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A new insight in desiccation tolerance and cryopreservation of mazzard cherry (*Prunus avium* L.) seeds

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Abstract: A variable response of *Prunus avium* L. seeds to desiccation and storage in liquid nitrogen (LN) has been reported in the literature. The majority of these experiments were conducted on initially dried seeds. The desiccation and LN exposure tolerance of fresh *P. avium* seeds is unknown. In the present study, fresh seeds were used to determine seed response to desiccation and cryopreservation. Desiccation of seeds from a moisture content (MC) of 19.7-20.2% to 10.1-10.9% or 07.6-8.5% reduced seedling emergence from approximately 73 to 19 and 16% for first provenance; and from approximately 89 to 10-12% for second provenance of seeds. After exposure to LN, seeds had the highest seedling emergence when seed MC was the highest (19.7 and 20.2%, respectively) prior to cryostorage. Results indicated that *P. avium* seeds should be classified as intermediate. For cryopreservation in seed banks, we recommend that seeds be dried directly after extraction from fruits in the range of 16.8-20.2% of MC (0.21-0.25 $g \cdot g^{-1}$ of WC) and directly immersed in LN in tightly closed cryovials.

Keywords: Gene banks, Liquid nitrogen, mazzard cherry, Seed desiccation

1 Introduction

There has been a significant decline in biodiversity during the past few decades compared with previous geological epochs, mainly due to human activity and climate change [1]. In view of the climate changes that are taking place on Earth, conservation and sustainable utilization of forest genetic resources are becoming increasingly important issues for maintaining the long-term homeostasis of European forests, and for the preservation of biodiversity on a large scale [2]. A loss of biodiversity in forests can reduce their ability to adapt to a changing climate. Therefore, preventing the impoverishment of ecosystems, including forest ecosystems, in a rapidly changing environment is a crucial challenge [3-6].

One of the approaches being utilized to protect plants against the consequences of climate change has been the collection and storage of genetic resources in local gene banks. Seeds are the primary material used for the purpose of reforestation and fortunately they are also the most useful material for the conservation of species [7]. Seeds are characterized by high genetic diversity and are therefore appropriate material for storage in gene banks [7].

So far more than 1.4 million germplasm accessions have been added to ex situ collections, bringing the total number now conserved worldwide to about 7.4 million. The majority of these are maintained in seed gene banks [8]. The most important factors affecting the viability of stored seeds are their water content, storage temperature [9], and the interaction between these two factors [10].

Cryopreservation is a method for storing biological material, including seeds, at the temperature of liquid nitrogen (–196°C, LN). This technique involves placing cells, tissues or organs of plants or animals in LN or LN vapour (about –160°C). Prior to LN exposure, the material often requires a pretreatment involving desiccation and/or the use of cryoprotectant. Based on research conducted at the National Center for Genetic Resources Preservation (NCGRP) in Fort Collins CO USA, the viability of material cryopreserved in LN vapours has been estimated to be as long as 500 years, and in the case of complete immersion in

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LN, as much as 3400 years. With respect to cryopreservation of seed material, lower temperatures result in longer storage life of seeds [11]. Cryopreservation has been successfully used for the conservation of many species originating from both tropical climate zones, e.g. *Amaryllis belladonna* L. and *Haemanthus montanus* Bake [12] and moderate climate zones, e.g. *Quercus robur* L., *Populus nigra* L., *Abies alba* Mill. or *Pinus nigra* Arn. [13-17].

Wild or mazzard cherry (*Prunus avium* L.) is a broad-leaved tree species that occurs naturally in Western Eurasia and Northern Africa. It has a mainly scattered distribution pattern and natural regeneration by seed prevails. It is considered highly important from an economic (timber production) and environmental perspective [18]. Seeds of mazzard cherry have been categorized as orthodox and are therefore stored in a dried state. Based on current literature, they tolerate drying to a MC of 10% (0.11 $g \cdot g^{-1}$ of WC), at which they can be stored without a loss of germination capacity for 15 years at a temperature of –3°C [19]. Successful cryopreservation of the genus *Prunus* has been reported for zygotic embryos and shoot tips derived from *in vitro* culture [20,21], embryogenic tissue [22] and dormant buds [23]. Studies were also performed on the cryopreservation of seeds still located in their stones [24,25]. Those studies have shown that mazzard cherry seeds in stones did not fully tolerate LN [25].

Currently there are no data indicating whether or not the pre-drying of fresh seeds can change the tolerance of seeds to the temperature of LN. This is an interesting issue, particularly in the case of seeds with high lipid content such as mazzard cherry [26], as changes in the germination capacity of seeds can be associated with lipid phase transition [27]. For this reason, we determined the sensitivity of fresh mazzard cherry seeds, still located in their stones, to desiccation and exposure to LN.

2 Methods

2.1 Plant material

Mature seeds of mazzard cherry (*Prunus avium* L.) from two provenances in southwestern (Bolków- N50° 55' 41.3089" E16° 6' 16.5015" – provenance A) and western (Runowo near Kórnik N52° 16' 53.7667" E17° 7' 12.4768" – provenance B) Poland were collected in June. Experiments (desiccation, cryopreservation, germination test and seedling emergence test) were conducted on whole seeds with the endocarp.

Mazzard cherry produces fleshy fruits with soft exocarpt and hard endocarp (stone). To obtain seeds, a procedure described by Suszka *et al.* [28] was applied. The first step was the manual extraction of endocarp, followed by cleaning of the stones carried using a strong current of water passing through a sieve in order to eliminate all fragments of the pulp from around the stone. Sand was added and the mixture was put into a sack of fine tissue and pressed from all sides. To eliminate all fragments of the pulp, it is necessary to avoid development of fungi during the period of warm stratification [28]. In accordance to Suszka *et al.* [28], seeds extracted from fruits were floated in water to separate the full seeds from the empty ones. Seeds were then air dried at ambient conditions (20°C) for 24 h. For experimental analyses only full seeds were used.

2.2 Moisture content of the seeds

Seed water content (WC) refers to the seed without the endocarp. WC was calculated on a dry weight basis (g $H_2O \cdot g$ dry $mass^{-1}$; $g \cdot g^{-1}$) and was expressed in brackets; and on a fresh weight basis expressed in % as a moisture content (MC). After cleaning, floating and air drying for 24 h seeds achieved a MC of 19.7 and 20.2% (0.24 and 0.25 $g \cdot g^{-1}$) for Bolków and Runowo provenance, respectively. To reach lower WCs they were dried further under the same conditions (20°C) for 2-10 days. The WC and MC of whole seeds, seeds without the endocarp and only endocarp are listed in Table 1. Three replications of 10 seeds each were used each time and seed WC and MC was assessed by drying them at 103 ± 2°C for 17 h. The calculation of water content for *Prunus* spp. was conducted according to ISTA Rules [29]. This method is commonly utilized for the calculation of water/moisture in experiments with oilrich seeds: i.e. previous work on *Prunus avium* seeds [25], *Camellia sinensis* seeds [30], *Corylus avellana* seeds [31], Citrus seeds: *C. sinensis* (sweet orange), *C. paradisi* (grapefruit), *C. reticulata* (mandarin) [32] or embryonic axes i.e.: C*arica papaya*, *Passiflora edulis* and *Laurus nobilis* [33].

2.3 Stratification

The substrate for seed stratification consisted of a moist [34] mixture (v/v, 1:1) of a 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 perforated lid 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

Table 1. Moisture content (MC; %) and water content (WC; $g \cdot g^{-1}$) of *P. avium* L. seeds after drying for 0, 2, 7, and 10 days

Provenance	Moisture content of seeds (%) (WC $g \cdot g^{-1}$)			Time of drying in days
	Seeds without endocarp	Seeds with endocarp	Endocarp	
Bolków	19.7	17.8	17.2	0
	(0.24)	(0.22)	(0.21)	
	16.8	17.0	17.0	2
	(0.21)	(0.21)	(0.21)	
	10.1	13.9	15.3	7
	(0.11)	(0.16)	(0.17)	
	7.6	11.9	13.6	10
	(0.08)	(0.13)	(0.15)	
Runowo	20.2	19.8	19.4	0
	(0.25)	(0.25)	(0.24)	
	14.1	17.2	19.2	2
	(0.16)	(0.21)	(0.23)	
	10.9	14.4	16.0	7
	(0.12)	(0.16)	(0.19)	
	8.5	11.6	13.1	10
	(0.09)	(0.13)	(0.14)	

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. Seeds were monitored for fungal infections and/or insect larvae and early germinated seeds were counted and removed (i.e. those with radicles 2-3 mm long which are visible signs of dormancy release). Stratification was done by placing seeds in a defined substrate and exposing them to the following alternating warm/cold temperatures: 2 weeks at 25°C, 2 weeks at 3°C, 2 weeks at 25°C, and 12 weeks at 3°C, until the appearance of the first germinating seeds (≤ 5%), [35,36].

2.4 Cryopreservation

Seeds were placed in cryovials (1.8 ml volume) prior to direct immersion in LN. Samples were kept in LN for 24 h and subsequently thawed in a water bath at 40°C for 15 min. After thawing, seeds were placed in stratification substrate and stratification was conducted.

2.5 Germination and seedling emergence tests

Seed germination tests were performed in the dark after seed stratification was complete (≤ 5% seed germination) utilizing a similar substrate and container as used for seed stratification. Optimum thermal conditions for seed germination were ensured by providing cyclically alternating temperatures of 3/20°C (16+8 h) [36]. Germinated seeds were counted weekly and water in the substrate was replenished at that time.

Seedling emergence tests were also conducted in a mixture of sand with peat, similar to that used for the stratification and germination tests. 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 h a day) [28], 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.

Germination and seedling emergence experiments included 4 replications of 50 seeds each. Germination and seedling emergence tests were conducted using separate seed samples.

2.6 Nomenclature utilized to describe seed evaluation and germination tests

In the present study, the term "cutting test" is used to denote the evaluation of seed viability after the stratification and germination tests were completed. True seeds were removed from the endocarp, and cut

along their longitudinal axis with a scalpel through the cotyledons and embryonic axis. Two groups of seeds (healthy and decayed) were distinguished among the non-germinated seeds that remained after the completion of the germination test. The quantity of seeds in each of these two groups is presented as the mean percentage of all replications. Healthy seeds had fleshy, white, shiny cotyledons and embryonic axes and were thus capable of further development into normal seedlings. Seeds that were partly or completely decayed as a result of a primary infection, originating from within the seed, were classified as decayed and did not develop into seedlings.

2.7 Statistical analysis

STATISTICA software (Stat-Soft Poland, 1995–2005) was used for all statistical analysisof the data. Analysis of variance (ANOVA) was used to assess the significance of different treatments, as was the Tukey's test for pair-wise comparisons. Before ANOVA analysis, percentage data were subjected to arc-sin transformation. Tukey's test was performed at a significance level of $P < 0.05$. Separate ANOVAs and Tukey's tests were performed for germination and seedling emergence. Error bars indicate standard errors (SEs) of the mean within an isolated treatment.

3 Results

3.1 Desiccation tolerance - Germination

As illustrated in Figure 1, mazzard cherry seeds from the A and B provenances germinated at 76% and 86%, respectively, when MC was 19.7% and 20.2% (WC 0.24 and 0.25 $g \cdot g^{-1}$). The germination capacity of seed from both provenances was significantly reduced to 62% and 54% when seeds were dried to MC of 7.6% and 8.5% (WC 0.075 and 0.085 $g \cdot g^{-1}$), respectively (Figure 1).

Germination curves showed that most of the seeds germinated within three weeks independently of MC (Figure 1). Only seeds with MC 14.1% (WC 0.16 $g \cdot g^{-1}$) showed an increase in germination after the fifth week (Figure 1B). Seeds with high MC of 19.7% and 16.8% (WC 0.24 and 0.21 $g \cdot g^{-1}$) (Figure 1A) or 20.2% (WC 0.25 $g \cdot g^{-1}$) (Figure 1B) had the highest germination rate during the first to third week in comparison to drier seeds (Figures 1A, 1B).

3.2 Desiccation tolerance - Seedling emergence

Seedling emergence tests (Figure 2) conducted in the laboratory showed some similarities with the results of the seed germination test (Figure 1). Seedling emergence for seeds from provenance A, at MC of 19.7% (WC 0.24 $g \cdot g^{-1}$) was 73% and those dried to MC of 10.1% (WC 0.11 $g \cdot g^{-1}$) exhibited only 19% seedling emergence. Further drying of seeds to MC of 7.6 % (WC 0.075 $g \cdot g^{-1}$) resulted in a non-significant decrease in seedling emergence to 16% (Figure 2A). The seeds of provenance B (Figure 2B) exhibited a significant decrease in seedling emergence, from 89% to 34%, when seed MC was reduced from 20.2% to 14.1% respectively (WC 0.25 to 0.16 $g \cdot g^{-1}$). Desiccation to MC of 10.9% or 8.5% (WC 0.12 or 0.09 $g \cdot g^{-1}$) resulted in further reduction in seedling emergence capacity to the level of 10-12% (Figure 2B).

Seedling emergence curves showed that at higher seed MC of 16.8% and 19.7% (WC 0.21 and 0.24 $g \cdot g^{-1}$)

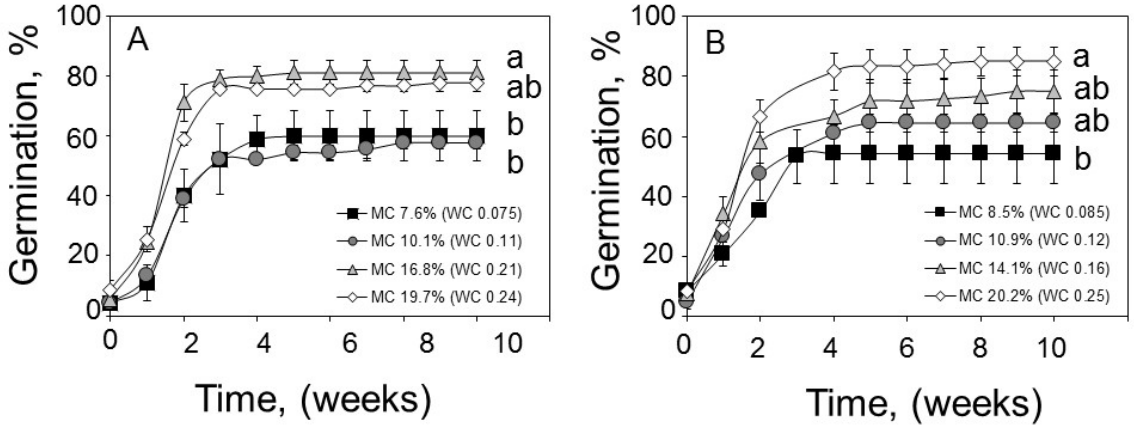

Figure 1. Germination time course of mazzard cherry (*Prunus avium*) seeds at different MC of 7.6%, 10.1%, 16.8% and 19.7% from provenance Bolków (A) and at MC of 8.5%, 10.9%, 14.1%, 20.2% from provenance Runowo (B). Tukey's test was performed separately for each provenance. Values with different lower case letters are significantly different at $P \leq 0.05$. Vertical bars indicate standard errors (SEs) of the mean within an isolate treatment.

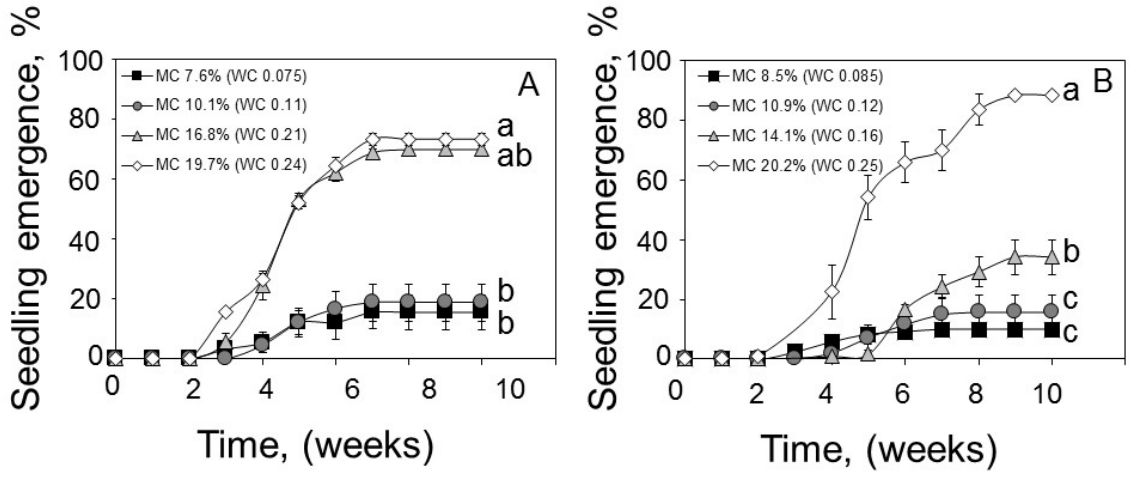

Figure 2. Seedling emergence time course for mazzard cherry (*Prunus avium*) seeds at different MC of 7.6%, 10.1%, 16.8% and 19.7% from provenance Bolków (A) and at MC of 0.085, 0.12, 0.16, 0.25 g·g^{-1} from provenance Runowo (B). Tukey's test was performed separately for each provenance. Values with different lower case letters are significantly different at $P \leq 0.05$. Vertical bars indicate standard errors (SEs) of the mean within an isolate treatment.

(provenance A) and 20.2% (WC 0.25 g·g^{-1}) (provenance B) seedlings started to emerge in the second week of the test and the highest rate was observed at the fourth week and maximum seedling emergence was achieved at the seventh (Figure 2A) and ninth week (Figure 2B).. Seeds with a MC ranging 7.6-10.9% (WC 0.075-0.12 g·g^{-1}) emerged one week later (during the third week of the test) and with a lower overall rate of emergence (Figures 2A, 2B). The latest seedlings to emerge (after 5 weeks) were derived from seeds with a MC of 14.1% (0.16 g·g^{-1}) (figure 2B). Seeds with a MC of 16.8-20.2% (WC 0.21-0.25 g·g^{-1}) had their highest rate of seedling emergence between the fourth and fifth week (Figures 2A, 2B).

3.3 Cryopreservation - Germination

After seed cryostorage, germination tests indicated that seeds (provenance A) that had MC of 7.6% or 10.1% (WC 0.075 or 0.11 g·g^{-1}) prior to immersion in LN did not differ from non-treated seeds (–LN) in their capacity to germinate (Figure 3A). Percent germination in the LN-treated and non-treated seeds was 60-63%, respectively (Figure 3A). Seeds at MC of 16.8-19.7% (WC 0.21-0.24 g·g^{-1}) after immersion in LN exhibited reduced germination capacity (46-47%) compared with the level of germination (78-82%) observed in seeds with the same WC that had not been immersed in LN (Figure 3A).

Seeds that had not germinated by the termination of the test were evaluated for decay. Among the non-frozen seeds, 18-30% were decayed depending on the MC. In the LN-exposed samples, 26-58% of them were decayed (Figure 3A). A small fraction (2-18%) of LN-exposed and non-exposed comprised viable, non-germinating seeds (Figure 3A).

Cryopreserved and non-cryopreserved seeds at MC 19.7% (WC 0.24 g·g^{-1}) had a similar germination rate. Seeds started to germinate at the beginning of the test and finished in the fourth week, however, these cryopreserved seeds germinated at significantly lower levels (Figure 3B). We observed that cryopreserved seeds at MC 7.6% (WC 0.075 g·g^{-1}) have not finished germination by the fourth week of testing (as non-cryopreserved seeds) and newly germinated seeds were still observed until the 11th week (Figure 3B).

Cryopreserved seeds from provenance B with MC in the range of 8.5-14.1% (WC 0.085-0.16 g·g^{-1}) exhibited germination in the range of 54-62% (Figure 4A). The germination capacity of cryopreserved seeds with MC of 8.5%, 10.9% and 14.1% (WC 0.085, 0.12 and 0.16 g·g^{-1}) did not differ significantly from control seeds with the same MC and not exposed to LN. Seeds with MC of 20.2% (WC 0.25 g·g^{-1}) stored in LN exhibited a significant reduction in germination capacity (41%) compared with seeds at the same MC, but not exposed to LN, had a germination totality of 86% (Figure 4A).

An evaluation provenance B seeds that had not germinated at the end of the germination test indicated that 4-22% of them were healthy and 7-55% of the seeds were decayed depending on the MC and whether or not they had been exposed to LN (Figure 4A).

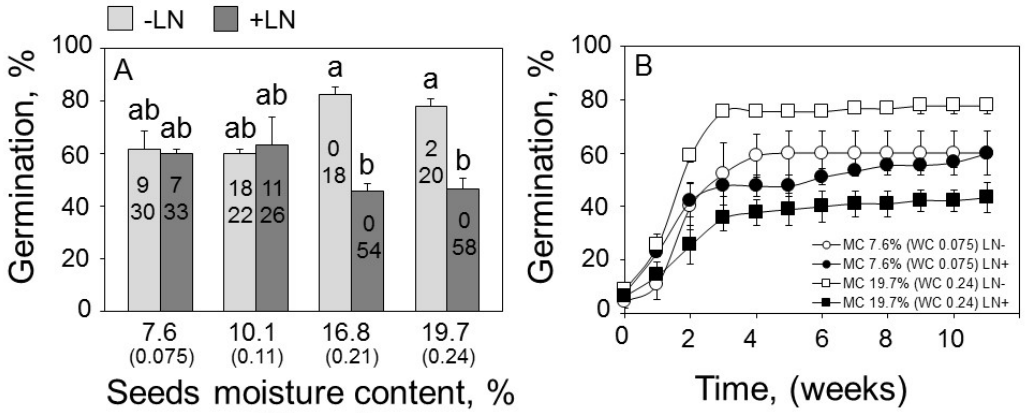

Figure 3. Effect of mazzard cherry (*Prunus avium*) seeds MC and cryostorage (24 h, +LN) on their germinability (A). Non germinated seeds: healthy seeds (upper numbers) + decayed seeds (lower number within each bar). Values with different lower case letters are significantly different at P ≤ 0.05, ANOVA, Tukey's test. Germination time course for mazzard cherry (*Prunus avium*) seeds within MC of 7.6% and 19.7% (B), untreated (-LN) and treated (+LN) for 24 h with liquid nitrogen. Vertical bars indicate standard errors (SEs) of the mean within an isolate treatment. Seeds collected from Bolków provenance.

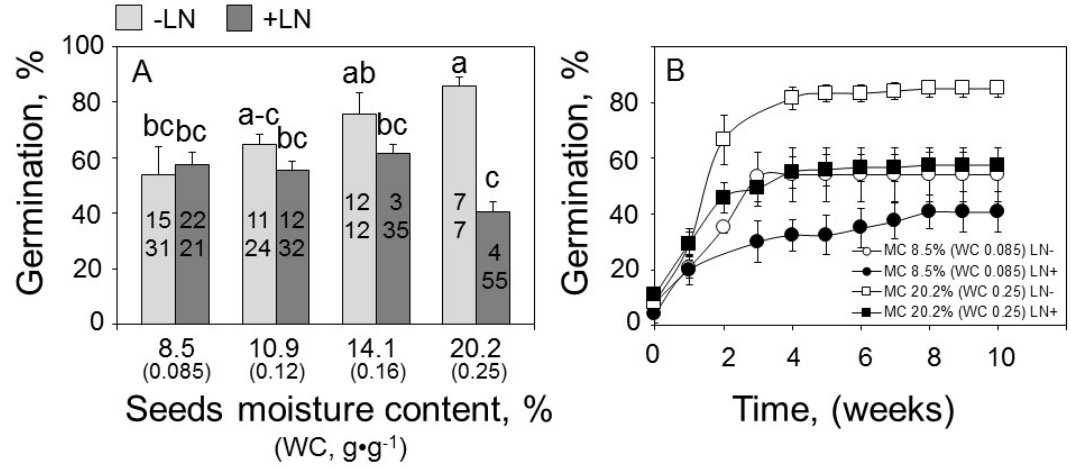

Figure 4. Effect of mazzard cherry (*Prunus avium*) seeds MC and cryostorage (24 h, +LN) on their germinability (A). Non germinated seeds: healthy seeds (upper numbers) + decayed seeds (lower number within each bar). Values with different lower case letters are significantly different at P ≤ 0.05, ANOVA, Tukey's test. Germination time course for mazzard cherry (*Prunus avium*) seeds within of 8.5% and 20.2%, untreated (-LN) and treated (+LN) for 24 h with liquid nitrogen. Vertical bars indicate standard errors (SEs) of the mean within an isolate treatment. Seeds collected from Runowo provenance.

Cryopreserved and non-cryopreserved seeds of mazzard cherry at the MC of 20.2% (WC 0.25 $g \cdot g^{-1}$) had similar rates of germination. The highest germination rate was observed from the first to third week of germination. Germination of seeds exposed to LN was 21% and 32% lower than of seeds not exposed to LN, respectively, at the third and fourth week (Figure 4B).

Cryopreserved seeds from provenance B at 8.5% (WC 0.085 $g \cdot g^{-1}$) did not complete germination in the fourth week contrary to non-cryopreserved seeds and the amount of germinated seeds was increasing until the eighth week of the germination test (Figure 4B).

3.4 Cryopreservation - Seedling emergence

Seedling emergence tests indicated that cryopreserved seeds with MC of 7.6% and 10.1% (WC 0.075 and 0.11 $g \cdot g^{-1}$) (provenance A) did not differ from control seeds (–LN) at the same MC as both were in the range of 12-22% (Figure 5A). Seedling emergence from seeds dried to MC of 7.6% or 10.1% (WC 0.075 or 0.11 $g \cdot g^{-1}$) was significantly lower compared with seedling emergence from seeds at MC of 16.8% or 19.7 % (WC 0.21 or 0.24 $g \cdot g^{-1}$), whether exposed to LN or not. Cryopreserved seeds with MC of 16.8% or 19.7% (WC 0.21 or 0.24 $g \cdot g^{-1}$) emerged at a significantly lower level

(41% and 47%, respectively) compared with seeds with the same WC but which were not exposed to cryogenic condition (70 and 73%, respectively) (Figure 5A).

Seeds exposed and not exposed to LN at MC 19.7% (WC 0.24 $g \cdot g^{-1}$) had a similar seedling emergence time course as they started to emergence at the third week and finished at the seventh week of the test (Figure 6A). We observed, however, that cryopreserved seeds at MC 19.7% (WC 0.24 $g \cdot g^{-1}$) had significantly lower total seedling emergence. No significant differences were observed in the seedling emergence curves of seeds at MC 7.6% (WC 0.075 $g \cdot g^{-1}$) non-immersed (–LN) and immersed (+LN) in LN for 24h (Figure 6A).

Seedling emergence obtained from cryopreserved seeds at MC 8.5%, 10.9% and 14.1% (WC 0.085, 0.12 and 0.16 $g \cdot g^{-1}$) (provenance B) was not statistically different from the emergence levels of non LN-exposed seeds at the same MC (Figure 5B). Cryopreserved seeds at MC in the range of 8.5-14.1% (WC 0.085-0.16 $g \cdot g^{-1}$) had a seedling emergence level of 12-30% depending on the MC. Seeds at MC of 20.2% (WC 0.25 $g \cdot g^{-1}$) stored in LN exhibited significantly lower levels of seedling emergence after thawing (57%) compared with controls (89%), (Figure 5B).

Seeds from provenance B exposed (+LN) and non-exposed to (–LN) had a similar rate of seedling emergence as they started to emergence during the second week and finished at the ninth week of testing (Figure 6B). We observed that cryostored seeds at MC of 20.2% (WC 0.25 $g \cdot g^{-1}$) had a lower (21-31%) seedling emergence rate (especially from the fourth week until the end of seedling

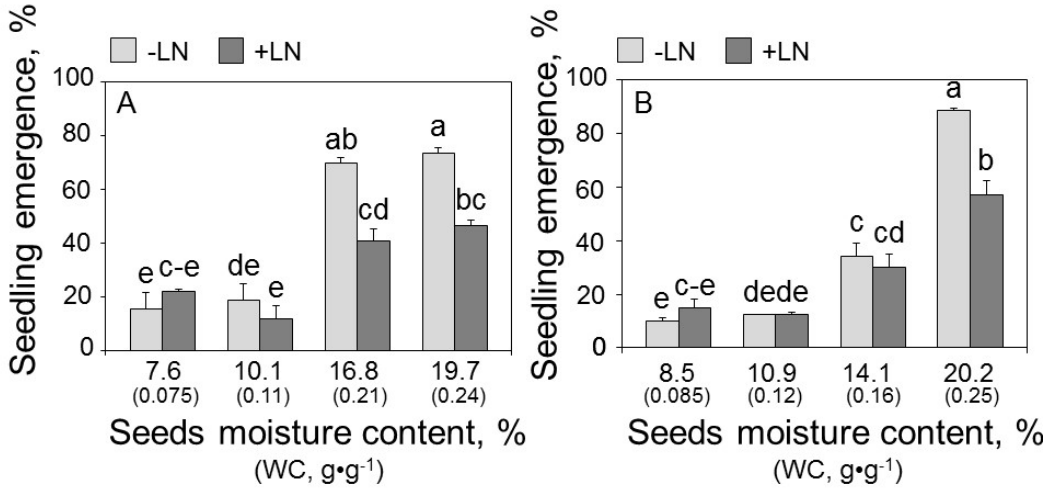

Figure 5. Effect of mazzard cherry (*Prunus avium*) seed MC and cryostorage (24 h, +LN) on their seedling emergence. Seeds collected from Bolków (A) and Runowo (B) provenances. Tukey's test was performed separately for each provenance. Values with different lower case letters are significantly different at $P \leq 0.05$

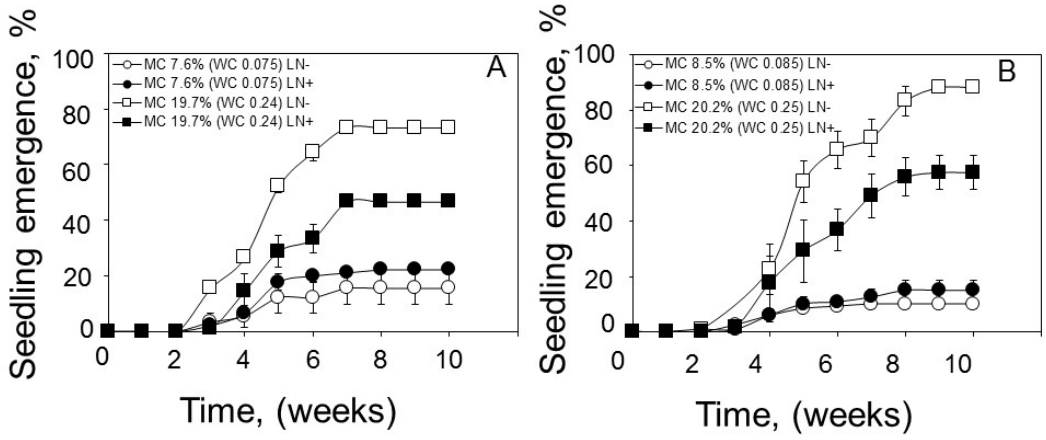

Figure 6. Seedling emergence time course for mazzard cherry (*Prunus avium*) seeds within MC of 7.6% and 19.7% from provenance Bolków (A) and MC of 8.5% and 20.2% from provenance. Runowo (B) untreated (-LN) and treated (+LN) for 24 h with liquid nitrogen. Vertical bars indicate standard errors (SEs) of the mean within an isolate treatment.

emergence) than non-cryostored seeds (Figure 6B). No significant difference was observed in the seedling emergence curves of seeds at MC of 8.5% (WC 0.085 g·g^{-1}) not immersed (–LN) and immersed (+LN) in LN for 24h (Figure 6B).

Seeds of mazzard cherry from both provenances A and B exhibited their highest capacity for seedling emergence after cryopreservation (47% and 57%, respectively) at a MC of 19.7% and 20.2% (WC 0.24 and 0.25 g·g^{-1}), respectively (Figures 5A,5B). Seedling emergence obtained from cryopreserved seeds with a MC of 19.7% and 20.2% (WC 0.24 and 0.25 g·g^{-1}) (provenance A and B, respectively) was significantly lower than that obtained from seeds at the same MCs, but not exposed to LN. Based on the analysis of germination and seedling emergence data of cryopreserved seeds from both the Bolków (A) and Runowo (B) provenance, it was difficult to find the safe range of MC for seeds stored in LN (Figures 3,5). However, in both provenances the trend was for decreased seedling emergence with decreasing of seed MC, whether stored in LN or not (Figures 5 and 6).

The conducted experiments showed that both cryostored and fresh seeds of mazzard cherry gradually exhibited reduced levels of germination and seedling emergence as the MC decreased from approximately 19.7-20.2 % (WC 0.24-0.25 g·g^{-1}) to 7.6-8.5% (WC 0.08-0.09 g·g^{-1}).

4 Discussion

Sensitivity of mazzard cherry seeds to desiccation

Mazzard cherry seeds are placed in the category of orthodox according to the literature [28, 37, 38]. It was shown that they tolerated desiccation to a very low MC (1.6%; WC of 0.016 g·g^{-1}) and sometimes actually exhibit an increase in seedling emergence [25]. However, in that particular study all seeds prior to experiments were dried to a MC of 13% (WC 0.14 g·g^{-1}) and germinated at around 50%; control seeds were also dried to a MC of 13% [25]. Such treatment (pre-drying), and use of a control with MC close to 10% (WC 0.10 g·g^{-1}), is speculated as the reason for observed differences between the previous and current study. The results of the present study indicate that the drying of fresh seeds of mazzard cherry at room temperature from MC 19.7-20.2% (0.24-0.25 g·g^{-1}) to 7.6-8.5% (WC 0.075-0.09 g·g^{-1}) causes a statistically significant decrease in their germination capacity and a significantly greater loss in the rate of seedling emergence. These results are consistent with other studies on cherry seeds reporting that drying of seeds to MC of 12% (WC of 0.13 g·g^{-1}) caused a reduction in germination from 88% to 32% [39]. That study also reported a loss in seed germination to 39-43% for seeds that were air-dried at room temperature for 3-5 days compared with fresh seeds that exhibited 93% germination [35]. In contrast, the opposite results were obtained by Wrześniewski and Tylkowski [40], who reported that germination of seeds desiccated to MC of 10.2% (WC of 0.11 g·g^{-1}) after stratification was at the high level of 96%. Nevertheless, after storage of seeds (at -3ºC) that had been dried to a MC of 10.2% (WC of 0.11 g·g^{-1}) for 1, 2 or 3 months, germination capacity gradually dropped to 90% and then 80% [40].

In the present study, we evaluated the non-germinated seeds after the germination test was completed by cutting them and showed that the percentage of viable, non-germinated seeds was low, indicating that the seeds had been properly stratified. Our results indicate that the observed sensitivity of mazzard cherry seeds to desiccation below MC of 20% (WC of 0.21 g·g^{-1}) places them in the suborthodox (intermediate) category of seed classification. However, further studies concerning seeds derived from different regions, especially from north and south edges of the natural range of occurrence, are needed to assess if similar desiccation tolerance will be also observed in different regions, as desiccation tolerance can vary in some species when they are collected from regions with different heat sum [41].

Interestingly, in the presented study we showed that there was a large difference between total germinability and seedling emergence levels in the case of mazzard cherry seeds dried to low level MC. When seeds were dried to a MC range of 7.6-10.1% (WC 0.075-0.11 g·g^{-1}) (for provenance A) the difference was 41-46%, whereas in the MC range of 8.5-14.1% (WC 0.085-0.16 g·g^{-1}) (for provenance B) it was 41-53%. In cases of higher MC > 16.8% (WC > 0.21 g·g^{-1}) we have not observed such difference. This shows that for the assessment of desiccation tolerance of seeds, the seedling emergence test should be conducted as the germination test is prone to give overestimated results. Only seedling emergence tests show how many regenerated plants can be obtained. The received seedlings are a reliable indicator of reproductive success for the plant and ensure the development of further generations. The difference between germinability and seedling emergence results from seed vigor which can be affected by multiple factors. For instance, *P. avium* seeds are rich in unsaturated fatty acids [26]. It was previously shown that seeds with high lipid content (*Fagus sylvatica* L.) are more sensitive to oxidative stress [42]. It is possible that the observed difference arises from increased activity of reactive oxygen species in desiccated seeds.

4.1 Cryopreservation of seeds

Stanwood *et al.* [43] demonstrated that seeds of mazzard cherry dried to MC of 9% (WC 0.1 $g \cdot g^{-1}$) did not tolerate temperatures below −40°C, while Kuhn and colleagues [24] found on the basis of viability staining tests that mazzard cherry seeds with MC of 10% (WC of 0.11 $g \cdot g^{-1}$) tolerated LN temperature.

Our results indicate a safe range of MC for cryopreserved mazzard cherry seeds may still be problematic, as seeds largely lost their viability when desiccated below the MC of 17-20% (WC of 0.21-0.25 $g \cdot g^{-1}$). Observed rates of seedling emergence drastically decreased when seed MC ranged from 7.6% to 10.1% (WC of 0.08-0.11 $g \cdot g^{-1}$) for provenance A and from 8.5 to 14.1% (from 0.09 to 0.16 $g \cdot g^{-1}$) for provenance B. This was true whether or not the seeds had been exposed to LN. The highest level of seedling emergence after LN treatment (47-57%) was obtained using fresh seeds extracted from the pericarp and dried to a MC of 16.8-20.2% (0.21-0.25 $g \cdot g^{-1}$). Compared with mazzard cherry seeds, broader and lower safe ranges of MC have been reported for seeds considered as orthodox i.e.: common hornbeam (*Carpinus betulus*; 3.2-16.5%; 0,032-0.2 $g \cdot g^{-1}$ WC) [44], wych elm (*Ulmus glabra*; 3.3-17.7%; 0.033-0.22 $g \cdot g^{-1}$ WC) [45], and Philippine date palm (*Phoenix hanceana* Naud. var. philippinensis Becc; 4.8-13.8% (0.048-0.15 $g \cdot g^{-1}$ WC) [28]. Also oil rich seeds such as European hazelnut (*Corylus avellana*) are considered orthodox species that tolerate storage in LN at lower levels of MC 7.2-9.1% (WC 0.08-0.10 $g \cdot g^{-1}$) [31].

Higher seedling emergence after LN treatment from seeds with high MC may be connected with their high lipids content [26, 46]. Similar results showing a higher seed tolerance for LN at higher MC was also observed for oil rich seeds considered as intermediate i.e.: citrus species (*C. grandis*, *C. madurensis*, *C. reticulate*, *C. sinensis*, *C. paradisi*, *C. reticulate*) [32, 47], and coffee species (*C. arabica*, *C. eugenioides*, *C. pseudozanguebariae*, *C. racemosa*, *C. sessiliflora*) [48].

In the literature there are data which suggest that the ability of oily seeds to survive temperature of LN depends strictly on avoidance of intracellular ice formation [30, 43]. Therefore for successful cryopreservation of oily seeds one of the crucial aspects is the amount of unfreezable water. Previous studies showed a negative relationship between seed unfreezable water content and lipid content for many different species producing lipid rich seeds [32, 46, 48]. *P. avium* seeds, as demonstrated by Lazos *et al.* [49], are also oil rich, with the kernel containing 26% of crude oil (% calculated to total dry weight) which is similar to total amount of lipids observed in coffea spp. [48]. Based on the quantity of unfreezable water and lipid content obtained for 16 different species [48], the predicted amount of unfreezable water in mazzard cherry seeds could be around 17-18% (0.21-0.22 $g \cdot g^{-1}$). This amount of unfreezable water is only slightly lower than that observed by us at the highest moisture content (19.7% and 20.2%) at which mazzard cherry seeds tolerate temperature of LN. However, we must take into consideration that the amount of lipid reported in mazzard cherry may vary between different research groups due to a different origin of seeds and differences in the environmental conditions at which maternal plants are growing [50-51]. Therefore, it is possible that *P. avium* seeds from Polish provenance contain a total lipid content that is slightly lower than seeds from Greece assessed by Lazos *et al.* [49]. Thus the amount ofwhole water observed was unfreezable allowing mazzard cherry seeds to tolerate temperature of LN at such high levels of MC 16.8-20.2% (WC 0.21-0.25 g g^{-1}). This is consistent with previous work on the cryopreservation of oil rich seeds [32, 46, 48].

Results obtained in this study are similar to this obtained by Dusser *et al.* [48] for five species of *Coffea* (*C. arabica*, *C. eugenioides*, *C. pseudozanguebariae*, *C. racemosa*, *C. sessiliflora*). They observed that there was a decline in seed survival after LN exposure with dehydrating seeds to water content below the specific HMFL. On the basis of our results and previous studies we could conclude that oil rich seeds better tolerate temperature of LN at higher MC and for long term storage they should be cryopreserved at their HFLM.

The presented data indicate that the mazzard cherry seeds should be classified as suborthodox (intermediate) rather than orthodox, due to the fact that these seeds are not resistant to drying. Also the response of *P. avium* seeds to temperature of LN is similar to seeds of species classified to intermediate category.

In conclusion the presented data showed that even initial drying below a level of 14.1% MC in the oil reach seeds of *Prunus avium* could have negative effect on their viability, especially in regard to seedling emergence. Our work confirms that the cryopreservation of mazzard cherry seeds could be applied as a successful method of long term preservation of genetic resources for this species. The highest viability after cryopreservation was obtained at HMFL (MC around 19.7%-20.2%). On the basis of desiccation tolerance and behavior after cryo-treatment *P. avium* seeds should be categorized as suborthodox (intermediate).

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