

Effect of Light and Moisture Conditions and Seed Age on Germination of Three Closely Related *Myosotis* Species

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Abstract Germination of three closely related species from the *Myosotis palustris* group (*M. nemorosa*, *M. palustris* subsp. *laxiflora*, *M. caespitosa*) differing in their habitats and capacity for clonal growth, was compared in two greenhouse experiments. To evaluate both inter- and intraspecific variation, each species was represented by seeds from several populations. Final germination percentage and germination rates T_{50} were compared both between species and populations within species. In the first experiment, we studied the influence of two external factors, moisture and light. Four moisture levels (dry, wet, periodically flooded and permanently flooded soil) and three types of shading (without shading, shaded with green foil, shaded with solid paper sheet) were combined in a complete factorial design. In all three species, total germination percentage was the same in the three wettest treatments, and decreased in the dry treatment. Germination in the treatments shaded with green foil (simulating vegetation cover, which changed light quality) was significantly slower than in treatments without shading and treatments shaded with a solid paper sheet. There were significant differences among species, but we also found very pronounced differences among populations within a species. *M. caespitosa* had the uniformly highest germination percentage (reaching in some cases 100%) and also fastest germination. Germination of *M. palustris* subsp. *laxiflora* populations was slower and reached lower final proportions, and medium variability among populations. Inter-population variability in the final germination

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percentage was highest, and the final germination the lowest in *M. nemorosa*. In addition, *M. nemorosa*, a species typical for permanent meadow communities was delayed by permanent flooding. In the second experiment, we studied the effects of seed age and storage conditions. Three combinations of seed age and storage were used: younger seeds (half year old) with no-chilling, younger seeds with chilling and old seeds (three years old) with chilling. *M. caespitosa* had again the highest final germination percentage and fastest germination rates T_{50} . In addition, final germination percentage of this species slightly increased with seed age, whereas it decreased in the other two species. The germination behaviour corresponded well to expectation based on species life histories and habitat preferences. Remarkably stable and high germination percentages and fastest germination rates T_{50} were ascertained in *M. caespitosa*, a species of disturbed habitats, with lowest capacity for clonal spread. *M. palustris* subsp. *laxiflora* (species with highest clonal capacity) and *M. nemorosa* (species with medium clonal potential) achieved lower, but still very high final germination percentage. In addition, *M. nemorosa* showed the highest inter-populations variability in our experiments.

Keywords Chilling · Flooding · Greenhouse experiments · Seed age · Seed storage · Shading

Introduction

A prominent goal of present ecological research is to understand the relationship between species traits and species habitat preferences (Lavorel and Garnier 2002), and to determine which of these relationships has an adaptive significance. There are evolutionary constraints to the development of species adaptation to environmental conditions (Silvertown and Charlesworth 2001), and consequently, not all species traits have adaptive significance. A convenient way to understand the ecological significance of traits is to study closely related species (Krahulec et al. 1999). Because they share a common evolutionary history, the differences in the traits of closely related species have more probably functional significance for the differences in their ecological behaviour.

Germination behaviour is an important part of a species regeneration strategy (Grubb 1977; Fenner 1992; Baskin and Baskin 1998). The germination stage is very sensitive to external conditions, and there exists a trade-off in the timing of germination. Often, seed ripening time is relatively dry, and/or is followed by a period unfavourable for seedling survival. In this case, germination immediately after seed ripening might subject the new seedling to highly unfavourable conditions. The longer a seed “waits” until it germinates, however, the higher the probability that it will be eaten, overgrown by other vegetation or seedlings, or destroyed in other ways. In addition, seed viability decreases over time (e.g., Leck et al. 1989). Also, the opportunities to establish might be rather rare, and to miss an opportunity often means seed death. To minimize the risk of failure, plants (and seeds too) have mechanisms to sense and perceive their surroundings and can respond to favourable external conditions; e.g., amount and quality of light (red/far red ratio), or temperature (Fenner 1992; Baskin and Baskin 1998). Dormancy and its

interruption are other mechanisms of regulation and timing of germination. For example, the chilling process during the winter season can interrupt seed dormancy, and then, following higher temperatures in the spring, can start seed germination. Species usually differ in their preferred combination of factors suitable for successful germination, with differences reflecting species' niches and behaviour. The species confined to disturbed habitats are often short-lived, lacking or with limited clonal propagation, but can germinate fast whenever the suitable conditions appear. Their population survival is vitally dependent on the seedling regeneration, and we can expect the genotypes with low seed germinability to be quickly eliminated from populations. Retaining seed germinability for a longer period is also profitable for the persistence of short-lived plant populations. Species of disturbed habitats often have small seeds that are more light-sensitive than large seeds (Milberg et al. 2000). Differences in germination behaviour between closely related species should be related to differences in their habitat preferences.

In fragmented landscapes (like the Central European one), individual populations are found in relatively discrete habitats and the gene flow to other populations is usually restricted. Consequently, differences between individual populations of the same species exist. The differences between the populations are of interest for at least two reasons – first, they might reflect interesting differentiation within a species, and, second, they are the baseline variation, against which the differences between species should be tested.

For our comparative study, we selected three species from the group of closely related species of swamp forget-me-nots, from the *Myosotis palustris* complex. Although the whole group is confined to wet habitats, the selected species differ considerably in their habitat preferences (Table 1). We have selected the south-western part of the Czech Republic as an area where all three species occur (although they rarely share a locality), and studied the populations there. We selected an area including South Bohemia and Českomoravská vrchovina highlands (ca. 150×100 km, with the altitude of seed collection localities ranging from 380 to 640 m a.s.l.). During our field observation from this area (ca. 100 phytosociological relevés, paper in prep.), we found that range and intensity of moisture (of substrate, presence of open water) and shading (by competitive vegetation and surrounding shrubs and trees) on sites differed between habitats occupied by adult plants of the three experimental *Myosotis* species. Therefore we designed experiments to test how these factors influence the germination of the studied species.

Some studies comparing species compare just one population of each. Because we expected some variability among populations of individual species, our first aim was to compare the differences between closely related species with the variability among populations within species. We have hypothesized that the species from the most disturbed habitat and with shortest life span (i.e., under highest selection pressure for successful seedling establishment) should exhibit uniformly high germinability; here we expected highest overall germination with small interpopulation variability. All the environmental responses are then interpreted with regard to the inter- vs intra- species differences.

The second aim of our paper is to compare how seed germination of three closely related species from the *Myosotis palustris* group (both of individual species and individual populations) is influenced by different combinations of two external

Table 1 Summary of published characteristics of the three *Myosotis* species investigated in this study

Species	<i>M. caespitosa</i> Schultz	<i>M. palustris</i> subsp. <i>laxiflora</i> (Reichenb.) Sychowa	<i>M. nemorosa</i> Besser
Ploidy	octoploid ($2n=88$)	hexaploid ($2n=66$)	diploid ($2n=22$)
Life history	annual (Grime et al. 1981; Štěpánková 2000) annual to biennial (Klimešová and Klimeš 1998), biennial semirosette hemicryptophyte (Klotz et al. 2002)	long-lived perennial hemicryptophyte (Klotz et al. 2002; Štěpánková 2000)	annual to biennial with no or only low clonal potential (Klimešová and Klimeš 1998), long-lived perennial hemicryptophyte (Klotz et al. 2002; Štěpánková 2000)
Clonality	non-clonal, does not form vegetative lateral shoots (Klimešová and Klimeš 1998)	strongly clonal, forms both below- and aboveground rooting lateral shoots of stem origin (Štěpánková 2000; Klotz et al. 2002)	clonal, forms short below-ground shoots of stem origin (Štěpánková 2000; Chaloupecká and Lepš 2004)
Ecological growth strategy	CSR (Klotz et al. 2002)	CSR (Klotz et al. 2002); CR (Grime et al. 1987, under the name <i>M. scorpioides</i>)	CSR (Klotz et al. 2002)
Altitudinal range in Central Europe (Štěpánková 2000)	from lowlands to mountains up to 630 m a.s.l.	from lowlands to mountains up to 650 m a.s.l.	from lowlands to mountains up to 1,400 m a.s.l.
Floristic zones (Klotz et al. 2002)	meridional, submeridional, S and N temperate and boreal	submeridional, S and N temperate and boreal	S and N temperate
Oceanity (Klotz et al. 2002)	weakly oceanic	weak suboceanic	suboceanic
Habitat preferences	marshlands, pond margins and exposed pond bottoms, alluvia of flowing waters, wet ditches, spring areas, reed and sedge margins (Grime et al. 1981; Štěpánková 2000)	marshlands, alluvia of rivers and streams: it is able to grow in waterlogged sites (Grime et al. 1987; Klotz et al. 2002; Lenssen et al. 2003), alluvia of rivers and streams (Štěpánková 2000)	wet meadows, alluvia of streams, spring areas and wet woods, wet ditches (Štěpánková 2000)
Flowering period	from May to July (Štěpánková 2000)	from May to September (Grime et al. 1987; Štěpánková 2000; Klotz et al. 2002)	from May to August (Štěpánková 2000)
Seed bank	abundant	transient and long-term persistent seed banks (Thompson et al. 1997; Jensen 1998)	abundant (Weiterová 2008)
Final germination percentages	high, up to 100% (Grime et al. 1981)	good germinability (52%–96%; Grime et al. 1981)	good germinability (65% in the field and 80% under greenhouse conditions; Kotorová and Lepš 1999)

factors: soil moisture (represented by four levels of water regime) and light intensity and quality (represented by three types of shading). We hypothesized that the sensitivity to environmental factors should correspond to species habitat preferences.

The third aim is to test changes in the germinability of seeds with age, in combination with effect of storage temperatures (both age and low temperatures are factors that can break down the possible seed dormancy and improve germination as well as destroy viability of seeds). We expected the species with the shortest life span to have the longest seed viability.

The species are known to differ in both their habitat preferences, and also in life-history characteristics, particularly life span. We are aware that the comparison of three species does not enable statistical testing of relationships between their life histories and seedling germination characteristics, nevertheless, we could compare the differences between species with our theoretical predictions.

Studied Species

Individual species of the *Myosotis palustris* complex (swamp forget-me-nots) look very similar, with the species within this group often not being correctly distinguished. In addition, the taxonomic concept of the *Myosotis palustris* group has been changed several times in the last decades and is presented differently in different floras. Here we adopt the classification and nomenclature used in both the Czech Flora (Štěpánková 2000) and in the recent key of the Czech Flora (Štěpánková 2002). Those publications and our field experience show that the species' habitat preferences differ consistently in Central Europe.

The *Myosotis palustris* complex includes the following taxa in the Czech Republic: *Myosotis palustris* subsp. *palustris*, *M. palustris* subsp. *laxiflora*, *M. brevisetacea*, *M. nemorosa* and *M. caespitosa*. Species of this group are considered to be annual or perennial with variable ability of forming below-ground or above-ground lateral shoots of stem origin. For our study, we have selected the species *M. palustris* subsp. *laxiflora* (referred to in pictures and tables as *M. laxiflora*), *M. nemorosa* and *M. caespitosa* because they are known to differ in their habitat preferences. They are also sufficiently common in the studied area, and consequently, each of them can be represented by several populations. All three species studied differ from each other in their ploidy level, and with some experience may be safely identified. In addition, all characteristics vary both among and within populations of the same species. Although these species grow in more or less wet sites (wet meadows, springs, river banks, swampy soils, etc.), they prefer different habitats and can rarely be found growing together in one locality. Their characteristics are summarized in Table 1.

Only a few taxonomic studies of the *Myosotis palustris* group include karyological investigations (Przywara 1983; Štěpánková 1993). Ecological studies of individual species, however, are rare and their interpretation is complicated by the changing taxonomy and nomenclature within the *Myosotis palustris* group; it differs among individual taxonomic classifications in various countries and times (e.g. Schuster 1967; Hegi 1979; Štěpánková 2000). No comparative ecological study of more species within the *Myosotis palustris* group is available.

There are ecological studies investigating ecological determinants of *M. palustris* (*M. scorpioides*, respectively) germination (e.g. Albrecht et al. 1997; Lenssen et al. 2003). Unfortunately, the identity of species is not completely clear there.

Phylogenetic study of the complex based on molecular data is not available. However, differences in morphological characteristics (e.g., presence and orientation of hairs on stem, calyx and underside of leaves, depth of calyx articulation, character of stem branching, ability to form lateral shoots, size and color of flowers and nutlets, etc.) separate *M. caespitosa* species as the most different from the rest of the *M. palustris* group (Štěpánková 2000, 2002) and in some floras it is separated into the *Caespitosae* group (e.g., Adler et al. 1994).

Methods

Experiment 1: Moisture and Shade Conditions

Five populations of each species were selected from the studied area. The locality characteristics of each population are summarized in Appendix 1.

Seeds of individual populations were collected during 2003. In each population, seeds were collected once. Only visually matured, dark seeds (i.e., those falling spontaneously from the calyces) were collected. Because the fruits mature gradually in each helicoid cyme, only a very low number of fruits mature in each inflorescence at a time. Consequently, the seeds were collected from hundreds of mother plants at each locality. Seeds were stored under dry conditions and room temperature (about 20°C) during the winter.

The experiment was established on May 2, 2004, in a greenhouse. Temperature in the greenhouse fluctuated between 10°C at night with daily temperatures ranging between 20–35°C. The experiment was established as a split-plot design, with the sets of pots as the whole-plots, moisture and light as the whole plot factors, and finally two split-plot factors, species, and population, nested in species. The sets of pots, referred to henceforth as set, is a block of physically connected pots, subjected to the same combination of external factors, and corresponds to the whole-plot in the split-plot design. Each of the 48 sets in the experiment contained 15 mini-pots (5 × 4 cm each), into which seeds of an individual population (five populations for each of the three species) were sown, ten seeds per one mini-pot. Mini-pot positions were randomized inside sets. A factorial combination of the two environmental factors, in several levels each, was applied on the level of the set. The first factor was moisture, and was applied in four levels: 1 – the surface of the soil was rinsed only once a day and was quite dry; 2 – soil was wet and drained regularly; 3 – soil was periodically waterlogged (flooded by water) and allowed to drain at approximately weekly intervals; 4 – soil surface was waterlogged (flooded by water) permanently. The second tested factor was light, applied at three levels: 1 – normal day light in the greenhouse; 2 – soil was covered and shaded by green foil; 3 – soil was covered and shaded with a solid paper sheet. Green foil changes both the light intensity (= photon flux density) and red/far red ratio in the transmitted light, with both reduced to 44% and 31% of that in the open, respectively, and have a similar effect as vegetation cover (complete spectral characteristics of this green foil are in Skálová and

Krahulec (1992). Solid paper sheet reduces light intensity to only a few percent of that in the open day light and did not change red/far red ratio (own measurement).

Seedling emergence was recorded daily over a 55-day period. Seed was counted as successfully germinating when it had produced a small seedling with two cotyledon leaves (if there were some seeds that died immediately after the seed breaks the seed coat, their germination was not spotted and they were counted as not germinating). Seedlings were immediately removed from the mini-pots after being counted, to minimize the possible effect on other seeds. The experiment was finished after 55 days; at this time, there was only negligible germination. Each pot was characterized by the proportion of seeds that emerged until the end of the experiment (called germination percentage thereafter). The rate of germination was characterized by the T_{50} , i.e., time in days when at least half of the finally germinated seed had already germinated (sometimes there was no germination in a pot; in this case, T_{50} was not calculated and these cases were omitted from analysis of rate of germination).

Experiment 2: Age and Chilling of Seeds

Twenty-eight populations originating from the studied area were selected. Numbers and identities of populations were different for each species and year (due to the short duration of some habitats, and high variability of flowering intensity in individual populations, the localities suitable for seed collection in 2003 often did not provide sufficient numbers of fruiting plants in 2005 for seed collection), with half collected in 2003 and half in 2005 (in the same manner as for the first experiment). The locality characteristics of each population are summarized in Appendix 2.

All seeds collected in 2003 were stored in a refrigerator under dry conditions and temperature of 4–6°C (chilling treatment). Seeds collected in 2005 were separated into two halves, with the first half stored dry at temperatures of 4–6°C (for three months; chilling treatment). The second half of the seeds was stored under dry conditions at room temperature (about 20°C; no-chilling treatment) throughout the winter.

The experiment was established on April 17, 2006, in a greenhouse. Temperature in the greenhouse fluctuated between 10°C at night, with the daily temperature between 20–35°C. The experiment consisted of nine sets (blocks of minipots). Each set contained 14 small pots (8×8 cm each) with 14 populations of the same treatment. Three combinations of seed age (younger and old) and kind of storage (chilling and no-chilling) were used: younger seeds without and with chilling and old with chilling (in nature seeds would not survive more than two years without experiencing low temperatures, so the combination of old seeds without chilling was not included). Each combination (= treatment) had three replications. Fifty seeds of an individual population were sown into each pot. Pot positions were randomized inside sets during the experiment.

Seedling emergence was recorded in the same way and for the same period as in Experiment 1.

Statistical Analyses

Differences between treatments in both experiments were evaluated using the appropriate ANOVA model with Statistica 7 (StatSoft, Inc. 2005), for the final

percentage of germination and germination rate T_{50} separately. The proportion of germinated seeds was subjected to angular transformation ($\arcsin \sqrt{\text{proportion of germinated seeds}}$) for analyses, but figures of final germination percentage show the original percentage data without transformation. In Experiment 1, population was the random effect factor nested in species, while set (block of the 15 minipots) was also a random factor nested in the interaction of the main-plot factors, i.e., moisture and shading. Cases with no germinated seeds were omitted from the T_{50} analysis (resulting in unbalanced ANOVA). In Experiment 2, population was a random factor nested in the species by treatment interaction, with set also being a random factor nested in treatment. Because there was no reason to compare the old seeds with chilling with young seeds without chilling, we have calculate two separate ANOVAs – one testing the effect of chilling in the young seeds, and one testing the effect of age in chilled seeds (the Bonferroni correction was not applied). Note that Statistica 7 uses a method that finds the linear combinations of random variation sources. These sources serve as appropriate error terms for testing the significance of individual effects of interest in mixed-model ANOVA designs and also in unbalanced hierarchical designs (StatSoft, Inc. 2005) – consequently, the “Denominator synthetic degrees of freedom” (Den.Syn.d.f.) need not necessarily be an integer. If the main effects were significant, the Tukey test at $\alpha=0.05$ (with the denominator mean square from corresponding ANOVA F -test used for standard error estimation) was used for post-hoc comparisons.

In the cases, where we wanted to stress the importance of variability (and ranges of the germinability values), we provide in the presented figures box and whisker plots with median, quartiles, outliers and extreme values, because we believe that those are more informative than classical mean and standard deviation for visualization of variability.

Results

Experiment 1: Moisture and Shade Conditions

There were significant differences in germination, both among treatments and also among species and populations within a species (Tables 2 and 3). No germination was recorded in 122 out of 720 mini-pots. There was however 100% germination in 65 mini-pots. Although there are significant differences between species, the differences between individual populations within a species are often much more striking (this is particularly true for *Myosotis nemorosa*, see Fig. 1).

Myosotis caespitosa had the highest germination, 70%, over all the treatments. *Myosotis palustris* subsp. *laxiflora* populations reached only about 40% mean germination and *M. nemorosa* even slightly (but not significantly) less. However, there were (sometimes large) differences among populations within a species. All five investigated populations of *M. caespitosa* had high final germination percentage (means over all treatments were from 61% to 80%, medians 63% to 100%). With the exception of population number 6, with a mean germination percentage of 21%, germination percentage of *M. palustris* subsp. *laxiflora* was quite uniform (mean germination was 46%–48%, medians 50% to 60%). The highest variability in final

Table 2 Results of ANOVA of final germination percentage for Experiment 1

	Effect d.f.	Den.Syn. d.f.	<i>F</i>	<i>P</i>
Species	2	12	6.2144	0.014
Population(Species)	12	636	44.7197	<0.001
Moisture	3	36	163.8562	<0.001
Shading	2	36	25.7021	<0.001
Moisture*Shading	6	36	17.9350	<0.001
Moisture*Species	6	636	6.9826	<0.001
Shading*Species	4	636	0.5565	0.694
Moisture*Shading*Species	12	636	1.5654	0.097
Set(Moisture*Shading)	36	636	1.5849	0.018

Brackets after a factor signify that this factor is nested in the factor named in the brackets. Tested dependent variable is arcsin transformed.

germination percentage was observed in populations of *M. nemorosa* (means were from 3.5% to 62%, medians 0% to 70%) (see Fig. 1).

A considerable part of the variability could be explained by moisture, less by shading and by their interaction (Fig. 2). The Tukey test on the moisture by shading interaction revealed that the three wettest treatments did not differ and shading had no effect at all in them (the average germination over all species was about 60%). Germination was lowest in the driest treatment (1), and depended on shading (being higher in shaded treatments, Fig. 2). The shading effect was significantly more positive with the solid paper sheet in comparison with the two other treatments (green foil and without shading).

The species reacted similarly to the experimental treatment, especially to the shading treatments (Table 2). The significant moisture \times species interaction indicates

Table 3 Results of ANOVA of germination rate, T_{50} for Experiment 1

	Effect d.f.	Den.Syn. d.f.	<i>F</i>	<i>P</i>
Species	2	14.02	10.2001	0.002
Population(Species)	12	515.00	8.0363	<0.001
Moisture	3	39.91	18.5440	<0.001
Shading	2	48.92	11.0732	<0.001
Moisture*Shading	6	37.86	1.0167	0.429
Moisture*Species	6	515.00	2.6121	0.017
Shading*Species	4	515.00	1.2963	0.270
Moisture*Shading*Species	12	515.00	1.6370	0.078
Set(Moisture*Shading)	34	515.00	3.1320	<0.001

Brackets after a factor signify that this factor is nested in the factor named in the brackets. Cases with no germination were excluded from the analysis. Effects of block are not shown.

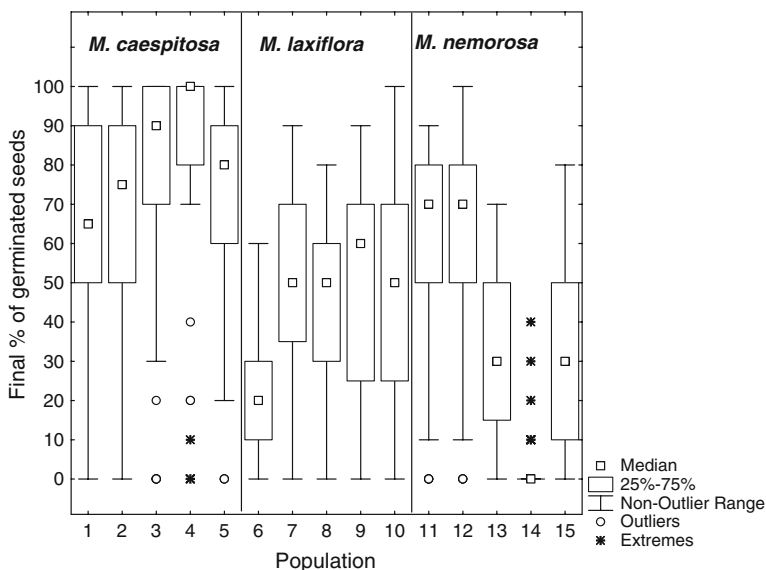


Fig. 1 Final germination percentage in three *Myosotis* species (15 investigated populations, five from each species), summarized over all treatments

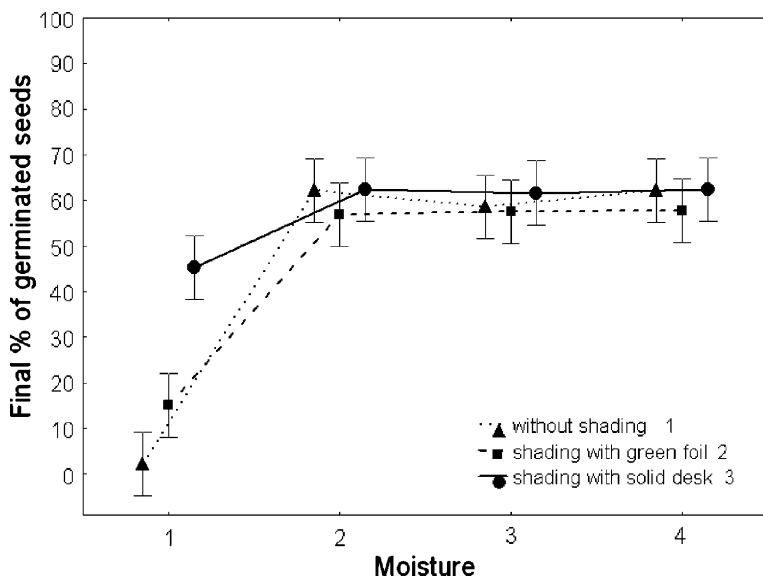


Fig. 2 Final percentage of germinated seeds – interaction of moisture and shading treatment averaged over all the species. Moisture treatments: 1 – driest, 2 – wet, 3 – periodically flooded, 4 – permanently flooded soil. Symbols indicate weighted means; vertical bars denote 0.95 confidence intervals. The lines connecting means under same shading treatment does not mean interpolation, but are added to better visualize interaction (non-parallelism signifies non-additivity)

that, although all of the *Myosotis* species had lower final germination percentage in the driest treatment, the effect is most prominent in *M. caespitosa* in comparison with the two other species (Fig. 3a,b,c). In addition, *M. caespitosa* (in contrary to other species) had at least some germination in all pots with moisture treatment levels 2, 3 and 4 (compare species in Fig. 3a,b,c).

Germination was faster for *M. caespitosa* (uniformly in all the populations) than the other two species (Tukey test) (Table 3). The average T_{50} for *M. caespitosa* was the 14th day, but the first germinated seeds were recorded as early as the 4th day after sowing. Variability of T_{50} between individual populations of *Myosotis* was smaller than the final germination percentage. Still, all five populations of *M. caespitosa* germinated very quickly (mean T_{50} was from the 13th to 17th day, median T_{50} was from the 10th to 14th day), and populations of *M. p. subsp. laxiflora* and *M. nemorosa* were quite similar (mean T_{50} was from day 16 to 20 and median T_{50} was from the 14th to 18th day in both, with the exception of population 14, which reached mean T_{50} by day 27, and median T_{50} by day 25, Fig. 4).

Germination rate T_{50} was affected differently by the moisture treatments. In germination rate T_{50} , all investigated *Myosotis* species reacted similarly to increasing moisture levels. Germination was the slowest in the driest treatment (1) for all species (mean T_{50} was day 25), improved in the wet treatment (2; mean was the 17th day) and was the fastest in the flooded treatments (4). The mean germination day

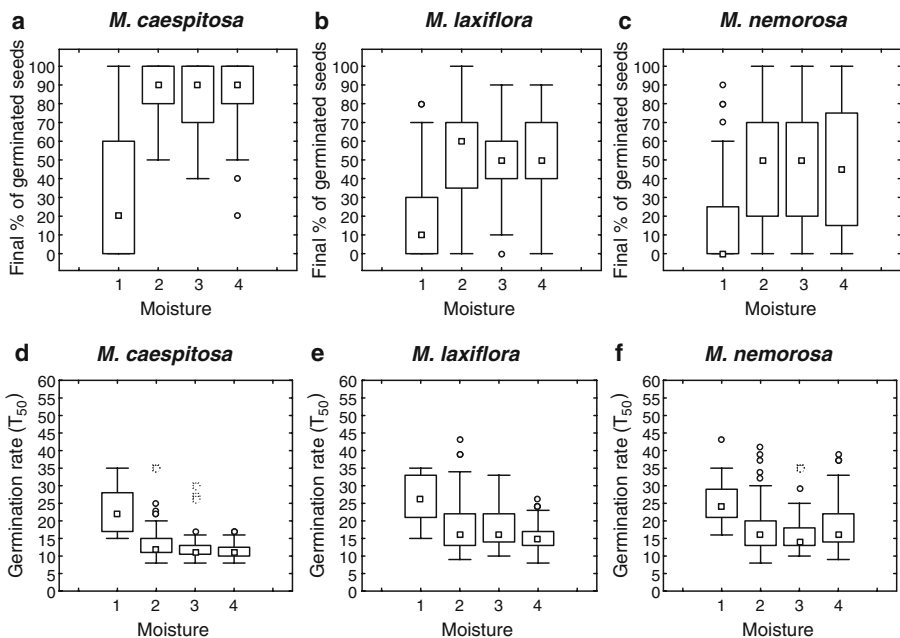


Fig. 3 Final percentage of germinated seeds (a, b, c) and germination rate T_{50} (represented by day, when half of the seeds had germinated) (d, e, f) in three *Myosotis* species at four moisture levels. Types of moisture treatments: 1 – driest, 2 – wet, 3 – periodically flooded, 4 – permanently flooded soil. Box and whisker plots: square – median; box – 25%–75% quartile; whiskers – non-outlier range; ○ – outliers; * – extremes

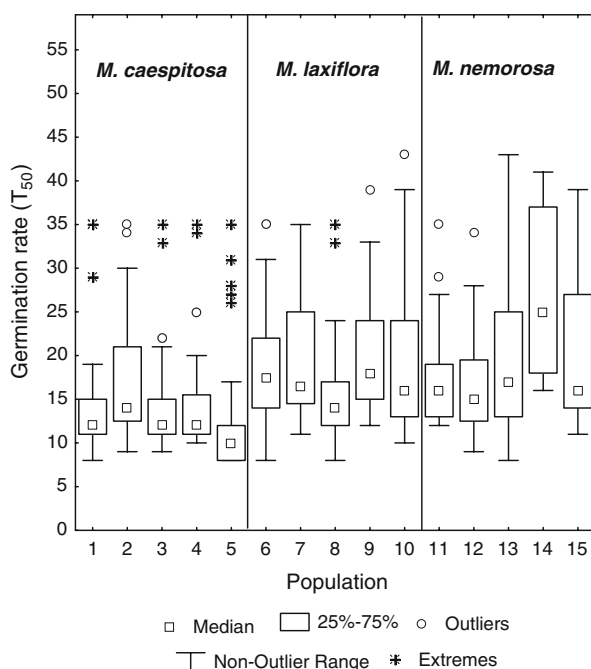


Fig. 4 Germination rate T_{50} of three *Myosotis* species (15 investigated populations, five from each species) is represented by day, when half of the seeds had germinated. Data summarized over all treatments

was the 15th day for both the periodically and permanently flooded treatments, except for *M. nemorosa* in the permanently flooded treatment, which had slower germination (Fig. 3d,e,f).

The shading treatment had an effect on germination rate T_{50} of all species. Whereas T_{50} was almost equal in the treatments without shading and shaded with solid paper sheet (mean=16th day), germination in the treatments shaded with green foil was significantly slower, although by only about three days.

There was no significant moisture \times shading interaction effect on germination rate T_{50} (see Table 3). However, the combination of the driest treatment without shading (1) seems to slow down germination speed.

Experiment 2: Age and Chilling of Seeds

Pronounced differences in germination existed both among species and among populations within a species (Tables 4 and 5). *Myosotis caespitosa* had the highest final germination percentage (mean of 78%, median 82%), in comparison with the two other species: mean germination was 55% for *M. palustris* subsp. *laxiflora* and 52% for *M. nemorosa* populations (medians 60% and 55%). However, germination percentage varied among populations within a species; for example, the most successful *M. caespitosa* populations reached 100% germination, but the worst only 26%.

Table 4 ANOVA of final germination percentage in Experiment 2

	Effect d.f.	Den.Syn. d.f.	<i>F</i>	<i>P</i>
a) effect of age (comparison of old chilling and younger chilling treatments)				
Species	2	22.00	11.20	<0.001
Population(Species*Treatment)	22	52.00	6.45	<0.001
Treatment	1	19.44	3.58	0.074
Species*Treatment	2	22.00	5.16	0.015
Set(Treatment)	4	52.00	1.00	0.418
b) effect of chilling (comparison of younger chilling and younger no-chilling treatments)				
Species	2	22.00	2.825	0.081
Population(Species*Treatment)	22	52.00	5.938	<0.001
Treatment	1	19.26	<0.001	0.990
Species*Treatment	2	22.00	0.217	0.807
Set(Treatment)	4	52.00	1.119	0.358

Brackets after a factor signify that this factor is nested in the factor named in the brackets. Tested dependent variable is arcsin transformed.

No significant main treatment effect (represented by the three combinations of age and type of storage) occurred, even when the effect of age (Table 4a) and effect of chilling (Table 4b) were tested separately. The interaction of treatment effect with species was significant for the effect of age (the interaction of storage type with species was not): the age affected final germination percentage, but differently according to species. Dry after ripening, old seeds of *M. caespitosa* germinated better (Figs. 5 and 6) than younger seeds (both with and without chilling), whereas

Table 5 Results of ANOVA of germination rate T_{50} in Experiment 2

	Effect d.f.	Den.Syn. d.f.	<i>F</i>	<i>P</i>
a) effect of age (comparison of old chilling and younger chilling treatments)				
Species	2	22.00	8.673	0.002
Population(Species*Treatment)	22	52.00	2.479	0.004
Treatment	1	9.84	2.463	0.148
Species*Treatment	2	22.00	0.919	0.414
Set(Treatment)	4	52.00	1.931	0.119
b) effect of chilling (comparison of younger chilling and younger no-chilling treatments)				
Species	2	22.00	41.89	<0.001
Population(Species*Treatment)	22	52.00	2.59	0.003
Treatment	1	6.09	0.01	0.932
Species*Treatment	2	22.00	2.51	0.104
Set(Treatment)	4	52.00	6.86	<0.001

Brackets after a factor signify that this factor is nested in the factor named in the brackets.

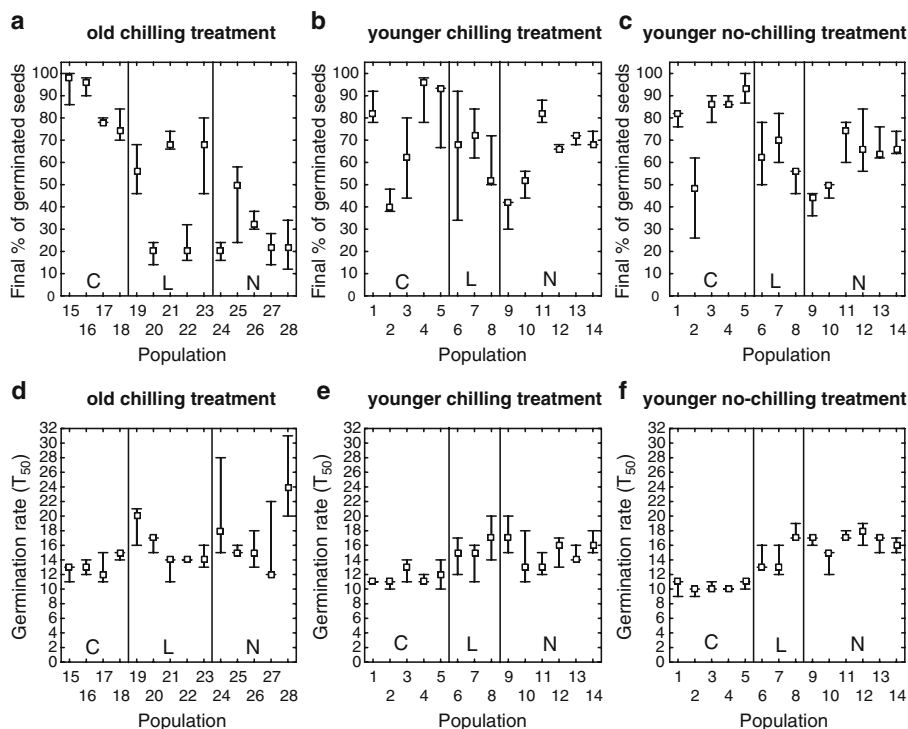


Fig. 5 Final percentage of germinated seeds (a, b, c) and germination rate T_{50} (represented by day, when half of the seeds had germinated) (d, e, f) in three age and type of storage treatments (younger no-chilling, younger chilling and old chilling). Abbreviations of species: C – *M. caespitosa*, L – *M. laxiflora*, N – *M. nemorosa*. Box and whisker plots: square – median; whiskers – minima and maxima

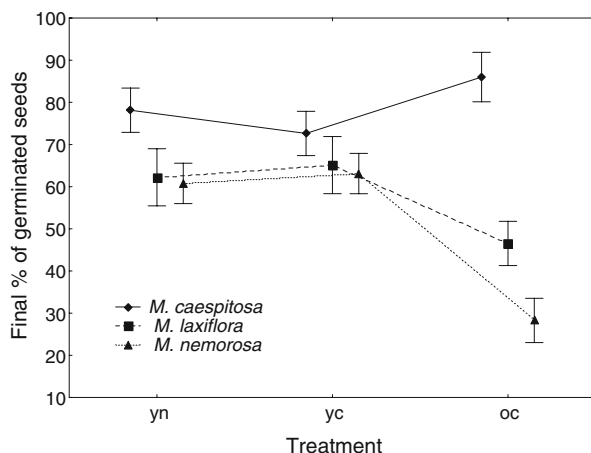


Fig. 6 Final percentage of germinated seeds of three *Myosotis* species in three age and type of storage treatments (yn – younger no-chilling, yc – younger chilling, oc – old chilling). Symbols indicate weighted means; vertical bars denote 0.95 confidence intervals. The lines connecting means for individual species do not mean interpolation, but are added to better visualize interaction (non-parallelism signifies non-additivity)

old seeds of *M. palustris* subsp. *laxiflora*, and, to a greater extent those of *M. nemorosa*, lost their viability.

The variability in germination rate T_{50} (Table 5) was highest among *M. nemorosa* populations, while it was rather uniform among populations of *M. caespitosa*. *M. caespitosa* germinated faster than the other two species (almost all populations of *M. caespitosa*; Tukey test), although the difference in T_{50} was only four days. The average T_{50} of *M. caespitosa* was day 11.5 (with minimum 9th and maximum 15th day), but the first germinated seeds were recorded as early as on the 7th day after sowing. Populations of *M. palustris* subsp. *laxiflora* did not have as rapid germination ($T_{50}=15$, and minimum at the 11th day and maximum at the 21st day). *M. nemorosa* germinated the slowest ($T_{50}=16$, minimum at the 11th day and maximum at the 31st day). Treatment did not significantly affect germination rate T_{50} (see Table 5), although younger seeds (with or without chilling) germinated slightly faster than older ones.

Discussion

We selected a restricted area (ca. 150×100 km), where all the three compared species occur (differing in their habitat preferences). All populations of all these species come from similar climatic conditions, and consequently, the observed interspecific differences were not caused by different climatic conditions of different species sites. However, the results should not be generalized to the whole area of distribution of the compared species (all three *Myosotis* species have a large distribution area; but *M. nemorosa* reach the highest altitudes and, e.g., in populations close to the tree-line the germination characteristics could differ from those studied here).

Predicted vs Observed Trends among the Species

The species comparison generally confirmed the trends, expected by the theoretical considerations based on habitat preferences and species life histories: *i*) The short-living species with the lowest clonal potential, confined to the temporary habitats (*Myosotis caespitosa*) exhibited the highest proportion of germinable seed, the highest speed of germination, the lowest variability among populations in germination characteristics and its germination was not negatively affected by seed age. *ii*) The species confined to the most constant habitats, with the ability to persist by short stolons in the community (*M. nemorosa*) exhibited relatively low and slow germination, with the highest variability among populations. *iii*) The two species typical for wet and often flooded habitats (*M. caespitosa*, *M. palustris* subsp. *laxiflora*) were never negatively affected by a high water level.

Environmental Responses

Although light was repeatedly reported to affect germination (e.g., Pons 1992), it did not appear to affect the final germination percentage in our experiment. The effect of changed light quality (changed red/far red ratio by green foil) was only a mild

decrease in germination speed, and did not differ among the species. This suggests that none of the species is able to prevent germination of its seeds when overgrown by extant vegetation. Contrary to expectation, we have found a positive effect of shading in the temporarily drying pots. Very probably, this effect was not caused by light quality, but was simply a protection against desiccation.

Germination was generally high in all the compared species, and chilling does not have any effect on it; however, good germination without any chilling suggests that there is no dormancy that would have to be broken. In comparison with high variability among populations within individual species, the interspecific differences are not very pronounced, nevertheless, they still might have adaptive significance, particularly in the case of *Myosotis caespitosa*, which is the most distinct in its habitat preferences, and also in its germination characteristics.

Seeds normally lose their viability with age (e.g., Leck et al. 1989). Moreover, germination from older seeds is sometimes delayed, which might reduce plant growth and final biomass in competition with other plants, as shown, e.g., by Rice and Dyer (2001) for *Bromus tectorum*. Whereas *Myosotis palustris* subsp. *laxiflora*, and to a greater extent *M. nemorosa*, followed this general trend, old seeds of *M. caespitosa* germinated slightly better in comparison with fresh seeds. This corresponds to the strategy of this species; *M. caespitosa* is a disturbance-dependent species, typical for temporary habitats, such as emerged fishpond bottoms. In this type of habitat, the species could be absent from the extant vegetation for several seasons (or, the habitat as such does not exist at all). Then, this species might survive for several seasons in the seed bank, maintaining high germinability for a long period. *M. nemorosa*, however, is a meadow species adapted for growth in the dense vegetation of meadow plant communities. In most of its localities, the species is always present in the extant vegetation, and consequently, seeds are shed (albeit with some variability) in all seasons. Similar to most meadow species, *M. nemorosa* is not crucially dependent on a permanent seed bank. Dependence of *M. palustris* subsp. *laxiflora* on the seed bank is probably also not crucial, because this species is strongly clonal, with clonal fragments enabling long-distance downstream dispersal.

Whereas the differences among species are not surprising, because the species differ in their habitat preferences, a striking and novel feature of our results is the relatively high variability among individual populations within species. They might be caused by genetic differences between individual populations, by the maternal effect, or even be the differences in the state of the populations (including the degree of ripening) in the time when the seeds were collected. However, as special care was paid to collect only well-developed ripe seeds, we expect that the differences are caused either by genetic differences, or by the maternal effect, generally the state of individuals in the various populations. At the time of seed collection, each individual provided a very limited number of seeds (few ripe seeds in each helicoid cyme). Consequently, it was neither feasible (nor our goal) to study the maternal effect of individual mother plants. However, each population is represented by seeds of many (hundreds of) mother plants, and consequently, the observed differences among populations correspond to the state of the compared populations (very probably affected by the habitat characteristics in the year of seed collection); with such a high number of seed providing plants, the possible

maternal effects of individual plants are averaged and the result characterizes the whole population. Interestingly, the variability within species differed among the species. *M. caespitosa* populations were rather similar in their germination characteristics, whereas *M. nemorosa* were the most variable. From a methodological point of view, the results suggest that any interspecific comparison based on single populations of each species should be interpreted with extreme caution (or avoided if possible).

Behaviour of Individual Species

Myosotis caespitosa is confined to short-term habitats (i.e., experiencing highest disturbance), and, consequently, is expected to be most dependent on seed regeneration. *M. palustris* subsp. *laxiflora* typically inhabits plots subject to temporary flooding, a disturbance that forms gaps in the community. In contrast, *M. nemorosa* is typical for permanent meadows and ditches, growing often in closed communities. (i.e., communities with cover reaching 100%). Habitats of both *M. palustris* subsp. *laxiflora* and *M. caespitosa* are often flooded, while flooding of *M. nemorosa* habitats is rather less frequent.

Myosotis caespitosa had the highest (mean 70%, in favourable conditions often 100%) and the fastest germination in both experiments. This species is sometimes considered to be an annual or short-lived perennial with no clonal potential (Grime et al. 1981; Klimešová and Klimeš 1998; Štěpánková 2000). However, results of our long-term field experiment (Koutecká and Lepš, in prep.) suggest that this species can survive several seasons and form lateral rosettes in permanent habitats. Nevertheless, among the studied species, *M. caespitosa* is undoubtedly the species with the most limited life span and ability of clonal propagation. Therefore, survival of this species is vitally dependent on generative reproduction. Probably, genotypes with deficiencies in seed germination are quickly eliminated. Fast germination (and establishment) might be of great importance for successful establishment, especially in changeable habitats. Although the difference again other two species in mean T_{50} was only five days (Fig. 3d,e,f), it might be of great importance. Rapid germination (and establishment) under favourable conditions might help the species to establish between two floods; our field observations confirm that young plants of this species can survive temporarily, and even flower, when under water. In favourable conditions, species germination often reached 100%, suggesting that most seeds germinate immediately. Nevertheless, older seeds of *M. caespitosa* exhibited even slightly higher germinability than freshly collected ones, suggesting the ability to be part of a seed bank. A seed bank, if formed, is probably composed of seeds having encountered less favourable conditions after being shed. A permanent seed bank is considered essential for the survival of disturbance-dependent species (e.g. Ritland 1983; Venable and Brown 1988; Grime 2001). On the contrary, germination of *M. caespitosa* is most susceptible to lack of moisture.

Unfortunately, studies of *M. palustris* subsp. *laxiflora* as an individual (sub) species are not available. There are some references to *M. palustris* (synonym *M. scorpioides* is sometimes used for this species), which can unfortunately mean either species *M. palustris* (which might, but need not, include *M. palustris* subsp. *laxiflora*) or even the *M. palustris* group.

Myosotis palustris subsp. *laxiflora* has a strong clonal potential: it forms both below- and aboveground rooting lateral shoots of stem origin. Severance from the mother plant often results in the formation of a new individual. The species can form floating mats at the edge of still or flowing water (Grime et al. 1987) and grow even in waterlogged sites (Lenssen et al. 1998; Štěpánková 2000). Propagation of *M. palustris* subsp. *laxiflora* from vegetative parts is obvious alongside rivers and streams, where this taxon spreads after floods (own observations). The severed parts are scoured by the streams, resulting in long-distance spreading. A relatively good germination percentage (40%–60% in our experiments) suggests that this species can reproduce both generatively and by clonal propagation. In the study of *M. palustris* (Lenssen et al. 1998), seeds germinated well in treatments where moisture was sufficient, including a waterlogged treatment. Seeds started to germinate at the 7th day and by the 15th day half of the seeds had germinated, which are similar results to our experiments. Seedlings were able to survive and grow after being waterlogged for eight weeks (until the end of the experiment), because of the physiological root and photosynthesis adaptations. Van den Broek et al. (2005) showed that seeds have good buoyancy (90% of seeds floated after 15 days, and 10% still floated after 124 days) both in stagnant and moving water, which explains the good propagation of this species along streams and rivers.

Myosotis nemorosa is sometimes considered to have low clonal potential (Klimešová and Klimeš 1998), but it forms short belowground shoots of stem origin and can be long-lived perennial even at dense vegetation meadow community (Chaloupecká 2003; Chaloupecká and Lepš 2004). In our experiment *M. nemorosa* achieved even lower mean final germination percentages than *M. palustris* subsp. *laxiflora* (mean about 40%), but populations of this species were the most variable in their final germination. In addition, *M. nemorosa* was the only species of the three exhibiting some negative response to water-logging, i.e., slightly delayed germination in the permanently flooded treatment. *M. nemorosa* occupies sites across a wide spectrum of moisture conditions, including drying soils (in our area, typically wet meadows of Molinion or Violion caninae in the phytosociological classification, but also ditches), i.e., drier habitats than the two other *Myosotis* species. These sites are sometimes flooded, but they are not waterlogged for longer periods. A good chance for successful establishment of *M. nemorosa* seedlings should probably be after flooding; therefore, it should be a good strategy for this species to delay germination when the sites are waterlogged.

An experimental study of seedling recruitment in the Ohražení oligotrophic wet meadow (a source locality of the *M. nemorosa* seeds in our experiment, Špačková et al. 1998; Špačková and Lepš 2004) showed that *M. nemorosa* was highly represented among seedlings. However, the year-to-year variability was high, with the species proportion among all seedling fluctuating between 4% and 30%, most probably because of the year-to-year variability in moisture conditions. Interestingly, the number of *M. nemorosa* seedlings was reduced in the treatment where a dominant species, *Nardus stricta*, was removed from the plant community. Zelený (1999) also confirmed the same behaviour in the same locality. This suggests that microhabitats in the *Nardus stricta* tussocks are suitable for seedling recruitment of *M. nemorosa*. Grass tussocks might appear to be a highly competitive environment.

Nevertheless, they might represent a microsite without extreme drought, and less affected by standing water, in the spring when the water level reaches a few centimetres above the ground surface. Those positive effects could outweigh competition. However, those studies also confirmed a high inter-annual variability in seedling establishment. This might correspond to the high sensitivity this species exhibited to temporary drying, but also to the high among-population variability in the final germination percentages found in this study. Our own observations confirm that germination of *M. nemorosa* seedlings has two main peaks (spring and autumn) both in nature and pot experiments (personal observation).

Soil Seed Banks

The study of Weiterová (2008) shows that *Myosotis nemorosa* is a most abundant herb in the upper 3 cm layer in the seed bank of a wet oligotrophic meadow “Ohrazení” (a collection locality for our experiment), although it is not dominant in aboveground vegetation.

There are some seed bank studies where *M. palustris* was present. Unfortunately, the name *M. palustris* may represent *M. palustris* sensu stricto, *M. palustris* subsp. *laxiflora*, as well as the *M. palustris* group, which includes *M. nemorosa* and sometimes also *M. caespitosa*, because of different taxonomic concepts of classification of the genus (for example, Grau and Merxmüller (1972) in Flora Europaea do not recognize any subspecific units within the species). Some studies suggest that seeds of *M. palustris* agg. can be abundant in the permanent soil seed bank, even in cases when the species was absent from the extant vegetation (Jensen 1998).

Bekker et al. (1998), in a long-term experiment, investigated the effect of groundwater level on the survival of seeds in the soil seed bank of a natural grassland community. Seeds of *M. palustris* did not germinate in the dry treatment (water level was 30 cm below soil surface), but germinated well in the high water-level treatment (water level was 5 cm below soil surface). Most germinated seeds were from the upper 5 cm of soil, and not from deeper soil, where anoxic conditions prevailed.

Conclusions

The comparison of the three related species mostly confirmed the predicted trends, in particular, that short-lived species confined to temporal habitats exhibit the uniformly high and fastest germination, and sensitivity to water conditions corresponds to the habitat preferences of individual species. However, the differences between species were rather small, particularly in comparison with differences between populations within a species. The germination of all the species exhibited very little sensitivity to shading, and no dormancy.

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Appendix 1. Localities of seeds collected for Experiment 1.

Abbreviations of species: C – *M. caespitosa*, L – *M. laxiflora*, N – *M. nemorosa*

Pop. nr.	Species	Coordinates	Altitude (m)	Locality	Habitat type
1	C	49°38' N, 15°54' E	610	Dářko	growth forbs at the margin of “Nový rybník” pond
2	C	48°46' N, 14°48' E	550	Veveřský rybník	margin of “Veveršský” pond
3	C	49°04' N, 14°21' E	385	U Vomáčeků	margin of “U Vomáčeků” pond
4	C	49°03' N, 14°23' E	385	Přední Topole	margin of “Přední Topole” pond
5	C	49°04' N, 14°22' E	385	Mydlovarský rybník	margin of “Mydlák” pond
6	L	48°54' N, 14°36' E	480	Trocnov	sandy deposit of brook
7	L	48°52' N, 14°22' E	440	Plešovice	bank of “Vltava” river
8	L	48°49' N, 14°20' E	460	Český Krumlov	alluvial deposit at “Vltava” river
9	L	49°00' N, 14°50' E	430	Stará řeka	alder carr near “Dušákovský” pond
10	L	49°01' N, 14°50' E	430	Lužnice	sandy alluvial deposit at “Lužnice” river bank
11	N	48°57' N, 14°36' E	515	Ohrazení	a wet, oligotrophic, species-rich meadow
12	N	48°58' N, 14°33' E	510	Třebotovice	a ditch separating road and wood
13	N	49°20' N, 15°01' E	640	Benešov	a wet, oligotrophic meadow
14	N	49°26' N, 15°18' E	585	Džbán	a meadow margin neighbouring with wood
15	N	49°25' N, 14°12' E	390	Zvíkov	a ditch along a road

Appendix 2. Localities of seeds collected for Experiment 2.

Abbreviations of species: C – *M. caespitosa*, L – *M. laxiflora*, N – *M. nemorosa*.

Abbreviations of age: Y – “younger” (collected in 2005); O – “old” (collected in 2003)

Pop. nr.	Species	Age	Coordinates	Altitude (m)	Locality	Habitat type
1	C	Y	49°04' N, 14°24' E	380	Munický rybník	margin of “Munický” pond
2	C	Y	49°04' N, 14°22' E	385	Mydlovarský rybník	margin of “Mydlák” pond
3	C	Y	48°46' N, 14°48' E	550	Veveřský rybník	margin of “Veveřský” pond
4	C	Y	49°23' N, 15°33' E	570	Rybník Lukáš	margin of “Lukáš” pond
5	C	Y	49°38' N, 15°54' E	610	Dářko	growth of forbs at the margin of “Nový rybník” pond
6	L	Y	49°00' N, 14°50' E	430	Stará řeka	alder carr near “Dušákovský” pond
7	L	Y	49°22' N, 15°35' E	520	Rančířov	margin of the meadow near “Jihlava” river
8	L	Y	48°54' N, 14°36' E	480	Trocnov	sandy deposit of brook
9	N	Y	48°57' N, 14°35' E	510	Kaliště-cesta	woody path
10	N	Y	48°57' N, 14°35' E	495	Kaliště-louka	wet meadow
11	N	Y	49°19' N, 15°28' E	580	Jezdkovické rašeliniště	small pond in the “Jezdkovické” mire
12	N	Y	49°22' N, 15°33' E	580	Vysoká	overgrown wet meadow along brook
13	N	Y	48°54' N, 14°36' E	660	Miličovské rašeliniště	“Miličovské” mire
14	N	Y	49°26' N, 15°23' E	610	Jankovský potok	margin of “Jankovský” brook
15	C	O	49°04' N, 14°21' E	385	U Vomáčeků	margin of “U Vomáčeků” pond
16	C	O	49°03' N, 14°23' E	385	Přední Topole	margin of “Přední Topole” pond
17	C	O	48°46' N, 14°48' E	550	Veveřský rybník	margin of “Veveřský” pond
18	C	O	49°38' N, 15°54' E	610	Dářko	growth of forbs at the margin of “Nový rybník” pond
19	L	O	48°52' N, 14°22' E	440	Hvízdal	bank of “Vltava” river
20	L	O	49°00' N, 14°50' E	430	Stará řeka	alder carr near “Dušákovský” pond
21	L	O	48°49' N, 14°20' E	460	Český Krumlov	alluvial deposit at “Vltava” river
22	L	O	48°54' N, 14°36' E	480	Trocnov	sandy deposit of brook
23	L	O	49°01' N, 14°50' E	430	Lužnice	sandy alluvial deposit at “Lužnice” river bank
24	N	O	48°57' N, 14°36' E	515	Ohrazení	a wet, oligotrophic, species-rich meadow
25	N	O	49°20' N, 15°01' E	640	Benešov	a wet, oligotrophic meadow
26	N	O	48°58' N, 14°33' E	510	Třebotovice	a ditch separating road and wood
27	N	O	49°25' N, 15°21' E	640	U Vyskytenské hájovny	an alder carr
28	N	O	49°25' N, 14°12' E	390	Zvíkov	a ditch along a road