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ORIGINAL PAPER

Optimal seed water content and storage temperature for preservation of *Populus nigra* L. germplasm

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Abstract
• ***Context*** Black poplar (*Populus nigra* L.) is an alluvial forest tree species whose genetic pool is decreasing in Europe. Poplar trees produce short-lived seeds that do not store well.
• ***Aim*** The feasibility of seed storage in conventional and cryogenic conditions after their desiccation from water content (WC) of 0.15 to 0.07 g H_2O g^{-1} dry mass (g g^{-1}) was investigated.
• ***Methods*** Seed germinability was evaluated (seeds with a radicle and green cotyledons were counted) after storage of seeds for a period of 3 to 24 months at different temperatures: 20°, 10°, 3°, −3°, −10°, −20° or −196°C.
• ***Results*** Seeds desiccated to a 0.07 g g^{-1} WC can be stored successfully at −10 °C and −20 °C for at least 2 years. A significant decrease in germination was observed only after 12 months of seed storage (WC 0.15 g g^{-1}) at temperatures above 0 °C. We demonstrated that both fresh (0.15 g g^{-1} WC) and desiccated (0.07 g g^{-1} WC) seeds can be preserved at −196 °C for at least 2 years.
• ***Conclusions*** Seed storage temperature and time of storage were statistically significant factors affecting seed storability. The presented data provide a foundation for the successful gene banking of *P. nigra* seeds.

Keywords *Populus nigra* · Black poplar · Seeds · Desiccation · Cryopreservation · Water content · Long term storage

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Contribution of the co-authors Jan Suszka: Coordinating the research project, designing the experiment, analysing data, supervising, writing the manuscript. Beata P. Plita: analysing data and writing the manuscript. Marcin Michalak: designing and running the experiment, analysing data, writing the manuscript. Barbara Bujarska-Borkowska: running the experiment. Tadeusz Tylkowski: designing and running the experiment. Paweł Chmielarz: designing and running the experiment, analysing data, writing the manuscript.

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1 Introduction

Black poplar (*Populus nigra* L.)—a member of the Salicaceae (Wyckoff and Zasada 2005)—is a tree species typical of alluvial forests along many European and Siberian rivers (Vanden Broeck 2003). It is the keystone species for softwood, floodplain, forest ecosystems and plays a crucial role in the initial phase of the development of riparian forests (Wyckoff and Zasada 2005). Unfortunately, many of the habitats occupied by black poplar have been lost since the seventeenth century through anthropogenic activities such as urbanisation, land drainage, and canalisation of rivers. It has been estimated that up to 99 % of the natural habitats of this species have disappeared (Cottrel 2004; Wyckoff and Zasada 2005). Riparian woodlands in Poland are also recognised as endangered habitats (Tylkowski 2010). For these reasons, *P. nigra* is one of the most threatened tree species in Europe. Therefore, in order to secure and preserve as much biodiversity as possible, ex situ conservation of the genetic resources of this species is needed.

The most efficient method for protecting the genetic diversity of plant material is seed storage (Linington and Pritchard 2001). For this purpose, there are more than 1,750 seed banks in the world established for the ex situ conservation (Hay and Probert 2013). Before *P. nigra* seeds can be deposited safely in a seed bank, the major problem to be solved is their short longevity. *P. nigra* seeds are described as a microbiotic seeds

(Stanton and Villar 1996). The life span of this seed group is known to not exceed 3 years under good storage conditions (Ewart 1908). Gosling (2007) classified them as recalcitrant and called them "suicidal" since they die shortly after being shed. Poplar seeds under natural conditions or when kept at room temperature and ambient humidity maintain viability from 2 weeks to a month, depending on the species, season, and microenvironment (Stanton and Villar 1996). At 4 °C and non-drying conditions, poplar seeds can be stored for only 4 weeks (Gosling 2007). Tylkowski (2010), however, demonstrated that desiccated *P. nigra* seeds can be stored much longer in appropriately selected thermal conditions. Bonner (2008) classified seeds of poplars (*Populus* L.) and willows (*Salix* L.) as sub-orthodox, and previous research reported that the viability of seeds can be maintained for several years when seeds are stored at below-freezing temperatures in a dry atmosphere (Stanton and Villar 1996). No single method of storage is generally acceptable for all *Populus* species (Wyckoff and Zasada 2005). Disagreement about the assignment of this species to a specific seed category (recalcitrant, intermediate, orthodox) and lack of comprehensive information about suitable storage protocols make any attempt to preserve the genetic resources of this endangered species difficult.

The objective of the current study was to determine optimum protocols for the successful ex situ conservation of *P. nigra* seeds. Since the relationship between water content and storage temperature is critical for optimising seed preservation (Walters 2003), the study was designed to identify optimal seed water content and to investigate if seeds could be stored for up to 2 years at 3 °C and sub-zero temperatures of −3°, −10° and −20 °C. These conventional storage regimes were compared with cryopreservation (−196 °C). The rate of seed decay at 10 °C and 20 °C was also assessed. The experiments were crucial to determine the storage potential of fresh and desiccated seed. We also checked whether the speed of seed drying affected seed viability during storage. The ultimate goal of the present study was to determine optimum protocols for the successful ex situ conservation of *P. nigra* seeds.

2 Materials and methods

2.1 Plant material

Mature seeds of *Populus nigra* mostly closed in catkins (5–10 % of capsules started to dehisce) were collected from several trees growing within 50–200 m of each other in floodplains located near Czeszewo (52°8′N and 17°30′E). The collection was made in 2010 (27 May–16 June) and in 2011 (19 May). No poplar plantations, or other poplar species, were located in the nearby vicinity, which ensured that the seeds used in the study were true seeds of *P. nigra*. After collection, catkins were placed in an environmental chamber at a constant temperature of 15 °C for 72 h to promote full opening. Seeds were then separated manually from the associated cotton very carefully using a sieve with mesh diameter 3 mm, avoiding seed testa damage. Seeds from individual trees were not mixed at any stage of the experiments.

2.2 Desiccation of seeds

Freshly extracted seeds had a WC in the range of 0.14– 0.17 g H_2O g^{-1} dry mass (g g^{-1}), which represents a moisture content (MC) of 12.5–14.4 % on a fresh weight basis. Prior to storage, seeds were dried by one of two methods: desiccation in a seed dryer at 20 °C for about 1 h to 0.071 g g^{-1} (7.1 % MC) or air-drying at ambient temperatures (approximately 20 °C) on a laboratory table for 3 days to 0.074 g g^{-1} (7.4 % MC), (one lot of seeds collected in 2010). A second lot of seeds collected in 2010 and all seeds collected in 2011 were dried in the range of 0.070 to 0.074 g g^{-1} WC. Seed WC (3 replications of 50 seeds each) was determined by drying seeds at 103±2 °C for 17 h (ISTA 2013).

2.3 Seed storage conditions

Fresh (0.14–0.17 g g^{-1} WC) and desiccated seeds (0.07 g g^{-1} WC) were stored at different temperatures: 10 °C, 3 °C, −3 °C, −10 °C, −20 °C in cold rooms or at 20 °C in an incubator. Seeds were packed in tightly closed polyethylene vials during storage. Both types of seeds (fresh and desiccated) were also cryostored at −196 °C in liquid nitrogen (LN). Seeds were placed in vials (Nunc 1.8 ml) and directly immersed in LN. After storage in LN, seeds in the cryovials were thawed at 40 °C in a water bath for 5 min. Seeds were stored conventionally or in LN for a period of 3, 6, 9, 12, 18 and 24 months.

2.4 Germination

Germination assays on fresh and desiccated seeds were conducted directly after cleaning or after a particular storage regime (time and temperature) in a Jacobsen germinator. One replicate consisted of 100 seeds, and four replicates were used for each treatment. According to information that growth and development of poplar seedling is correlated closely with the relative abundance of light and soil moisture (Mahoney and Rood 1991, 1992; Rood and Mahoney 1990), seeds were placed on moist filter paper (70 mm in diameter) and covered with a plastic lid; cool white light was provided on a 12-h cycle (irradiance of 22 μmol m^{-2} s^{-1} at the level of blotting paper) with illumination occurring during the period with the highest daily temperature. The temperature in the Jacobsen incubator was maintained at 23 °C for 22 h and 27 °C for 2 h per day. All seeds with a radicle and green cotyledons were considered as germinated. Each germination assay was

conducted over a period of 14 days. After the germination assay non-germinated seeds were evaluated visually and mechanically (pressed), all non-germinated seeds were soft and decayed therefore were counted as a dead. Results of germination test from individual trees were used for statistical calculation. In order to keep the fully orthogonal experiment scheme, for each germination test individual control (germination of non-treated seeds) was made.

2.5 Statistical analysis

JMP software (version 7.0.2; SAS Institute, Cary, NC) was used for statistical analyses of the data. Analysis of variance (ANOVA), using a mixed model with "tree" as a random effect, was used to assess the influence of storage temperature, storage time, and seed drying on the level of seed germination. This method of analysis allowed us to investigate the general germinability of *P. nigra* seeds without distinction of individual trees. All percentage data were arc sin transformed prior to statistical analyses. A Tukey's test was used to determine significant differences between sample means at $P=0.05$.

3 Results

Fresh seeds (0.15 g g^{-1} WC) collected in 2010 germinated in 90 %. Desiccation of seeds to 0.07 g g^{-1} WC exhibited 86 % of germination. Results for seeds desiccated to 0.07 g g^{-1} WC indicated that both time (3, 6, 9, 12, 18, and 24 months) and temperature (3°, −3°, −10°, −20°, and −196 °C) of storage had a significant influence on seed germination ($P<0.05$). Moreover, there was a significant interaction between time and temperature of storage (Table 1, Fig. 1).

No significant changes in the level of germination were observed for desiccated seeds (0.07 g g^{-1} WC) stored at 3 °C for 3 to 12 months. Longer periods of storage, however, significantly decreased the germination of desiccated seeds. The germination of such seeds stored for 18 and 24 months was 57 and 40 %, respectively (Fig. 1). Other not germinated seeds were dead (soft and decayed).

Storage of seeds (0.07 g g^{-1} WC) at −3 °C for up to 18 months did not affect their level of germination, which was 82–94 % (Fig. 1). Seeds stored at −3 °C for 24 months exhibited a slight decline in germination to 73 %. These changes were not statistically significant. No changes in the level of germination were observed with storage time when seeds were stored at −10 °C and −20 °C, even after 24 months. Germination of seeds stored at −10 °C and −20 °C ranged from 87 to 90 % and 83 to 90 %, respectively, over the entire storage period. Lastly, cryostorage for 3, 9, 12 and 18 months also did not have any impact on the germination. The observed decrease in germination after 6 and 24 months of storage to 76 % and 79 %, respectively, was not significant and was considered the result of technical issues (Fig. 1).

Table 1 Analysis of variance (ANOVA) of the effect of seed storage time (3, 6, 9, 12, 18 and 24 months) and temperature (3 °C, −3 °C, −10 °C, −20 °C, −196 °C) on *Populus nigra* seed germination. Seeds were desiccated to a water content of 0.07 g H_2O g^{-1} dry mass. ANOVA mixed model with "tree" as a random effect. Seed from harvest in 2010. *ST* Storage time, *STE* storage temperature, *DF Den* degrees of freedom in the denominator of the test

Source variance	DF	DF Den	F	*P*
ST	6	18	4.51	0.0059
STE	4	12	6.59	0.0048
ST*STE	24	72	3.33	0.0001

Desiccated seeds were stored at 20 °C in order to determine how quickly seeds lost viability. This condition resulted in a significant and dramatic decrease in total germination (Fig. 2). After 1 month, germination dropped from 91 % to 22 % and after 3 months only 1 % of the seeds germinated.

Results indicated that the rate of drying had no significant effect on germinability (Fig. 3). The germination level for slow and rapidly dried seeds was 80 % and 81 %, respectively.

Analysis of the results of germination assays of seeds collected in 2011 indicated that both the time and length of storage had a significant impact on seed germination. Moreover, there was a strong interaction between time and temperature of storage, time of storage and WC, and between time of storage, temperature of storage and WC (ANOVA test, $P<0.05$), (Table 2; Figs. 4, 5).

A germination of 86 % was observed for fresh seeds, collected in 2011, with an average WC of 0.15 g g^{-1} (13.5 % MC) (Fig. 4). Storage of these seeds for 12 months at temperatures of 10 °C or 3 °C caused a 100 % reduction in the germination. When seeds were kept at either −20 °C or −196 °C, similar levels of germination (77 % and 95 %, respectively) were observed. These levels were similar to that obtained for non-stored seeds (86 % germinability) (Fig. 4).

Seeds dried to a WC of 0.07 g g^{-1} (7 % MC) germinated at the level of 88 %. Storage of such seeds for 12 months at 10 °C caused a significant reduction in seed viability with germination dropping to 17 %. The lower temperature of 3 °C impacted germination to a lesser extent. The germination was 68 %, and the difference between stored and non-stored seeds was not statistically significant. No differences were observed between stored and non-stored seeds for seeds maintained at −20 °C or immersed in LN (−196 °C) (Fig. 4).

4 Discussion

Storage of seeds is the primary method of choice when attempting to preserve the gene pool of a plant species. Stored

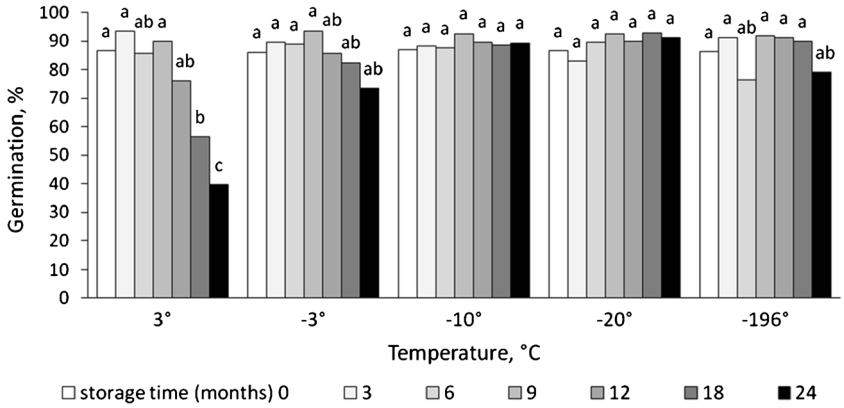

Fig. 1 Effect of storage temperature (3 °C, −3 °C, −10 °C, −20 °C, and −196 °C) and time of storage (0, 3, 6, 9, 12, 18 and 24 months) on *Populus nigra* seed germination. Seed water content was 0.07 g H_2O g^{-1} dry mass. Means with the same letter are not significantly different at $P<0.05$, according to the Tukey's test. Seeds were collected in 2010

seeds represent an excellent reservoir of preserved genetic diversity that is convenient and cost effective compared to in situ preservation. Other advantages include the ability to store seeds at multiple locations, thus providing a safeguard against accidental loss, and the ability to propagate the material in a greenhouse regardless of the season (http://www.britishecologicalsociety.org).

While the length of seed viability varies between and within tree species, poplar seeds are one of the prime examples of seeds that have a short span of viability once they are shed from their parent tree (Wyckoff and Zasada 2005). In the case of *P. nigra* seeds, the optimum storage conditions needed to preserve seed viability for as long as possible still needed to be determined. Therefore, the objective of the present study was to identify the optimal thermal conditions and seed water content for the long-term storage of *P. nigra* seeds.

Most preservation procedures are based on the premise that drying and cooling will slow deterioration by creating kinetic barriers. Drier or colder conditions of seed storage are thought to extend life spans, as slower molecular motion at lower temperatures decreases the rate of biochemical reactions (Walters 2003). In contrast, high temperatures accelerate biochemical reactions, thus accelerating the rate of degradation and reducing seed viability. As expected, our results are in accordance with this assumption. Seeds from a Polish provenance, located near Czeszewo, with a WC of 0.07 g g^{-1} (7 % MC), exhibited an 80 % reduction of germination after 1 month of storage at ambient temperatures and were dead (decayed on a germinator) after 3 months of such storage (Fig. 3).

The lack of *P. nigra* seeds ability to withstand longer storage at higher temperatures may be connected with seed shedding strategy. In nature, these small seeds (1–2 mm) germinate quickly after shedding in May/June. The storage products accumulated in seeds are related to their longevity and protect them against environmental stresses (Kermode 2011). Probably, as *P. nigra* seeds do not have enough storage compounds, it is difficult to supply energy to keep seeds in good condition for a long time at temperatures that trigger seeds germination. Secondly, faster deterioration of *Populus* seeds stored in higher temperatures is associated with the aging process. Metabolism is at a high level at temperatures

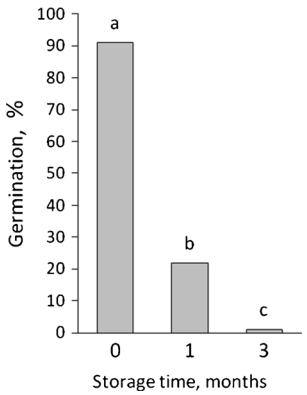

Fig. 2 Effect of time of seed storage for 1 and 3 months at 20 °C on the germination of *P. nigra* seeds (mean of each storage time 0, 1 and 3 months are compared within each temperature). Seed water content (WC) was 0.07 g H_2O g^{-1} dry mass. Means with the same letters are not significantly different ($P<0.05$). Seeds were collected in 2010

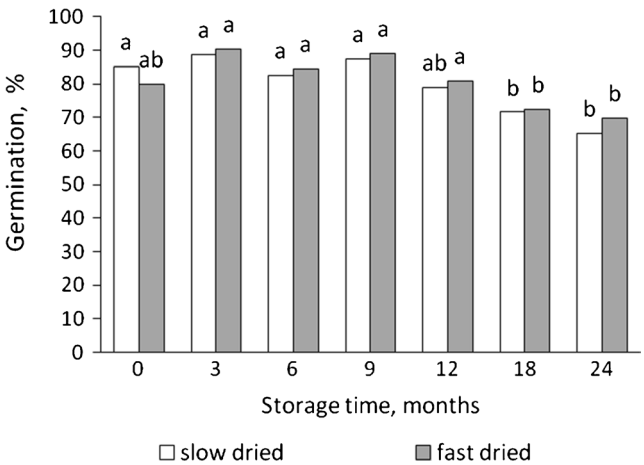

Fig. 3 Effect of seed drying: slow (3 days) and fast (1 h) at 20 °C on the germination of *P. nigra* seeds after storage (0, 3, 6, 9, 12, 18 and 24 months). Seed WC was at 0.07 g H_2O g^{-1} dry mass. Means with the same letters are not significantly different ($P<0.05$). Seeds were collected in 2010

Table 2 ANOVA of the effect of storage time (0, 12 months), storage temperature (10°, −3°, −20 °C, −196 °C) and water content on *P. nigra* seed germination. Seeds had a WC of 0.15 g g^{-1} (non-desiccated) or 0.07 g g^{-1} (desiccated). ANOVA mixed model with "tree" as a random effect. Seeds from harvest in 2011. *WC* Water content

Source variance	DF	DF Den	F	*P*
ST	1	2	65.04	0.0115
STE	3	6	66.26	0.0001
Seed WC	1	2	10.42	0.084
ST*STE	3	6	71.45	0.0001
ST*WC	1	2	10.31	0.0848
STE*WC	3	6	9.87	0.0098
ST*STE*WC	3	6	7.74	0.0174

above zero and the production of reactive oxygen species (ROS) is much higher than in temperatures below zero. Probably, the antioxidative system is not able to avoid damage caused by ROS species. In similar *Salix* seeds the increment in the level of ROS was shown to be one of the principal causes of the rapid loss of normal germination in seeds (Causin et al. 2012). In comparison, seeds of *P. balsamifera* L. from Bowness (United States) deteriorated after 4 months of storage at 21–24 °C (Hellum 1973). Similar results were obtained by Maroder et al. (2000) for *Salix alba* and *Salix matsudana*. A complete loss of germination was observed after 2 weeks of storage at 25 °C. These examples illustrate how detrimental storing seeds at ambient conditions can be when trying to preserve the genetic resources of these species in the family Salicaceae.

We proved in our research that conventionally stored seeds of *P. nigra* should be kept at −10 °C or −20 °C and have a WC of approximately 0.07 g g^{-1}. According to other studies, Salicaceae seeds will lose viability most sharply at 8–10 weeks of storage at 5 °C (Popova et al. 2012). We decided to extend our research and *P. nigra* seeds were stored for up to 2 years. We prooved that, at −10 °C or −20 °C, they can maintain high levels of viability (Fig. 1). Similar results were reported by Zasada and Densmore (1977), who showed that *Salix alaxensis*, *Salix glauca*, *Salix bebbiana* and *Salix novae-angliae* maintained a high percentage of germination during 2 years of storage at −10 °C. Sato (1955) also reported that the viability of *Salix urbaniana* seeds can be maintained for 535 days when they are stored at −8 °C. Tylkowski (2010) successfully stored *P. nigra* seeds desiccated to a WC of 0.1 g g^{-1} (9.6 % MC) at −3 °C for 1 year. At the same WC of 0.1 g g^{-1} seeds of *Populus alba* x *Populus glandulosa* showed high germinability (88–100 %) after conventional storage for 7 and 10 weeks at 5 °C. However, further drying diminished their viability. Germinability of *Populus alba* seeds also showed a decrease after storage at 5 °C for 7 (85 %) and 10 weeks (62 %), (Popova et al. 2013). Our present results indicate that seeds desiccated to 0.07 g g^{-1} WC and stored at 3 °C significantly decreased germinability after 18 months of storage. But at −3 °C they exhibited only a slight (not statistically relevant) reduction in the level of germination after storage for more than 12 months (Fig. 1). Contradictory to our results, seeds from *S. alba* lose viability after 5 months of storage at −20 °C (Maroder et al. 2000).

The present study also compared the effect of cryopreservation on seed viability. Results indicated that storage at an ultra-low temperature in LN can be used successfully as a method for long term preservation for desiccated seeds of *P. nigra*. Seeds of *P. nigra* were not affected by cryopreservation at WCs of either 0.15 g g^{-1} or 0.07 g g^{-1}. These results were in agreement with other experiments conducted in our laboratory. *P. nigra* seeds desiccated to a WC of 0.1 g g^{-1} (9.6 % MC) were successfully cryostored for 24 h with only a slight delay but no decrease in germination (Tylkowski 2010). Popova et al. (2013) showed that hybrid poplar seeds (*P. alba* x *P. glandulosa*) survived 2 weeks of storage in LN at a WC

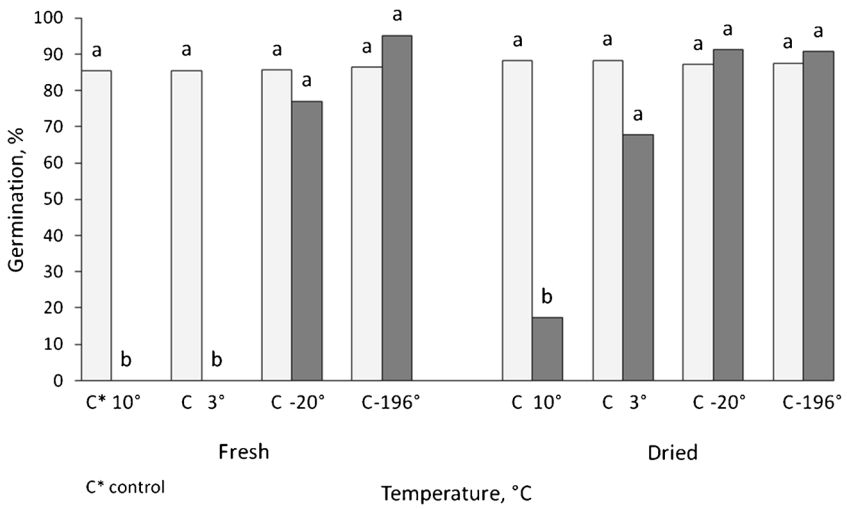

Fig. 4 Effect of storage at different temperatures on the germination of *P. nigra* seeds after 12 months. The WC of fresh and dried seeds was 0.15 g g^{-1} and 0.07 g g^{-1}, respectively. Means with the same letters are not significantly different ($P<0.05$). Seeds were collected in 2011

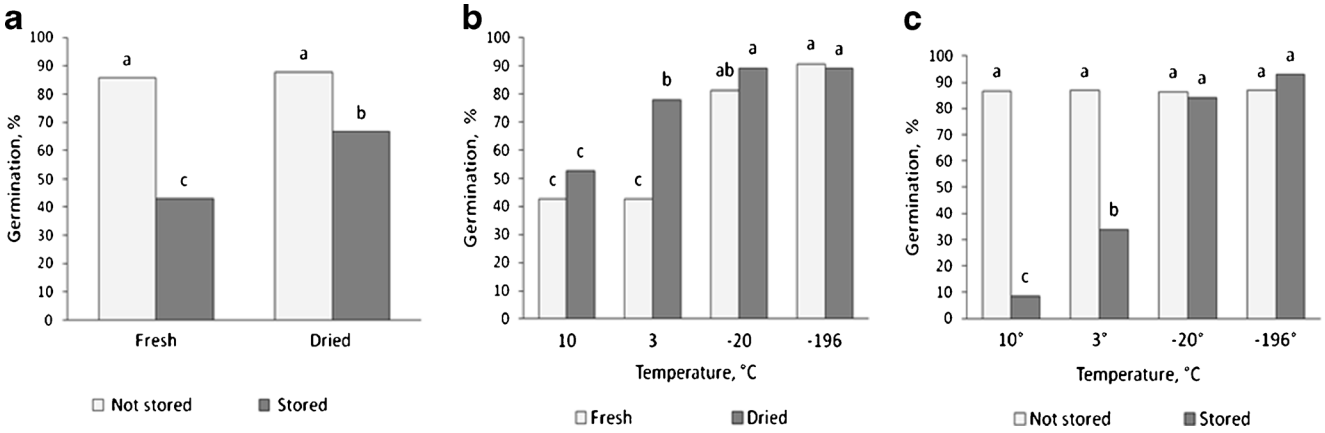

Fig. 5 Effect of seed storage conditions on the germination of *P. nigra* seeds. **a** Interaction between seed WC (fresh 0.15 g g^{-1} and dried 0.07 g g^{-1}) and time of storage (not stored or stored 12 months). **b** Interaction between temperature (10 °C, 3 °C, −20 °C, −196 °C) and seed WC (fresh and dried). **c** Interaction between temperature and time of storage (not stored or stored 12 months). Means with the same letters are not significantly different ($P<0.05$). Seeds were collected in 2011

range of 0.07–0.10 g g^{-1} without significant loss in germinability. *Salix caprea* seeds were also cryopreserved successfully at WC 0.09–0.3 g g^{-1} (Popova et al. 2012). In the presented study, we demonstrate that seeds of *P. nigra* maintain high levels of germination even after 2 years of cryostorage. Maroder et al. (2000) reported that 11 months of cryostorage did not adversely impact the germination of *S. matsudana* seeds. Pence (1996) reported on the cryopreservation of *Populus deltoides*. According to that report, seeds of *P. deltoides* can be cryostored when their WC is in the range of 0.09–0.17 g g^{-1}. These reports provide convincing evidence that cryostorage can be considered as a reliable method for the long-term storage of many short-lived seeds from various species in the Salicaceae family.

The current study also demonstrated that low temperature is a more important factor than WC in the storage of *P. nigra* seeds. Seed germination was similar after 1 year of storage at −20 °C or −196 °C whether WC 0.15 g g^{-1} (fresh seeds) or 0.07 g g^{-1} (dried seeds), (Fig. 5b). Additionally, if seeds were stored at high temperature (10 °C), they lost viability whether they were desiccated or fresh, although seeds stored at 3 °C deteriorated more rapidly at WC 0.15 g g^{-1} WC than at 0.07 g g^{-1} WC (Fig. 5b).

Statistical analysis indicated that the most important factors affecting seed viability are time and temperature of storage. Significant interactions between time of storage and temperature, temperature and seed WC, and between all these three factors were also evident from the ANOVA analysis. Nevertheless, an analysis of WC alone indicated no effect on the results obtained ($P>0.05$). The interaction between storage time and water content was also insignificant (Tables 1, 2). Similar results were obtained in studies examining the effect of thermal conditions on seed viability of *S. alba* and *S. matsudana*. Storage at lower temperatures resulted in higher levels of germination in both species (Maroder et al. 2000). Similar observations were reported by Wood et al. (2003) for *Salix rehderiana* x (*Salix x capreola*) and *Salix x sericans* x *Salix viminalis*. The highest levels of germination were obtained by storage at the lowest temperature when seeds had a WC of 0.05 g g^{-1} or 0.11 g g^{-1} (5 % and 10 % MC, respectively).

This study did not definitely solve the problem of *P. nigra* seed classification; however, on the basis on available data, we can make some assumptions. The results indicate that *P. nigra* seeds should not be classified as recalcitrant because they can be stored for 2 years after desiccation to 0.07 g g^{-1} at −10 °C and −20 °C. Nevertheless, in the present research we did not consider desiccation tolerance in the broader aspect that would be necessary to assign the proper category. However, the observed germinability decline at 3 °C and beginning of germinability decease at −3 °C after 24 month of storage suggest that these seeds could be classified as intermediate rather than as orthodox. These findings are also supported by our other data that indicate that *P. nigra* seeds did not tolerate severe drying (M. Michalak et al. manuscript in preparation). Moreover, *P. nigra* seeds are non-dormant, and dormancy is known to be common feature of orthodox seeds (Hay and Probert 2013).

5 Conclusions

Populus nigra seeds can be stored successfully by both conventional and cryogenic techniques. In the first approach, low, sub-zero temperatures (−10 °C, −20 °C) are strongly recommended since no changes of seeds germinability were observed after 2 years of storage under these thermal conditions in seeds with a WC of 0.15 g g^{-1} or 0.07 g g^{-1}. Cryopreservation of seeds can be used as a backup for traditional seed storage in gene banks and is the method of choice for long term conservation, theoretically for very long periods of time, even

hundreds of years (Walters et al. 2013). In the present study, cryostorage of *P. nigra* was as good as conventional storage since no changes in seed germinability were observed during cryostorage. Poplar seeds should not be kept at ambient temperatures (about 20 °C) prior to storage at low temperatures (−20°, −10°, −196 °C) since this may cause a rapid loss in viability. This research provides a variety of protocols suitable for the ex situ conservation of *P. nigra* genetic resources. We also suggest that *P. nigra* seed can be classified as intermediate.

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