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Seed dormancy and germination of an endangered coastal plant *Eryngium maritimum* (Apiaceae)

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Abstract. *Eryngium maritimum* is a coastal plant of the Apiaceae family. This species is threatened in several countries where it reaches the northern limits of its distribution area. Existing populations in the Baltic Region and Scandinavia are potentially affected by the low efficiency of its generative reproduction. We studied physiological aspects of its germination and dormancy breaking requirements using seeds collected from two Latvian populations. Seeds were subjected to cold and warm stratification and treatment with gibberellic acid. We monitored the development of seed embryos throughout the process of stratification at different temperatures and built a size-class structure of embryo development within a batch of seeds to visualize the developmental progress and to compare different treatments. The final germination percentage and germination rate increased after cold stratification at 5 °C in seeds germinated at 25/10 °C. Maximum germination was achieved after four months of cold stratification. Treatment with gibberellic acid had a similar dormancy breaking effect that was enhanced by previous warm stratification. The relative size of embryos increased during cold stratification, although this increase was not uniform. We conclude that in seeds of *E. maritimum*, embryo growth during stratification is required before germination can start and that growth is enhanced by warm stratification. Breaking the physiological component of seed dormancy requires cold stratification that can be substituted by treatment with gibberellic acid. We suggest that germination characteristics of seeds and the structure of embryo development within seeds in a particular population may reflect the state of the plant population itself and provide valuable information for research on the biology of this species.

Key words: cold stratification, dormancy, gibberellins, seed embryos, size-class.

INTRODUCTION

Eryngium maritimum L. is a littoral species growing on sand dunes and shingle beaches. Although it is listed among the species widespread in western and southern Europe, overall its population is declining (Van der Maarel & Van der Maarel-Versluys, 1996). The species is included in the Red Data Book of Latvia (Fatare, 2003) and is protected in several other European countries. In northern Europe and in the Baltic Region it grows near the limits of its current area of distribution

and therefore is at a greater risk of extinction because in small and isolated populations there is a risk of inbreeding depression. For example, in *Eryngium alpinum* partial self-incompatibility causes lower seed set in selfing plants and selfing negatively affects seed mass and germination (Gaudeul & Till-Bottraud, 2003).

A survey of *E. maritimum* populations along the Skagerrak coast was conducted and the persistence prospects of this species were evaluated as low in these localities due to the small size and fragmentation of individual populations (Curle et al., 2007). Population surveys were also conducted in Poland and Lithuania (Olsauskas, 1996; Labuz, 2007). While in some cases the decline of *E. maritimum* is linked to habitat disturbance, a population can be threatened also due to limited generative reproduction, affected both by low seed production and low germination as well as high juvenile mortality (Curle et al., 2007; Aviziene et al., 2008). It was suggested also that a decrease of the physiological fitness of *E. maritimum* individuals in northern populations is associated with lower photosynthetic productivity due to high precipitation and low air temperature (Andersone et al., 2011).

Seeds of the Apiaceae family are often morphologically or morphophysiologically dormant (Finch-Savage & Leubner-Metzger, 2006). Seeds with morphological dormancy have small, differentiated embryos that need time to develop before a seed can start to germinate (Baskin & Baskin, 2004). Morphophysiologically dormant seeds also have a physiological component of dormancy and therefore require a dormancy-breaking pretreatment. Depending on the type of the physiological component, different combinations and length of warm and cold stratification can be required (Baskin & Baskin, 2004). Preliminary research on *E. martimum* seeds (J. Necajeva, unpublished results) confirmed that seeds of this species have underdeveloped embryos at the time of maturation and seed dispersal and, in addition, seeds require dormancy-breaking treatment (cold stratification) to germinate. Other researchers also reported that cold stratification is necessary to break the dormancy of *E. maritimum* seeds (Walmsley & Davy, 1997; Curle et al., 2007).

The results of previous studies probably give sufficient information to develop an effective method of germinating *E. maritimum* seeds. However, to our knowledge, there have not been any detailed studies of the physiology of the germination process in *E. maritimum*. The physiological component of seed dormancy is related to the effect of temperature on dormancy breaking and germination. From the point of view of seed physiology, this action of temperature is related to changes in the activity of gibberellins and abscisic acid in the seed. This is why exogenous gibberellins can in some cases substitute for the effect of dormancy-breaking temperatures (Nikolaeva et al., 1985; Finch-Savage & Leubner-Metzger, 2006). When seeds have complicated physiological mechanisms of dormancy release and germination, it is difficult to predict or model germination in natural conditions because the effects of temperature and soil moisture are not straightforward. The morphological component of dormancy can be studied using embryo size-class structure, and is a way to understanding the dynamics of germination within a batch of seeds. We can put forward the hypothesis that the germination rate

depends on the degree of embryo development at the time of maturation. At the same time, we suggest that the size-class structure can be a tool to compare seed development in different populations and study the effects of different environmental factors on seed development. However, this particular research is limited to the only two populations of *E. maritimum* found in Latvia, so wider research is necessary.

MATERIALS AND METHODS

Seed collection

Fruits (schizocarps) of *E. maritimum* were collected in September 2008 in Užava (57°14′N, 21°25′E) and Ziemupe (56°47' N, 21°03' E). Both sites are located on the Kurzeme coast of Latvia on the Baltic Sea. The plants grow in small groups situated on white dunes. Fruits were considered mature if they were brown and loosely attached to the infructescence. Fruits from central and lateral branches were collected separately. For stratification and germination tests fruits were divided into individual mericarps, further referred to as 'seeds'. The length of seeds (with the pericarp removed) from the two types of infructescences (central and lateral) was measured under a stereomicroscope using millimetre paper.

In the Ziemupe population there were 25 separate groups of plants, monitored from 2005 to 2009. The number of individuals in each group changed over this period and reached up to seven individuals. In Užava there were 15 similar groups (Andersone et al., 2011).

Pre-germination treatments of the seeds

For cold stratification treatment, seeds from central and lateral branches were separately imbibed and incubated in Petri dishes between moist filter paper layers at 5 °C (in darkness). Stratification lasted for one to four months (cold stratification) with and without one month of warm stratification before the start of cold stratification (Table 1). Germination was tested after each month of cold stratification.

Table 1. Pre-germination treatments of seeds from Užava and Ziemupe

Warm stratification (25/10 °C), months	Cold stratification (5 °C), months	Treatment with gibberellic acid	Seed position
0	1–4	0	Central and lateral
1	1–3	0	Central and lateral
0	0	0.1, 1.0, and 10.0 mM	Central
1	0	0.1, 1.0, and 10.0 mM	Central and lateral

For warm stratification treatment seeds were incubated in the same way at 25/10 °C (12/12 h). After a month of warm stratification seeds were treated with gibberellic acid (GA_3) or transferred to 5 °C for one to three months.

To evaluate the initial germination and possible effect of storage at room temperature, a batch of seeds was germinated at 25/10 °C without cold stratification or GA_3 treatment one week after collection and another batch was germinated in the same conditions after three months of storage at room temperature. Germination tests lasted for five and nine weeks, respectively.

In GA_3 treatments seeds were soaked overnight in 0.1, 1.0, or 10.0 mM GA_3 solution (Duchefa Biochemie) without pre-treatment or after one month of warm stratification. Seeds were germinated on top of a double filter paper layer moistened with the same GA_3 solution.

Germination tests

Each treatment had three or four replications of ten seeds (except in cases where there were not enough seeds and fewer seeds had to be used). The total number of seeds per individual treatment was 15–40, therefore the total number of seeds per collection site (both seed positions) was 30–80, the total number of seeds per stratification or GA_3 treatment was 60–160 (both collection sites and both seed positions).

Germination tests lasted at least one month, but in treatments where the germination rate was low, the test was extended up to three months if there was no fungal infection. Germinated seeds were counted three times a week; a seed with an at least 1-mm-long visible radicle was considered germinated.

Seeds were germinated at 25/10 °C (12/12 h) (Narva luminofluor 58 W fluorescent tubes, average photon flux density of 35 ± 1 μmol m^{-2} s^{-1}). Seeds were soaked overnight in distilled water and germinated in glass Petri dishes on top of a double filter paper layer moistened with distilled water. Seeds were stored at 5 °C and at room temperature, at uncontrolled relative humidity.

The germination rate was calculated as $T50^{-1}$. To calculate the value of $T50$, i.e. the time in days required for the germination of 50% of the seeds, the proportion of germinated seeds was plotted against the germination time. A linear trend was fitted to the linear part of the germination curve and the equation of this trend was used to calculate the time for 50% germination; R^2 for the fitted trend line was at least 0.8 (Table 2).

Germination data (the number of germinated seeds) was analysed using the chi-squared test ($P = 0.05$) to test the effect of different pre-germination treatments (cold stratification, warm stratification, and GA_3), collection site (Ziemupe or Užava), and seed position (central or lateral). When the difference between collection site and seed positions was not statistically significant, the data were pooled and the effect of cold stratification and GA_3 was analysed using the total

Table 2. Germination rate in seeds germinated after cold stratification (CS) and seeds treated with GA_3 with and without warm stratification (WS) before cold stratification or GA_3 treatment and linear regression models used to calculate $T50$ (time to 50% germination calculated as x at $y = 50$)

CS, months	WS, months	Linear regression model	R^2	$T50$	Germination rate
1	0	$y = 0.16x - 1.44$	0.91	332	0.003
2	0	$y = 0.76x - 4.19$	0.95	71	0.014
3	0	$y = 7.60x - 67.85$	0.82	15	0.067
4	0	$y = 8.57x - 14.64$	0.93	9	0.111
1	1	$y = 0.46x - 0.77$	0.87	110	0.009
2	1	$y = 2.76x - 3.6$	0.92	15	0.067
3	1	$y = 9.86x - 32.69$	0.89	8	0.125
GA_3, mM	WS, months	Linear regression model	R^2	$T50$	Germination rate
0.1	0	$y = 2.63x - 58.56$	0.98	177	0.006
1	0	$y = 0.72x - 34.05$	0.91	117.1	0.009
10	0	$y = 0.41x - 22.28$	0.98	41.35	0.024
0.1	1	$y = 0.27x - 4.0$	n/c	199	0.005
1	1	$y = 2.57x - 19.72$	0.97	27.14	0.037
10	1	$y = 4.43x - 15.93$	0.84	14.88	0.067

n/c– not calculated.

number of germinated seeds from both sites and neglecting the seed position. A chi-squared test on 2 × 2 contingency tables with Yates' continuity correction and linear regression was performed using Microsoft Excel.

Embryo : seed ratio

To describe the development of seed embryos during cold stratification a batch of seeds from Užava was stratified at 5 °C during six months. A sample of seeds (15–32 seeds in a sample) was taken each month to assess embryo development. Seeds were dissected under a stereomicroscope and the degree of the development of each embryo was assessed visually using a six-point scale (1 – embryo length less than 1/4 of the seed length, embryo is heart-shaped; 2 – embryo length ca 1/4 of the seed length, cotyledons and radicle of equal length; 3 – embryo length ca 1/3 of the seed length, pronounced cotyledons and radicle; 4 – embryo length ca 1/2 of the seed length, radicle twice as long as the cotyledons, cotyledons leafy; 5 – embryo length 2/3 of the seed length; 6 – embryo length nearly equal to the seed length).

Before the measurements, the seeds were incubated for two months at 5, 15, or 20/10 °C (12/12 h) in darkness in Petri dishes on 10 g L^{-1} agar medium. Each

sample contained 24 to 30 seeds. A sample of 32 seeds stored dry, and imbibed overnight in distilled water before dissection, was used as the control. To measure the embryo : seed length ratio (E : S) the imbibed seeds were dissected and photographed using a stereomicroscope (Zeiss AxioCam). Then the length of each seed and embryo was determined using a camera software package (AxioVision). After that E:S was calculated and a size-class rank was assigned to each embryo using a 6-point scale. Size-classes were assigned as follows: class 1 – E : S = 0.0–0.19; class 2 – E : S = 0.20–0.29; class 3 – E : S = 0.30–0.39; class 4 – E : S = 0.40–0.59; class 5 – E : S = 0.60–0.79; class 6 – E : S = 0.80–1.0. Incubation and measurements were done at the Millennium Seed Bank seed germination laboratory (Royal Botanic Gardens, Kew, UK) in May–July 2010.

Seed stratification and germination tests were performed in the laboratory of the Department of Plant Physiology, Faculty of Biology (University of Latvia) in September 2008–March 2010.

RESULTS

Seed collection

Although Ziemupe is located only approximately 54 km to the south of Užava, at Ziemupe the aboveground parts of the plants were already discoloured and withered, while at Užava living branches were still present on the largest plants at the time of seed collection in September. Compared to Ziemupe in plants from Užava seeds were more firmly attached to the infructescence, which suggests that the fruits were less mature. However, in both locations seeds were not yet dispersed. Spikes situated under each flower (and later, fruit) forming a rack-like structure supported even loosely attached seeds. In plants from both locations seeds from lateral branches were smaller than those from central branches: mean ± SE 5.00 ± 0.05 and 5.80 ± 0.09 mm, respectively.

Effect of cold stratification

No seeds germinated within two months without cold stratification or GA_3 treatment immediately after collection. Likewise, no seeds germinated after three months of dry storage at room temperature. Few seeds germinated after only one month of cold stratification and the germination percentage increased further after two, three, and four months of cold stratification (Fig. 1a).

Neither collection site nor seed situation on the central or lateral branch had a significant effect on final germination percentages. The germination rate was higher in treatments with longer periods of cold stratification. Seeds stratified at 25/10 °C for one month before cold stratification reached similar germination percentages as those that were only stratified at 5 °C (Fig. 1).

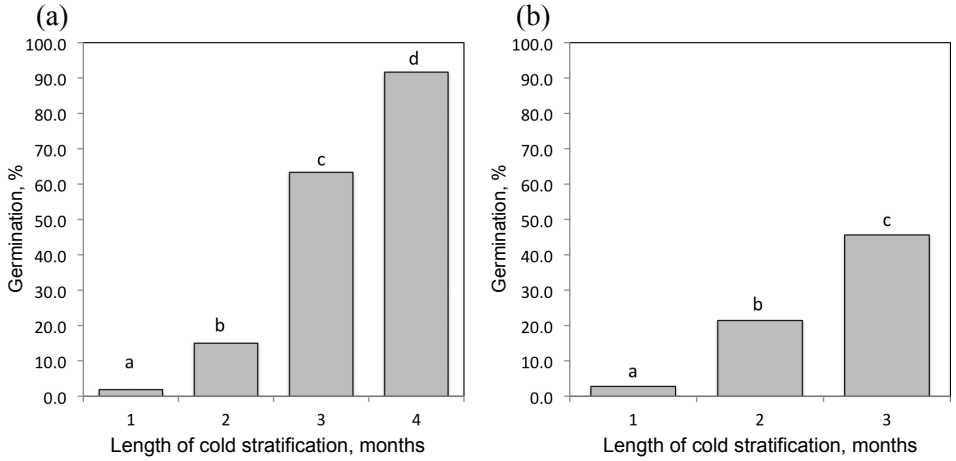

Fig. 1. Effect of cold stratification on the germination of *Eryngium maritimum* seeds. Seeds were stratified at 5 °C without previous warm stratification (a) and with previous warm stratification at 25/10 °C (b). Data for two seed collections (Užava and Ziemupe, both central and lateral branches) were pooled. Columns marked with different letters show results that are significantly different at $P = 0.05$.

Effect of treatment with GA_3

Treatment with GA_3 significantly increased germination and the effect depended on previous warm stratification. Among the seeds collected at Ziemupe many lost viability in the process of germination tests (tissues within the pericarp became soft or even liquid), up to 78% in seeds from central branches. Therefore in GA_3 treatments with warm stratification (Fig. 2b) germination is shown only for seeds from Užava.

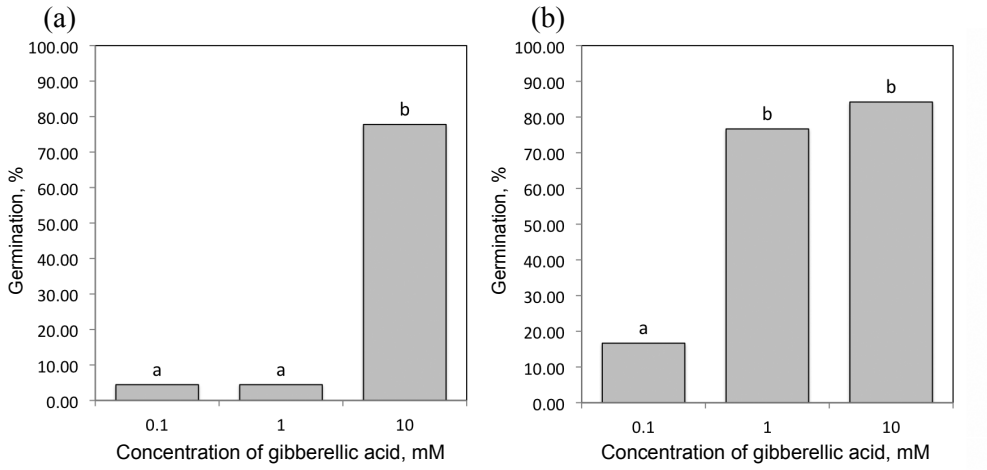

Fig. 2. Effect of treatment with gibberellic acid on the final germination percentage of *Eryngium maritimum* seeds: (a) without previous warm stratification and (b) with previous warm stratification at 25/10 °C (the data for the seed collection from Užava are shown). Columns marked with different letters show results that are significantly different at $P = 0.05$.

In seeds treated with GA_3 without previous warm stratification the final germination percentage was low at the two lower concentrations of the acid but significantly higher at the highest concentration (Fig. 2).

Many of the seeds collected at Ziemupe lost viability during the germination tests; as a result, no differences were detected between GA_3 treatments. In seeds from Užava the germination percentage and germination rate increased with increasing GA_3 concentrations (Fig. 2).

In treatments with warm stratification the germination rate was higher than in similar treatments without previous warm stratification. This was true for both cold stratification treatment and GA_3 treatment (Table 2).

Changes in the E:S ratio

The distribution of embryos within size-classes, assigned according to the degree of development, changed with the increased length of the period of cold stratification. There was a marked shift towards a higher class after four months of cold stratification (Fig. 3).

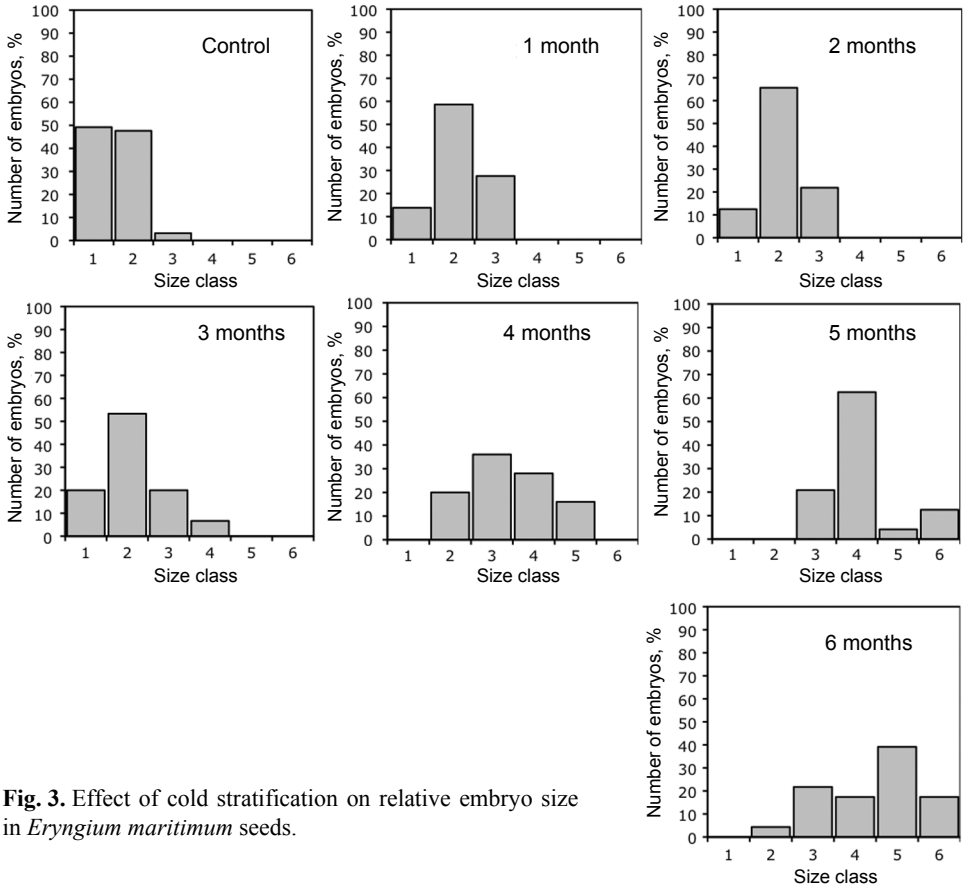

Fig. 3. Effect of cold stratification on relative embryo size in *Eryngium maritimum* seeds.

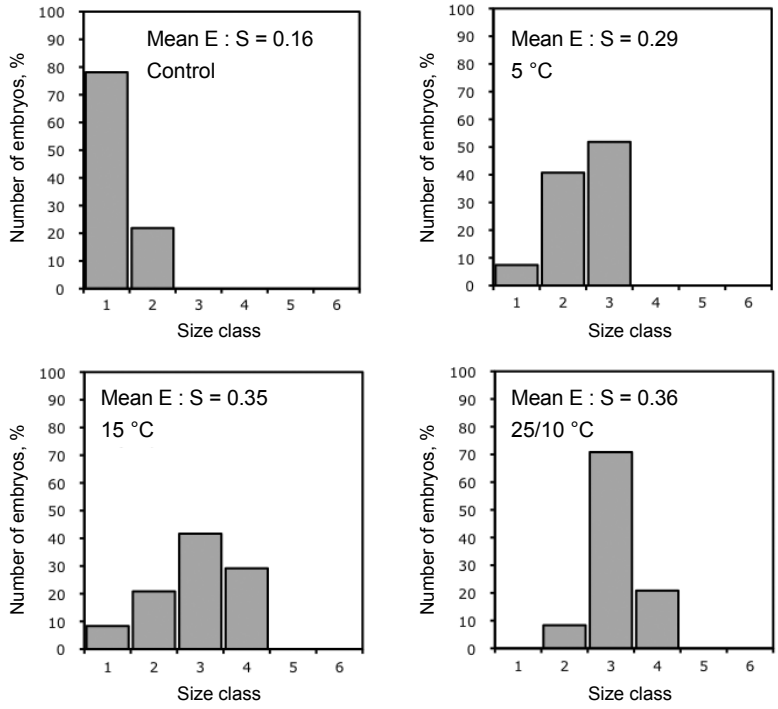

Fig. 4. The effect of incubation at different temperatures on relative embryo size in *Eryngium maritimum* seeds. E : S is embryo : seed (length) ratio.

The E : S ratio increased significantly ($p < 0.0001$) after two months of incubation in all temperature treatments in comparison with non-incubated seeds. The mean E : S ratio was significantly ($p < 0.0001$) larger at higher temperatures, i.e. at 15 and 20/10 °C (Fig. 4).

DISCUSSION

The high germination percentage in *E. maritimum* seeds after both cold stratification and treatment with GA_3 is in agreement with the results of previous germination research (Royal Botanic Gardens Kew, 2008), but other researchers report much lower numbers of germinated seeds (Walmsley & Davy, 1997; Curle et al., 2007). In some cases it is hard to compare the results because the proportion of empty fruits is not mentioned. In one of the previous studies cold stratification of *E. maritimum* seeds for a period longer than six weeks did not further increase germination (Walmsley & Davy, 1997), but in our experiment seeds germinated to a higher final germination percentage after three and four months of cold stratification than after one or two months (Fig. 1). This suggests that there can be considerable differences between populations or between seed batches collected in different years.

The highest final germination percentage after four months of cold stratification corresponds to a larger proportion of more developed embryos after four months of cold stratification (Fig. 3). The size of embryos also increased after a period of incubation at warm temperatures (Fig. 4). However, it appears that a sufficiently long period of cold stratification is also required for germination, since seeds that were stratified at 5 °C for only two or three months and did not germinate during the first month, failed to germinate even after three months of germination tests. Similarly, the germination percentage was low in seeds treated with a low concentration of GA_3 (0.1 mM) (Fig. 2). If treatment with GA_3 is a substitute for cold stratification, low concentration of GA_3 is comparable with too short a period of cold stratification. It has also been reported that seeds can germinate after warm stratification (at 25 °C) alone if subsequent germination temperatures are sufficiently low (15/5 °C) (Walmsley & Davy, 1997). This agrees with our conclusion that *E. maritimum* seeds require an impact of cold temperature to germinate.

Enhanced germination in seeds treated with GA_3 compared to untreated seeds means that GA_3 can substitute for cold stratification. This indicates non-deep physiological dormancy. The seed information database of Royal Botanic Gardens Kew (2008) contains a report that 83% of seeds germinated at 23/9 °C after 56 days of cold stratification and treatment with 250 mg L^{-1} GA_3 (which is approximately 0.7 mM and therefore roughly corresponds to 1.0 mM used in our experiments). This also agrees with other reports, although in this case it is hard to separate the effect of GA_3 from the effect of cold stratification.

Germination rate and sensitivity of seeds to GA_3 were higher in seeds treated with GA_3 after previous warm stratification: seeds germinated faster and to a higher final germination percentage at lower GA_3 concentrations than at similar concentrations without warm stratification (Fig. 2, Table 2). While one month of warm stratification did not increase the germination percentage of seeds after subsequent cold stratification, it did increase the germination rate (Table 2). Possibly, this is because embryos develop faster at higher temperatures (Fig. 4) and become more sensitive to GA_3 treatment.

The proportion of embryos in classes 1 to 3 remained almost constant during the first two months of cold stratification. After five and six months of cold stratification more than 50% of the embryos moved to classes 5 and 6; however, some remained in class 2 (Fig. 3). Authors of one of the previous studies described that some *E. maritimum* seeds germinated in the second year after seed dispersal (Curle et al., 2007). Possibly this happened because of non-uniformity of embryo development and/or the physiological state within the seed population. It has been proposed that within a seed population some seeds can be only morphologically dormant and embryos grow immediately upon seed imbibition while others are morphophysiologically dormant (Adams et al., 2011). Morpho-physiologically dormant seeds require a dormancy-breaking treatment to germinate as well as conditions suitable for the growth of the embryo. The depth of the physiological component may be a variable as well.

It is interesting to compare seed germination and embryo development of *E. maritimum* with those of another species from the Apiaceae family, *Aegopodium podagraria*. In *A. podagraria* seed embryos are also underdeveloped at maturity and require cold stratification for embryo growth and the breaking of dormancy (Vandelook et al., 2009). In both species incubation at warm temperature (23 °C in the case of *A. podagraria* and at 20/10 °C in the case of *E. maritimum*) before cold stratification influences germination, but in *E. maritimum* warm stratification did not increase the final germination percentage, although it increased the germination rate. Another difference is that the effect of GA_3 is insufficient to induce germination in *A. podagraria* (Vandelook et al., 2009). It is possible that seed dormancy is deeper in *A. podagraria* and therefore GA_3 does not break the physiological dormancy in its seeds as it did in *E. maritimum*. Treatment with GA_3 increases embryo growth in *A. podagraria* (Vandelook et al., 2009). In this study no E : S measurements were made with seeds treated with GA_3, so more research is needed to find out whether GA_3 also promotes embryo growth in *E. maritimum*.

Variations in seed properties depending on seed position on the plant or within an inflorescence are characteristic of Apiaceae (Fenner & Thompson, 2005). Although the position and seed size do not influence dormancy and germination as such, there may be an impact on seedling emergence and establishment because larger seeds potentially have an advantage in case they are buried deeper in sand or soil. The embryo size-class structure is a useful tool for studying seed development and the physiological stage in Apiaceae species and other species with similar seed morphology.

Possibly at Ziemupe aboveground plant parts complete growth and wither earlier in the vegetation season due to the effects of the local microclimate. The earlier death of aboveground plant parts and higher moisture can cause infection by pathogenic fungi or increase the action of saprophytic fungi (spores of *Alternaria* sp. were found on dry stems and leaves surrounding infructescences collected at Ziemupe, Dr E. Vimba, personal communication). The causes of seed damage and increased seed mortality during germination tests have to be further investigated.

Our results prove that *E. maritimum* plants in both Latvian populations produce viable seeds that are able to germinate well. A period of three to four months at cold temperatures is probably required for embryo growth and the breaking of dormancy. The timing of germination depends on the embryo's state of development, which is uneven among the seeds that mature at the same time in a population of plants. This creates a variation in germination timing. However, the success of generative reproduction may also depend on other factors. The number of seeds produced can depend on the success of pollination and, therefore, on weather conditions in a particular year. The possible effects of climate change should also be taken into account. Further studies of the impact of climatic conditions, as well as the onset of dormancy, on seed development and maturation will help to understand differences in seed germination between populations and wider differences between regions.

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