples of *T. cordata* often vary in the proportion of seeds with hard seed coats (Tylkowski 1998). Apart from hard seeds, which do not increase in weight after 24 h of soaking in water, there are seeds with more permeable seed coats, which increase their weight by about 8–9%. Thus it can be expected that the seed samples used in our experiment also included both types of seeds. Table 7 shows that about 35% of seeds were hard, as they were healthy but did not germinate. This means that the measured moisture content of each sample was probably a mean of a slightly lower value of hard seeds and a slightly higher value of seeds lacking hard seed coats.

The safe moisture range of seeds frozen in LN after scarification was assessed as 9.0–17.4% on the basis of germinability, and as 11.1–20.1% on the basis of seedling emergence (Table 5). Similar differences were observed for seeds frozen before scarification: 9.1–16.2% (germinability) and 7.3–17.8% (emergence). It is difficult to explain the significant decrease in germinability after freezing at 17.8% m.c., as compared with 16.2% m.c., and the lack of such a decrease in seedling emergence. This could result from the different conditions of the germination test (plastic bottles with a mixture of sand and peat) and the emergence test (boxes with a mixture of sand and peat, where seeds were covered with a layer of sand).

In conclusion, the safe moisture content for seeds frozen in LN (before or after scarification) is 9.0–15.3%. This range is similar to that reported by Schönborn (1964): 9.7–15.1% for seeds frozen to –70°C. Such a moisture content protects seeds against damage both during scarification and during LN treatment. It should be emphasized that the val-

ues of germinability of frozen seeds for the closest levels outside the safe moisture range, i.e. 7.2% and 20.1% m.c., are not significantly different from those for 9.0% and 17.4%, respectively. Even the germinability value for seeds frozen at 5.2% does not significantly differ from that for 9.0%. Thus, further research is needed to compare the sensitivity of *T. cordata* seeds from several localities to freezing in LN.

References

Ashwood-Smith M. J., Grant E. 1977. Genetic stability in cellular stored in the frozen state, in: The freezing of mammalian embryos. Ciba Foundation Symp. 52 Elsevier Excepta Medica, North Holland, 251.

Chmielarz P. 2000. Cryopreservation of subotrhodox and recalcitrant seeeds of three tree species. Cryobiology 41: 4, p. 381.

Gordon A.G., Rowe D.C. 1982. Seed Manual for ornamental trees and shrubs. Forestry Commission, Bulletin No. 59.

Harrington J. F. 1972. Seed storage and longevity, in Seed Biology, Kozlowski T.T., Ed., Academic Press, New York, 1972, chap. 3.

Kartha K.K. 1985. Cryopreservation of Plant Cells and Organs, CRC Press, Bocaraton, Florida, USA.

Lang G.A, Early J.D., Martin G.C., Darnell R.L. 1987. Endo-, Para-, and Ecodormancy: Physiological Terminology and Classification for Dormancy Research. Hort. Science, 22(3): 371–377.

Roberts E. H. 1972. Viability of Seeds, Chapman and Hall, London, 253.

Schönborn A. 1964. Die Aufbewahrung des Saatgutes der Waldbäume. BLV München.

Suszka B. 1966. Dormancy, storage and germination of *Fagus silvatica* L. seeds. Arboretum Kórnickie 11: 221–240.

Suszka B. 1975. Cold storage of already after-ripened beech (*Fagus silvatica* L.) seeds. Arboretum Kórnickie 20: 299–315.

Suszka B., Muller C., Bonnet-Massimbert M. 1994. Nasiona leśnych drzew liściastych od zbioru do siewu [Seeds of broad-leaved forest trees from collection to sowing]. Wydawnictwo Naukowe PWN, Warszawa-Poznań.

Tylkowski T. 1994. Przysposabianie spoczynkowych nasion do siewu przez cyklicznie powtarzane moczenie w wodzie. II. Lipa drobnolistna *Tilia cordata* Mill. [Presowing treatment of dormant seeds by ciclically repeated soaking in water. II. Small-leaved lime *Tilia cordata* Mill.] Sylwan 139 (7): 15–21.

Tylkowski T. 1998. Przezwyciężanie spoczynku nasion jesionu wyniosłego *Fraxinus excelsior* L. i lipy drobnolistnej *Tilia cordata* Mill. [Overcoming of dormancy of *Fraxinus excelsio* L. and *Tilia cordata* Mill.)]. Wyd. Z. Bartkowiak. Poznań. 1–64.