EMBRYO DEVELOPMENT AND DESICCATION TOLERANCE OF *HEPATICA NOBILIS* SCHREB. SEEDS

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Abstract

Hepatica nobilis seeds exhibit deep morpho-physiological dormancy and contain undeveloped embryo at the time of dispersal. The objective of our study was to investigate the development of *H. nobilis* embryo in relation to the natural vegetation season in Poland, and to test the sensitivity of seeds to desiccation. Achenes were harvested on May 20, 2011, at the time of natural dispersal, and were sown immediately. Our observations, with the use of paraffin sectioning method, revealed that at the moment of seed dispersal, embryos was in the globular phase. Five weeks after sowing they developed into the early torpedo stage (mid-June). Then, during the summer, embryo growth slowed down and cotyledons continued their expansion in the endosperm during the hot season. From mid-October, radicals started to grow and emerge from the seeds, but did not fully germinate before winter. Germination of *H. nobilis* seeds was observed in the spring in week 43 after sowing. In the second experiment, the seeds were desiccated under laminar air flow for 0 (control) to 9 h to estimate the influence of the seed water content on germination. Fresh seeds contained 62% of water and germinated in 73%. The seeds dried for 7.5 and 9 hours contained 34-32% of water and did not germinate. Hepatica seeds are sensitive to dehydration and are not a suitable plant material for storage in gene banks.

Key words: *Hepatica nobilis*, Embryo, Achenes and Desiccation tolerance.

Introduction

Hepatica nobilis Schreb. is a rosette hemicryptophyte, an important component of the herbaceous layer of mesophytic, deciduous forests of the northern hemisphere of temperate climate zone. It is blooming early spring in March, and the seed occurred at the end of May. Hepatica has three-lobbed leaves creating compact undergrowth that remains over winter. Numerous stands of this plant can be found in nature reserves and national parks in Europe (Piękoś-Mirkowa &Mirek, 2006, Żuraw *et al.*, 2013, Jagel, 2014). An increasing demand for conservation of natural resources will continue to degrade habitat and push a growing number of plants towards extinction. Therefore we have to face this challenge and efficiently protect and preserve biodiversity (Havens *at al*., 2006, Ashraf *et al*., 2012, Zielińska *at al.*, 2018).

Early-spring flowering, abundant and evergreen foliage and shade-tolerance make hepaticas very valuable perennials for parks and gardens with naturalistic or forest zones, when planted together with other native groundcovers (Jagel, 2014, Kameoka *et al.*, 2016). Unfortunately, due to problems with efficient propagation, hepaticas are rarely present in the commercial offer of nurseries. Traditional methods of vegetative propagation are rhizome cuttings and mother plant division (Szewczyk-Taranek & Pindel, 2008) used mainly for varieties and forms. There are known micropropagation methods involving organogenesis by axillary buds and the induction of adventitious buds and somatic embryos on leaves (Nomizu *et al*., 2003, Szewczyk-Taranek & Pindel, 2009). Somatic embryogenesis was also successfully performed on anthers (Nomizu *et al*., 2005) and seedlings (Szewczyk-Taranek & Pawłowska, 2014).

In a nursery practice, fresh seeds of hepaticas are best sown immediately after harvest, collected in late May, early June, and germinated next spring. Hepaticas fruits are numerous, hairy achenes forming the fructification. When the seeds are ripe, the flower stalk with fructification bends and seeds flake off on the ground. Seeds have elaiosomes and are collected and spread by ants (myrmecochory). Seeds are dispersed when they are still green, indicating that they may not be fully mature at this point (Barykina & Gulanian, 1974, Listl *et al.*, 2017). Hepatica seeds are characterized by complex mechanisms of morpho-physiological dormancy. Typically, morphologically dormant seeds are defined as having small, underdeveloped embryos, and need dormancy with an appropriate temperature treatment to allow differentiation and growth of embryos before radicals emerge and germination can occur, significant for many species from temperate climate zone (Baskin & Baskin, 2004, Erken & Özzambak, 2017). Baskin & Baskin (1985) reported that the seeds of *Hepatica acutiloba* (North American *Hepatica* species) during dispersal had rudimentary or underdeveloped embryos, and they started to grow during the period of hot weather, and developed radicles before winter, but epicotyls and cotyledons emerged only after the chilling period. Later study by Nomitsu *at al.* (2004) on *Hepatica nobilis* var. *japonica* brought similar results with respect to the developmental stage of embryos after particular temperature treatments.

Hepaticas seeds are not commercially available because they have to be sown fresh immediately after harvest. For this reason, the development of a method for long-term ultra-low-temperature preservation (cryopreservation) appears to be an optimal solution. The evaluation of seed tolerance to desiccation is a key factor decisive for successful cryopreservation (Chandel *et al*., 1995). Hence, it is necessary to classify hepatica seeds to one of the groups distinguished by Roberts (1973) and Elis *et al*., (1990): orthodox–surviving desiccation below 5% humidity; suborthodox (intermediate)–tolerating desiccation in the range of 6-10%, and recalcitrant–tolerating 12-50% hydration. The distinction between recalcitrant and orthodox seeds provides a useful determination of cryopreservation technique applied (Walters *et al.*, 2013).

The aim of this study was to clarify the process of post-dispersal embryo development in *Hepatica nobilis* seeds. We put special emphasis on the sensitivity of embryos to desiccation in order to initiate further research on adequate cryopreservation procedures for *ex situ* protection of this valuable plant.

Materials and Methods

Collection site and plant material: *Hepatica nobilis* seeds (achenes) were collected from a population grown in a deciduous forest in Krzeszowice Forestry District 50°07'14.0"N 19°38'25.1"E, the area of the Kraków-Częstochowa Upland in Poland (Fig. 1a). Whole fructifications were collected on May 20, 2011, just before the dispersal of achenes (Fig. 1b). After the harvest, the achenes were husked from frutifications in laboratory conditions, and well developed ones were selected for further research (Fig. 1c, d). The weight of 1000 fresh seeds was estimated on the same day.

Seed desiccation and dry mass assessment: Seeds were exposed to dry, sterile air in the laminar flow chamber for 0 (control), 1.5, 3, 4.5, 6, 7.5 and 9 hours, at room temperature (± 23°C). In each treatment, there were 200 seeds (4 replicates of 50 seeds) for further germination evaluation, and additionally 3 replicates of 20 seeds for water content assessment. The dry mass of seeds was determined at the time of removal from the fructification (control) as well as after each drying time. The samples were placed in weighing dishes, 20 seeds per dish and dried at 103°C (± 2°C) in a Sanyo MOV-112S oven, for 17 hours. The dry mass content (%) was calculated according to the following equation: $DM = (m_2 – m / m_1 – m) \times 100$, where: m – weight of empty weighing dish, m_1 – weight of weighing dish with plant samples before drying, m_2 – weight of weighing dish with plant samples after drying. Seed water content (WC) is expressed as the proportion of dry mass (DM).

Germination trial: Fresh seeds after harvest (control) and batches of seeds after drying treatments were sown in a 12-cm diameter pots filled with substrate (Klasmann KTS2, Poland) and sand mixture 2:1, and covered with a thin (5 mm) layer of sand. The pots were placed into trays and were protected with a double shading mat and cultivated at the field collection of ornamental plants, Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow. The pots with seeds were regularly watered during the vegetation season. To investigate the effect of dehydration on germination, four pots were used for each drying period containing 50 seeds each.

During the experiment, the temperatures were recorded at a meteorological station at the greenhouses of The Faculty of Biotechnology and Horticulture, University of Agriculture in Kraków (Fig. 2). Germination (percentage of germination) was assessed at the spring time, (week 10-12 of 2012). The seeds that grew into seedlings and developed cotyledons were considered as germinated. The results from the experiment were subjected to analysis of variance (ANOVA). Comparisons of the means were made using Duncan's multiple range test, (p=0.05) implemented in the Statistica software (Stat Soft).
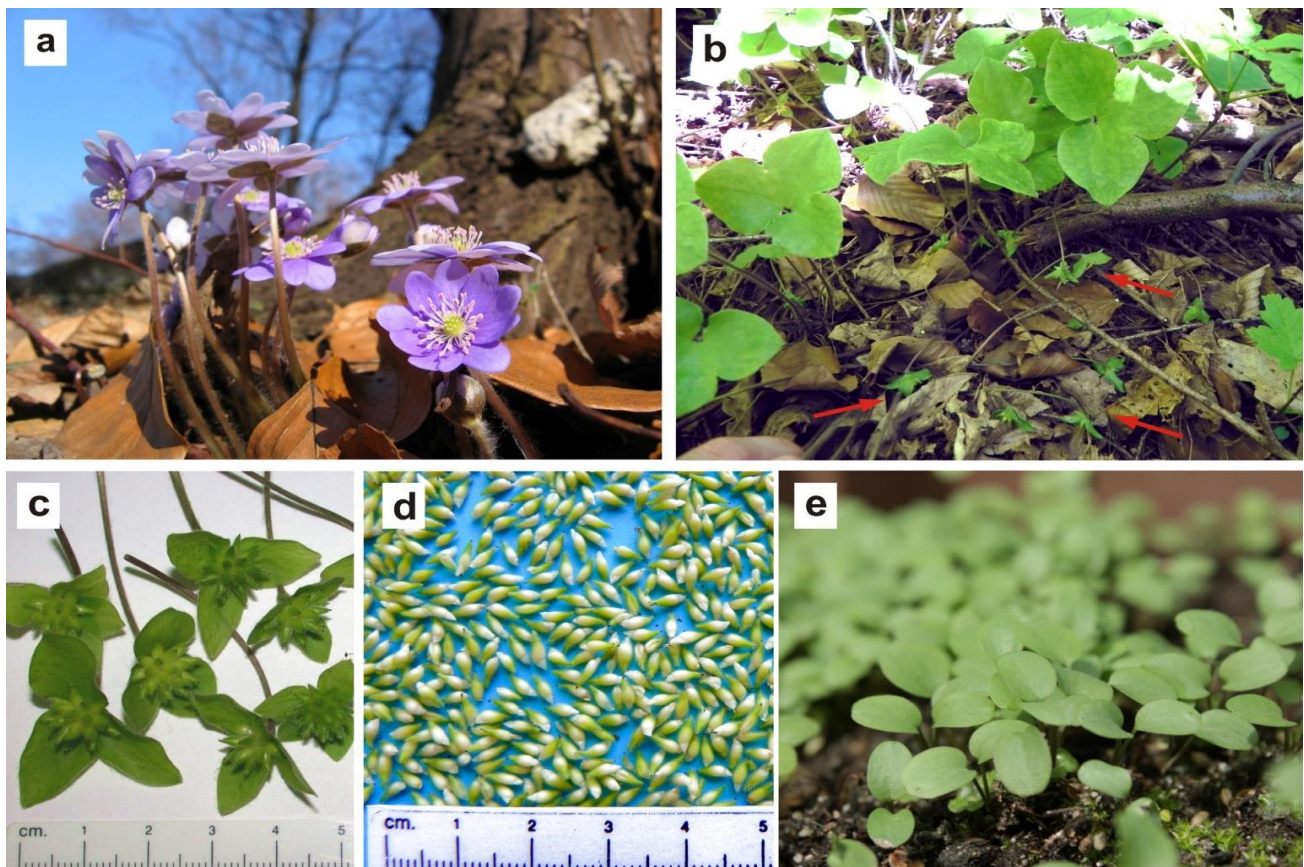

Fig. 1. *Hepatica nobilis*: a – flowering in the natural environment in deciduous forest, of April 8, 2011, southern Poland; b – plants with fructifications (red arrow), May 20, 2011, day of seed collection; c –fructifications; d – fresh achenes detached from frutifications, before sowing (May 21, 2011); e – hepatica seedlings, 45 weeks after sawing, April 15, 2012.
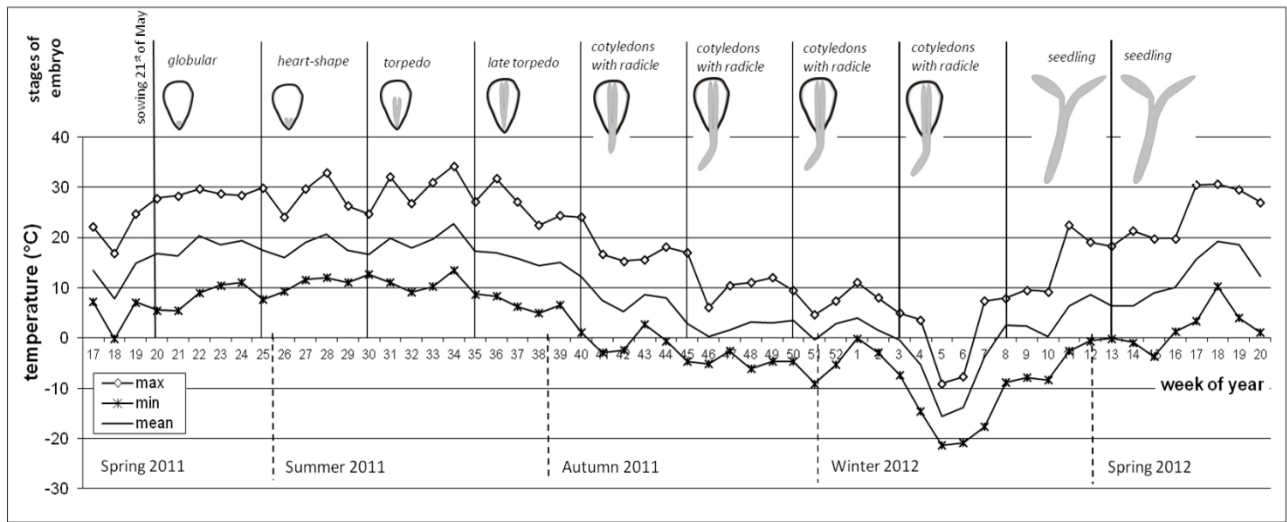
Fig. 2. Developmental stage of embryos of *Hepatica nobilis* seeds in relation to the vegetation season 2011/12 and temperatures. Data recorded during the field experiment from May 2011 to June 2012 were provided by the meteorological station at the Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, Poland
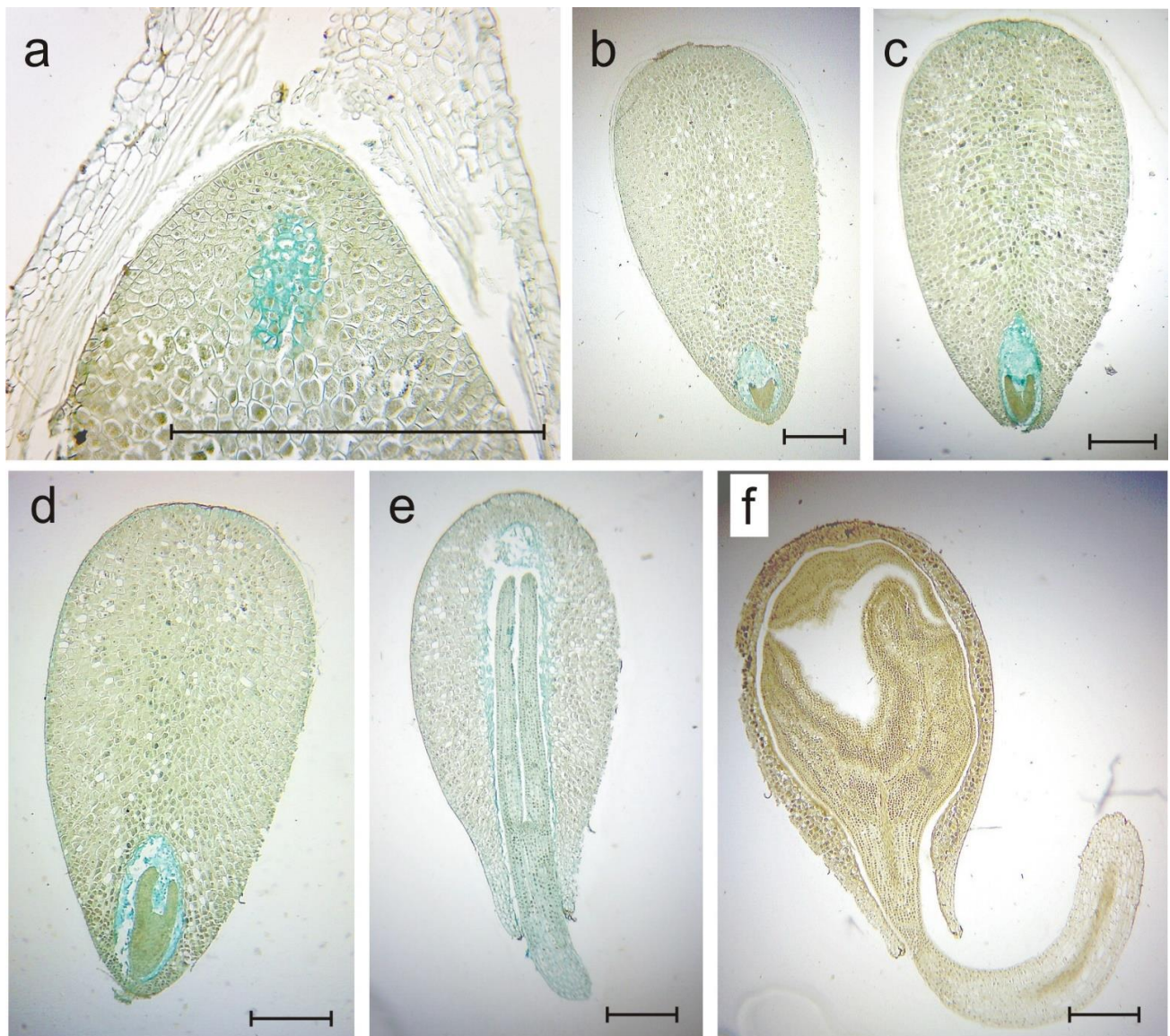

Fig. 3. Embryo development in *Hepatica nobilis* seeds – paraffin sections: a –time of dispersal (20th of May); b – heart-shaped embryo (after 5 weeks); c – early torpedo stage (after 10 weeks); d – torpedo stage (after 15 weeks); e – cotyledon shaped with developing radicle (after 20 weeks); f – cotyledon stage with growing root (after 30 weeks). Bar = 0.5 mm.
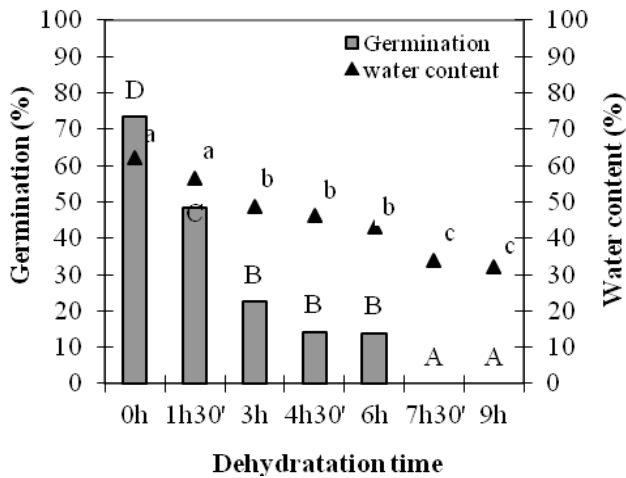
Fig. 4. The effect of air dehydratation of *Hepatica nobilis* seeds on the water content and germination.
Means followed by the same letters, for each sample separately, are not significantly different according to the Duncan multiple range test ($\alpha = 0.05$)

Histological observations: For the paraffin method, the seeds were collected after sowing at five-week intervals (10 seeds per each observation) and were fixed in a solution of FAA for 1 day, following dehydratation with a gradual ethanol series (from 80% to 99.8%) and then with a four-step graded series of ethanol-chloroform (33-100%). The samples were kept in a paraffin heater at 62°C for min 6 months in order for paraffin to fully infiltrate the dehydrated samples. Then they were embedded in solid paraffin blocks, sectioned at 10 μm on a rotary microtome (Leica RM 2145) and stained with haematoxylin (Filutowicz & Kużdowicz 1951). Sections were examined and photographed using a Zeiss Axio Imager M2 stereoscope microscope equipped with a digital camera.

Results and Discussion

Seed longevity and germination are useful for the plant conservation. Research on seed development and desiccation sensitivity reported here arose from the problems with the storage of hepatica seeds and concerns related to seed banking possibilities. Ranunculaceae are very variable in terms of fruits and seeds, but most species exhibit morphlogical dormancy, with rudimentary or linear embryos, and many of them are known as short-lived seeds (Baskin & Baskin 1998). Ranunculaceae is a family with the lowest seed viability (Godefroid *et al.*, 2010).

Hepatica nobilis seeds at dispersal have embryos in the globular stage, the remaining portion is developed endosperm (Fig. 3a). Optimum seed harvest time for hepaticas is the time of natural dispersal (6-8 weeks after flowering in Poland). The only difference is that the ripe achenes very easily detach from receptacle, and within one week they are scattered on the ground and dispersed by ants, and thus it is impossible to collect them later. To start the experiments, achenes were collected when ripe and then they were weighed (Fig. 1d). The weight of 1000 fresh seeds was 4.22 g (data not shown). Our results are similar to those reported by Mark & Olesen (1996), who collected seeds of *H. nobilis* in Hestehaven, Denmark

(56°3'N, 9°54'E). The mass of 1000 seeds was 4.93 ± 1.03 g. In other study by Michael *et al.*, (1988), the mean weight of one *H. nobilis* seed was 3.003 ± 0.021 mg. The seeds were harvested at the collection of the University of Ilionis, United States (6°40'N, 88°13'W), but those differences might be due to the lack of precise information about the time of seed harvest.

Histological observations of achenes, embryo development and germination, were carried out every 5 weeks after sowing during the next 50 weeks. The results showed that embryo growth stages are strictly connected with the time of the year and temperature (Fig. 2). The embryos were in the globular stage on the day of seeding (May 21, 2011) (Fig. 3a). After 5 weeks (June 29, 2011), the embryos reached the heart-shape stage (Fig. 3b). The average temperature during this period was 12.4°C and was above 5°C at all times. Ten weeks after sowing (Fig. 3c), we observed that the embryos reached the torpedo stage and the average temperature was 18°C. During the summer, the embryos remained at the torpedo stage, reaching half of the size of the seed (Fig. 3d). The average temperature at this time was 19.5°C. The cotyledon stage was observed on October 12, 20 weeks after sowing (Fig. 3e) and the hypocotyl started to grew when the average temperature significantly decreased to 10.9°C. After another 5 weeks (November 14), cotyledons extended further and the radicle grew out of the seed reaching an average of 3-12 mm. The average temperature during this period declined to 6.4°C. On December 19 (30 weeks after sowing), the embryos developed cotyledons, but still were covered in the endosperm; hypocotyl began to expand (Fig. 3f). The average temperature at that time was 2.3°C. The development of the seedlings was arrested when they survived 12 weeks of winter with a minimum average temperature of -2.1°C. In mid-March 2012, when the average temperature rose to 6.4°C, 43 weeks after sowing, cotyledons and epicotyls resumed their growth and the emergence of cotyledons above the substrate surface was regarded as successful germination (73% seeds germinated) (Fig. 1e, 2). The observations lead to the conclusion that the mechanisms of hepatica seed dormancy are characterized by two distinct periods of physiological dormancy, and are an adaptation to the moderate climate; in this manner, the process of germination is optimized in time. Similar results were obtained for *H. acutiloba* (Baskin & Baskin, 1985), *H. nobilis* var. *japonica* (Nomizu *et al.*, 2004) and *H. asiatica* (Chon *et al.*, 2015). During the seed dispersal, the embryos were in the globular stage and occupied approx. 1/8 of their length, and were surrounded by the endosperm. Similar underdeveloped embryos are also found in many other Ranunculaceae species, such as *Cimicifuga racemosa*, *Helleborus niger*, *Anemone nemorosa* (Baskin & Baskin, 1985, Niimi *et al.*, 2006, Ali *et al.*, 2007). The dormancy of morpho-physiological seeds in those plants manifested as germination arrest, which continued until the seeds were exposed to both hot and cold stratification (Baskin & Baskin, 1985). In a study on *H. nobilis* var. *japonica* embryo development, Nomizu *at al.* (2004) observed that temperatures had an effect on seed dormancy and the transition of embryos to next stages. Globular embryos developed into the torpedo stage 9 weeks after sowing. When the temperature rose (in summer), the growth of embryos stopped - the first stage of

physiological dormancy. The cotyledon stage of embryos was observed in the autumn, at 25 weeks of cultivation, when the average weekly temperature dropped below 20°C. The roots appeared in week 29 after sowing.

Comparing the results of Nomizu *et al*., (2004), we can say that the seeds of *H. nobilis* in our climate reach successive stages of development in similar time periods, but germination appears one month later than in Japan. This difference is probably due to the fact that in our latitude the average temperature falls below 10°C in October, but in Japan, in the area of the University of Niigata, where the experiment was conducted (37°55'N 139°03'E), the temperature falls below 10°C in mid-November. Our observations confirm the fact that the development of the radicle preferably occurs at a temperature of 15°C, while it is arrested at lower temperatures. First of all, the development of the root system in the autumn provides better supply of water and nutrients for seedlings growing in the spring (Baskin & Baskin, 1985). Cotyledons covered at that time are not exposed to the autumn-winter frosts. This temperature cycle is an adaptation of plants to the temperate climate with four seasons. Baskin & Baskin (1985) studied the phenomenon of epicotyl dormancy in *H. acutiloba*. The plants were cultivated in an unheated plastic tunnel at the University of Kentucky (38°01'N 84°29'W). Most of the roots sprouted in the period from October 26 to November 2, when average daily temperatures ranged from 21.9 to 12.4°C. Cotyledons developed between February 22 to April 4, when daily temp. ranged between 18.2 and 5.4°C. In that case, the temp. in autumn were higher than in our climate (the average daily temp. of approx. 8°C), which resulted in an earlier root growth. The control group of seeds was kept all season at a temp. of 10-20°C. After 165 days, no roots were observed, and later, even after 345 days, no development of cotyledons was detected. This is consistent with the notion that cotyledons need cold stratification to expand and start to grow at a temp. above 5°C, followed by a period of low temp. below 0°C.

Estimation of hepatica seeds sensitivity to drying is the first step for selecting *ex situ* conservation strategy. Dry matter and water content were estimated both in fresh seeds, and those subjected to drying under laminar air flow for 1.5, 3, 4.5, 6, 7.5 and 9 hours. It was found that the reduction of seed water content had a significant effect on germination. Three-hour drying reduced water content to 48.6%. Desiccation for 6 hours caused a decrease in the water content down to 43.0% and further drying for the next 3 hours reduced it to 32.0%, which constituted 30% of the total water content of the freshly collected seeds (Fig. 4). Control part of the seeds sown next day after harvest (May 21, 2011) - without dehydration - germinated in 73% during the spring next year (Fig. 4). The seeds dried for 3 hours germinated in 22.5%, but the germination was not observed in samples treated with dry air for 7.5 and 9 hours (Fig. 4). The results proved that the seeds of hepatica were dehydratation-sensitive, and showed characteristics of recalcitrant seeds. Drying the seeds greatly decreased their germinability. Maturation drying is absent or significantly reduced in recalcitrant seeds, and at dispersal time, the seeds show little water loss and are metabolically active.

The seeds of hepatica contained approx. 62% water after harvest. According to Berjak *et al*., (1990), fresh weight of recalcitrant seeds contained 30-80% water, while orthodox seeds contained only 10% of water (Gumilevskaya & Azarkovich, 2007). The relatively high water content suggests that hepatica seeds might be considered as a recalcitrant type. This was also demonstrated in our experiment regarding the effect of desiccation on germination. Seeds that loose viability at a 30-50% water content reduction are classified as short-viable seeds (Gumilevskaya & Azarkovich, 2007).

Cryopreservation in seed banks is one of the possibilities of storing this kind of seeds, and it is the main form of *ex situ* conservation of plant genetic resources. Seed preservation methods are important not only for conservation, but also for breeding programs, because genetic diversity is most efficiently sustained through seeds. Future experiments on preservation of hepatica seeds in liquid nitrogen are very important in terms of *ex situ* conservation of these valuable species.

Conclusions

Hepatica nobilis seeds show morpho-physiological dormancy and seeds are dispersed when the embryo is in the globular stage. The embryo requires a lower temperature period (about 15°C) to develop the radical and then needs a cold period (about 4°C) to initiate the growth of cotyledons. Seed germination occurred in week 43 after sowing. Hepatica seeds are sensitive to dehydratation, as drying inhibits their germination. Seeds are not a suitable plant material for storage in gene banks.

Acknowledgment

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