

Performance of three closely related *Myosotis* species in an experiment in which substrate quality and competition were manipulated

Reakce tří blízce příbuzných druhů *Myosotis* v experimentu s manipulovanou kvalitou substrátu a konkurencí

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Dedicated to the memory of Leoš Klimeš

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Closely related species can be used for studying the ecological significance of their traits. The response in terms of survival, clonal growth and vegetative and generative characteristics of three related *Myosotis* species to competition and soil characteristics were studied in a three year pot experiment. Plants from four populations per species were cultivated in a factorial combination of substrate (nutrient-rich soil and mixtures with sand) and competition (with or without *Holcus lanatus*) treatments. Survival, clonal growth and the majority of the growth characteristics of all three *Myosotis* species were reduced by competition. The effect of substrate was less pronounced, and variable for various traits: the soil with sand mixture was more suitable for survival, clonal growth and seed germination whereas in the nutrient-rich soil plants were taller, but this effect was modified by competition. The differences among species corresponded well to expectation based on their known habitat preferences. *Myosotis caespitosa*, a species typical of short-term habitats such as emerged bottoms of ponds, exhibited the shortest life span and was also the most sensitive to competition: all plants of this species died in the competition treatment before the end of the second season. Nevertheless, the surviving plants (in the no-competition treatment) were able to form several daughter rosettes or stolons; some of them spread clonally till the third year. *Myosotis palustris* subsp. *laxiflora*, which inhabits the banks of rivers and brooks often disturbed by torrential floods, survived best and had the highest potential for clonal growth and spreading. Most plants of this species produced rhizomes and stolons and spread the furthest of all the three species. *Myosotis nemorosa*, which lives mostly in meadows, the most stable habitat of the studied congeners, but also a habitat with a strongly competitive matrix of species, was intermediate in terms of survival, and clonal growth, forming mainly short rhizomes. This species exhibited the highest among-population variability in all recorded characteristics, which might be due to its local adaptation to a wide spectrum of habitats. We argue that the details of prevailing disturbance regime, rather than some general disturbance intensity explain the clonal behaviour of the species compared.

Key words: among-population variability, daughter rosettes, life span, pot experiment, rhizomes, stolons, trade-off

Introduction

One of the more important goals of present ecological research is to understand the relationship between the traits of species and their habitat preferences (Lavorel & Garnier

2002). Some plant characteristics have no adaptive significance; there are evolutionary constraints on the ability of a species to adapt to environmental conditions (Silvertown & Charlesworth 2001) and some of the traits might come from a species' "phylogenetic past" and have no or very limited direct significance for their present ecology. Because closely related species share a common evolutionary history, comparing them is considered a convenient way of understanding the ecological significance of their traits (Krahulec 1994, Krahulec et al. 1999). For example, Rünk et al. (2004, 2010) and Rünk & Zobel (2009) demonstrate that various growth characteristics help to explain the local abundance of three closely related *Dryopteris* species. Stöcklin & Zoller (1992) relate the difference in clonal behaviour of two related *Epilobium* species to their altitudinal preferences. The situation can be further complicated in cases where species of particular ploidy levels (e.g. triploids) are limited in their generative reproduction, as shown by Urbanska-Worytkiewicz (1980) for *Cardamine*.

The potential for clonal growth is an important trait, which is connected with life strategy and influences the competitive ability of individual species (Herben et al. 1994, van Groenendael et al. 1996, Klimeš et al. 1997). Both the relative representation of clonal species in a community and also the clonal behaviour of individual species differ according to environmental conditions, including competitive pressure from neighbouring vegetation.

In this study, we focused on three closely related *Myosotis* species from the *M. palustris* group: *M. palustris* subsp. *laxiflora*, *M. nemorosa* and *M. caespitosa*. Although they look very similar, and all grow in wet habitats (wet meadows, springs, river banks, swampy soils), they are known to differ in their life span, clonal potential and habitat preferences (Table 1). In particular, the optimal conditions for the species can be ordered from less disturbed habitats (*M. nemorosa*) to the most disturbed short-term habitats of *M. caespitosa*. We have already demonstrated (Koutecká & Lepš 2009) that the seeds of the species living in the most disturbed habitat, *M. caespitosa*, are consistently more viable and germinate faster than those of the congeners. Although data on life span and clonal potential are available in various databases and floras they are based on occasional observations (as is the case for many other species) and sometimes differ among individual sources. In addition, our experimental study of *M. nemorosa* (Chaloupecká & Lepš 2004) suggests that data are not always accurate, the clonal behaviour of *M. nemorosa* is modified by competition and it is likely that other species of this group will show a similar reaction.

To test the differences in reaction of these closely related species to different treatments, we conducted a pot experiment under controlled conditions, with transplants affected by different soil conditions, and grown alone or together with *Holcus lanatus* as a typical competitor. The main objective of this study was to compare the differences between species with those expected on the basis of their known habitat preferences. We expected that the species confined to short-term habitats should have the shortest life span, and also, that it will be the most negatively affected by competition. All the species were expected to be positively affected by increased levels of nutrients. Moreover, we expected the positive effect of nutrients to be more pronounced in the competition-free environment, because *Holcus lanatus* is a strong competitor, well adapted to nutrient-rich conditions (experience of a fertilization/mowing factorial experiment, Lepš 1999) and consequently could counteract the possible positive effect of nutrients. Previously, no trade-off was found between flowering and clonal spreading in *Myosotis nemorosa* (Chaloupecká & Lepš 2004), but we expected that this trade-off might be found in the shortest living

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

Species	<i>M. caespitosa</i> C. F. Schultz	<i>M. palustris</i> subsp. <i>laxiflora</i> (Reichenb.) Schübler et Martens	<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 & Klimeš 1998), biennial semirosette hemicryptophyte (Klotz et al. 2002)	long-lived perennial hemicryptophyte species (Štěpánková 2000, Klotz et al. 2002)	annual to biennial with no or only low clonal potential (Klimešová & Klimeš 1998), long-lived perennial hemicryptophyte (Štěpánková 2000, Klotz et al. 2002, Chaloupecká & Lepš 2004)
Clonality	non-clonal does not form vegetative lateral shoots (Klimešová & Klimeš 1998)	strongly clonal, forms both below-ground and above-ground rooting lateral shoots of stem origin; (Štěpánková 2000, Klotz et al. 2002)	clonal, forms short below-ground shoots of stem origin (Štěpánková 2000, Chaloupecká & Lepš 2004)
Plant strategy (according to Grime 1977)	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 middle altitudes to mountains up to 1400 m a.s.l., missing from warm lowlands
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 bottoms of ponds, alluvia associated with 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 woodland, 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)	no data
Germination rate	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 & Lepš 1999)

species of the group, i.e. *M. caespitosa*, because resources should be most limiting during its short life span; we suspected that during this short time the resources can either be invested into flowering, or into clonal growth (we expected a trade-off in resource allocation, not in meristem allocation; see Huber & During 2001).

The morphological (and very probably, also physiological) characteristics of all *Myosotis* species vary both among and within populations (we have already demonstrated this for germination, see Koutecká & Lepš 2009) and thus any comparison among species has to be done against the variability of populations within a species (not against the variability among individuals within a single population of each species). Our study aimed to determine both the variability among populations of individual species, and then, the variation among species. Germination of seedlings in different substrates and at different levels of competition was observed as an additional characteristic.

Materials and methods

Studied species

In our study we have adopted the classification used in both the Flora of the Czech Republic (Štěpánková 2000) and the recent key of the Czech flora (Štěpánková 2002). Three closely related species from the *Myosotis palustris* group were selected for the pot experiment: *M. caespitosa*, *M. palustris* subsp. *laxiflora* (referred to as *M. laxiflora* in figures) and *M. nemorosa*. The species were selected because they are sufficiently common in southern Bohemia (our aim was to compare populations from a single restricted area), so that each of them can be represented by several populations. The three related *Myosotis* species differ in life span and clonal growth (see Table 1).

Experimental design

Four populations of each species, all originating from the southern and south-eastern part of Bohemia (western part of the Czech Republic), were selected for the pot experiment (Table 2). Seeds of individual populations were collected from hundreds of mother plants at each locality during 2003. Seeds were stored under dry conditions and at room temperature (about 20 °C) during the winter. Germination of seeds started on 2 May 2004 in a greenhouse. Temperature in the greenhouse fluctuated between 10 °C at night and a daily maximum temperature between 20–35 °C. Preplanted young plants (one month old) were selected when they had about six leaves.

The pot experiment was established on 3 June 2004 in a greenhouse, when the selected young plants of *Myosotis* were planted into pots (one plant per 16.5 × 16.5 cm pot). Two environmental factors were applied in factorial design: substrate (two levels differing in nutrient content and texture) and competition (the competitive treatment pots contained also young plants of *Holcus lanatus*, one month old, five plants per pot). *Holcus lanatus* is a tufted perennial grass, a CSR-strategist according to Grime et al. (1987). CSR strategy means that a species' strategy is somewhere in the middle among the three primary strategies of Grime (1977), i.e. between competitive, ruderal, and stress-tolerant. *Holcus lanatus* grows in various types of habitats, is most abundant in meadows and pastures, but can be found also growing on stream banks and in marshy ground. It encounters all three

Table 2. – Localities at which seed was collected. Abbreviations of species: C – *M. caespitosa*, L – *M. palustris* subsp. *laxiflora*, N – *M. nemorosa*. Note that all the localities for *M. caespitosa* are rather unstable fishpond margins (often temporarily flooded), localities of *M. palustris* subsp. *laxiflora* are fairly stable localities such as (deposits on) the banks of rivers and brooks – often disturbed during flooding, and the localities of *M. nemorosa* are ditches and meadows, probably the most stable habitats (often temporarily water logged, but also drying out in summer).

Species/ Population	Coordinates	Altitude (m a.s.l.)	Locality	Habitat type
C1	49°03'N, 14°23'E	385	Přední topole	margins of the Přední topole pond
C2	49°04'N, 14°21'E	385	U Vomáčků	margins of the U Vomáčků pond
C3	49°38'N, 15°54'E	610	Dářko	growth of herbaceous plants at the margins of the Nový rybník pond
C4	48°46'N, 14°48'E	550	Veveřský rybník	margins of the Veveřský rybník pond
L1	49°00'N, 14°50'E	430	Stará řeka	alder carr near the Dušákovský rybník pond
L2	48°49'N, 14°20'E	460	Český Krumlov	alluvial deposit of the Vltava river
L3	48°52'N, 14°22'E	440	Plešovice	bank of the Vltava river
L4	48°54'N, 14°36'E	480	Trocnov	sandy deposit of a brook
N1	49°25'N, 14°12'E	390	Zvíkov	a ditch along a road
N2	49°20'N, 15°01'E	640	Benešov	a wet oligotrophic meadow
N3	48°57'N, 14°36'E	515	Ohrazení	a wet oligotrophic species-rich meadow
N4	48°58'N, 14°33'E	510	Třebotovice	a ditch separating road and wood

experimental *Myosotis* species, in various vegetation types; however, it most frequently occurs with *M. nemorosa* in meadows (E. Koutecká & J. Lepš, personal observation). Pots were filled with a commercial nutrient-enriched garden substrate (240–400 mg of N/l; 275–450 mg P₂O₅/l; 300–500 mg K₂O/l; 80–120 mg MgO/l; pH(H₂O) 5.5–6.5), hereafter called “soil” vs pots filled with a 1 : 3 mixture of this garden substrate with sand; hereafter called the “sand mixture”. These two substrates differed in nutrient content and texture; the soil desiccated faster. The two substrates should be seen as two contrasting soil environments, not just a difference in nutrient content. One-time measurement of C and N contents of substrates in the second year of the experiment revealed that their contents were 30× and 16×, respectively, lower in the sand mixture than in the soil (i.e. many times more than expected from 25% content of garden substrate in the sand mixture), probably because the nutrients were more easily leached. Presence of competitor (*Holcus lanatus*) had no effect on the C and N content of substrates. *Holcus lanatus* was cut three times during the season to keep its height at 10 cm. This led to tillering and higher density, particularly during the first season, which mimicked the situation in a real meadow (where regular mowing also leads to higher densities of competitive grasses). As the clipping was mainly a means of producing a dense tuft of tillers, the target plants were not clipped. We expect that the presence of *Holcus* affected the target individuals by shading, but also by under-ground competition. Based on our (unsuccessful) attempt to extract under-ground biomass, we know that the soil space was filled with *Holcus* (but also *Myosotis*) roots; we expect that the physical presence of roots can affect the competing species, as has been shown, e.g. by experiments of McConnaughay & Bazzaz (1992). Starting in the second season, the *Holcus* tillers started to die, which resulted in a decrease in density.

This design was replicated in five blocks, with each block containing all of the populations of all the species planted into all four combinations of competition/substrate. Position of the pots was randomized inside the blocks during the experiment. All five blocks were placed in a continuous and homogeneous area in the greenhouse, and after the first month of

the experiment in a continuous and homogeneous area in an experimental garden, where they remained till the end of the experiment. The blocks were adjacent to each other in a homogeneous area. We did not expect that block would have an effect on the measured characteristics (we used them mainly to spread the individual species and treatments evenly over the entire area), and, consequently, we did not use block as explanatory variable in the analyses. In the greenhouse, the pots were watered as needed to keep the soil wet, when in the garden, they were watered only by precipitation (the pots were in shallow trays, which stored water and prevented desiccation; the whole experimental area was partially shaded by shrubs and saplings growing in an adjacent area); no additional nutrients were applied during the course of the experiment. Survival and selected growth characteristics of the target *Myosotis* individuals were measured several times during the 2004 and 2005 seasons, and once during the 2006 season. The number of clonal shoots (stolons or rhizomes) or daughter rosettes in one pot was measured regularly. The size of clones was characterized by height of the longest shoot (cm) (hereafter called “shoot height”; measured once in the 2004 and 2005 seasons) and by the furthest distance between shoot tips on the soil surface of a pot (cm), i.e. width of a clone, hereafter called “distance of shoots”; this characteristic was measured only once (in 2005). The distance of shoot tips was considered to be a characteristic of the ability to spread clonally. Flowering of clones (yes/no) was recorded in both seasons (as a potential generative characteristic showing a trade-off with the ability to produce shoots and spread vegetatively). Number of flowers were counted only once in 2005.

Seedlings of the *Myosotis* species were found in the pots starting in autumn 2004. As the pots were close to each other, they might have originated from various mother plants; it is not possible to distinguish seedlings of the three species. Seedlings were removed from the pots, and the numbers recorded as an additional characteristic.

The experiment was terminated in spring 2006.

Statistical analysis

The data were evaluated using various ANOVA models (StatSoft 1998, 2005), taking into account the repeated observations of individual plants (repeated measurements ANOVA) and the hierarchical nature of the experiment; individual populations are considered as a random factor, nested within the species (fixed factor), while competition and substrate are fixed factors (not nested).

Some plants died during the course of the experiment. The dead plants can be considered in the analyses in two ways; either as missing observations, or as zeroes. In the first case (missing observations), we compared the state of surviving plants. Assigning zero to the dead plants was used when comparing all plants. We used this approach when considering, e.g. the clonal potential of a plant: if we ask, how many shoots originating from a single seedling are alive on a particular date, then zero is the correct answer for a dead plant, and inclusion of dead individuals characterized by zero shoots leads to a correct estimate of the average number of surviving shoots per single seedling planted. In this case, our design was balanced and complete, and enabled the use of repeated measurement ANOVA (calculated using Statistica ver. 5), with individual populations a random factor, nested within the species, while competition and substrate were fixed factors, and not nested. We used this analogical approach to also evaluate survival. In this case, live individuals were assigned a value of 1, and dead individuals a value of 0. We are aware that the Bernoulli distribution of ones

and zeroes does differ from normality. Nevertheless, the tests that are of interest are those on the averages of survival of individual populations and particularly about individual species. For individual populations, the estimates of p (proportion of surviving individuals) are closer to normality (thanks to the Central Limit Theorem); further averaging of population means within a species leads to further improvement in normality (and tests on species averages are the most important ones in the experiment). Consequently, we believe that the results of the test can be considered as a reasonable approximation in this case.

In the case where we were interested in the properties of surviving plants, we conducted tests for (selected) individual observational dates, and all surviving individuals were included in the analyses. In this case, the design was necessarily unbalanced, and were evaluated using Statistica 7. Note that Statistica 7 uses a method which finds the linear combinations of sources of random variation that serve as appropriate error terms for testing the significance of the respective effect of interest in mixed-model ANOVA designs (accounting for varying variability of means calculated from differing numbers of observations in unbalanced hierarchical mixed models). As a consequence of this approach, the “denominator synthetic degrees of freedom” (Den.Syn.df) need not necessarily be an integer. If the main effects were significant, the Tukey test at $\alpha = 0.05$ (with the corresponding error mean square used for s.e. estimation) was used for post-hoc comparisons of species. The numbers of shoots, flowers and seedlings were subjected to square root transformation ($\sqrt{\text{variable} + 0.5}$) for analyses, but all figures show the original non-transformed values.

Further, we tested for a possible trade-off between flowering and production of new shoots. If there was a negative effect of flowering on survival or production of new shoots, then this effect would manifest itself as a significant interaction between time and flowering in the repeated measures ANOVA, with the number of shoots in autumn 2004 and spring 2005 being the two repeated observations, and flowering in 2004 being the explanatory variable. The interaction term tests the null hypothesis that temporal change is the same in the flowering and non-flowering individuals. The interpretation of this ANOVA, however, has to take into account that flowering was not the manipulated factor, and we expected that flowering might be affected by the prior state of the individuals.

Results

Plants of all species survived better and produced more shoots in the absence of competition and in the sand mixture substrate; although the differences between the competition and control treatments gradually decreased (mostly because the *Holcus lanatus* tillers died), the effect of competition was much more pronounced than the effect of substrate (Table 3: both the main effects and the interaction with time). A balanced experimental design was used, and so both the factors (i.e. competition and substrate) had the same number of replications. Each factor has one df, and also, both are tested against the same denominator MS in the F-test. Consequently, the ratio of their F-values in individual tests corresponds to the ratio of explained variability by each of them (i.e. F-values are proportional to the corresponding effect SS). The response to substrate type did not differ among species (non-significant interactions: species by substrate and species, substrate and time). Survival was best in *M. palustris* subsp. *laxiflora*, followed by *M. nemorosa*. The lowest survival was recorded for *M. caespitosa*, particularly in the competitive treatment, where

Table 3. – Significance of effects and their interactions for variables: survival of plants and number of shoots, repeated measures. Summary of all effects, × means interaction. The Population was a random factor nested in the Species factor. Statistically significant results ($P < 0.05$) are in bold.

Effect(s) and interaction(s)	df		Survival		Shoots	
	Effect	Error	F	P-level	F	P-level
Substrate	1	9	11.53	0.008	10.86	0.009
Competition	1	9	39.59	< 0.001	61.01	< 0.001
Species	2	9	30.72	< 0.001	13.88	0.002
Population	9	192	2.52	0.010	3.54	< 0.001
Time	6	54	83.42	< 0.001	6.46	< 0.001
Substrate × Competition	1	9	1.07	0.328	0.00	0.992
Substrate × Species	2	9	2.11	0.177	1.32	0.315
Competition × Species	2	9	0.35	0.711	4.16	0.053
Substrate × Population	9	192	1.29	0.245	0.75	0.663
Competition × Population	9	192	1.11	0.354	1.51	0.148
Substrate × Time	6	54	2.53	0.032	1.93	0.092
Competition × Time	6	54	11.80	< 0.001	17.71	< 0.001
Species × Time	12	54	9.48	< 0.001	6.04	< 0.001
Population × Time	54	1152	2.05	< 0.001	3.95	< 0.001
Substrate × Competition × Species	2	9	3.86	0.062	0.43	0.661
Substrate × Competition × Population	9	192	0.81	0.611	0.90	0.527
Substrate × Competition × Time	6	54	0.52	0.792	1.89	0.100
Substrate × Species × Time	12	54	1.47	0.167	1.37	0.208
Competition × Species × Time	12	54	3.88	< 0.001	3.89	< 0.001
Substrate × Population × Time	54	1152	1.30	0.074	0.85	0.779
Competition × Population × Time	54	1152	0.92	0.642	1.50	0.012
Substrate × Competition × Species × Time	12	54	1.50	0.151	0.44	0.939
Substrate × Competition × Population × Time	54	1152	1.25	0.109	0.91	0.667

all of the plants died before the end of the second year of the experiment; only two plants survived in the without-competition treatments until the end of the experiment (significant interaction between species, time and competition, Fig. 1). Individual populations within species also differed in their survival, with *M. nemorosa* exhibiting the highest variability in comparison with the congeners (Fig. 2). There were also consistent differences in the production of shoots among species (Fig. 3), with the highest numbers recorded for *M. palustris* subsp. *laxiflora*, followed by *M. nemorosa* and the lowest for *M. caespitosa*. Again, the highest variability was recorded for *M. nemorosa*.

The differences between living plants were analysed for individual dates only, taking into account only plants that survived to a particular date. However, the power of the test decreased over the course of the experiment as the number of surviving plants decreased (and this resulted in an unbalanced design due to unequal survival). The shoot number was the only characteristic significantly higher in the no-competition treatment in this experiment. Differences among species were significant only in the first season, when the highest number of secondary shoots was produced by *M. palustris* subsp. *laxiflora*, followed by *M. caespitosa*, with *M. nemorosa* producing the least ($P = 0.029$). The greatest among-population variability was again found in *M. nemorosa*. Our results confirmed the superior clonality of *M. palustris* subsp. *laxiflora* and comparison of the analyses with either all or only the surviving plants suggests that *M. nemorosa* has rather low clonal potential, but better survival. The low survival of *M. caespitosa* outweighs its ability to form daughter rosettes or stolons.

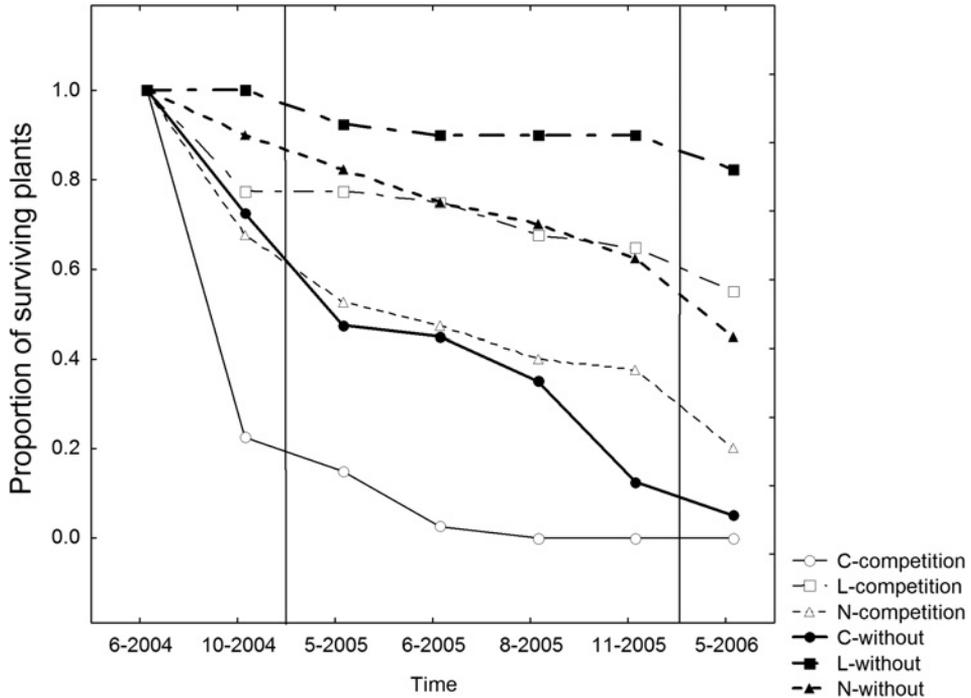


Fig. 1. – The proportions of the plants of three *Myosotis* species that survived in treatments with and without competition from *Holcus lanatus*. Species \times Competition \times Time interaction: $F_{12,54} = 3.89$; $P = 0.00027$. Abbreviations of species: C – *M. caespitosa*, L – *M. palustris* subsp. *laxiflora*, N – *M. nemorosa*, with combinations of competitive treatments: competition – with *Holcus lanatus*, without – without competitor.

Distances between shoot tips (as a characteristic for clonal spreading) were measured on living plants in July 2005 (Fig. 4). Shoot distances were greater in the treatment without competition ($F_{1,97} = 4.62$; $P = 0.034$) and were greatest for *M. palustris* subsp. *laxiflora* than the other two species ($F_{2,12.25} = 5.25$; $P = 0.023$). Unfortunately, maximal distances between shoot tips were restricted by pot size diameter in our experiment, although it was obvious that shoots of *M. palustris* subsp. *laxiflora* grew around the pot borders, and had the potential to spread over much greater distances (this was not the case in the other two species; consequently, the difference between *M. palustris* subsp. *laxiflora* and the other two species is even greater than suggested by the results of this test). Again, there were significant differences among populations within individual species ($F_{9,97} = 3.74$; $P < 0.001$).

Shoot height of all living plants was measured in August 2004 and May 2005. Plants of all species were taller in treatments without competition in August 2004 ($F_{1,202} = 78.70$; $P < 0.001$, Fig. 5). Plants of *M. caespitosa* and *M. palustris* subsp. *laxiflora* were taller than those of *M. nemorosa* (Tukey post-hoc comparison, $F_{2,9.03} = 4.73$; $P < 0.039$ for overall ANOVA). There was an interesting interaction between substrate and competition treatments: clones in the without-competition treatment were taller in the sand mixture than in the soil treatment, while in competition with *Holcus lanatus*, clones of *Myosotis* were a little taller in the soil and shorter in the sand mixture (substrate by competition interaction:

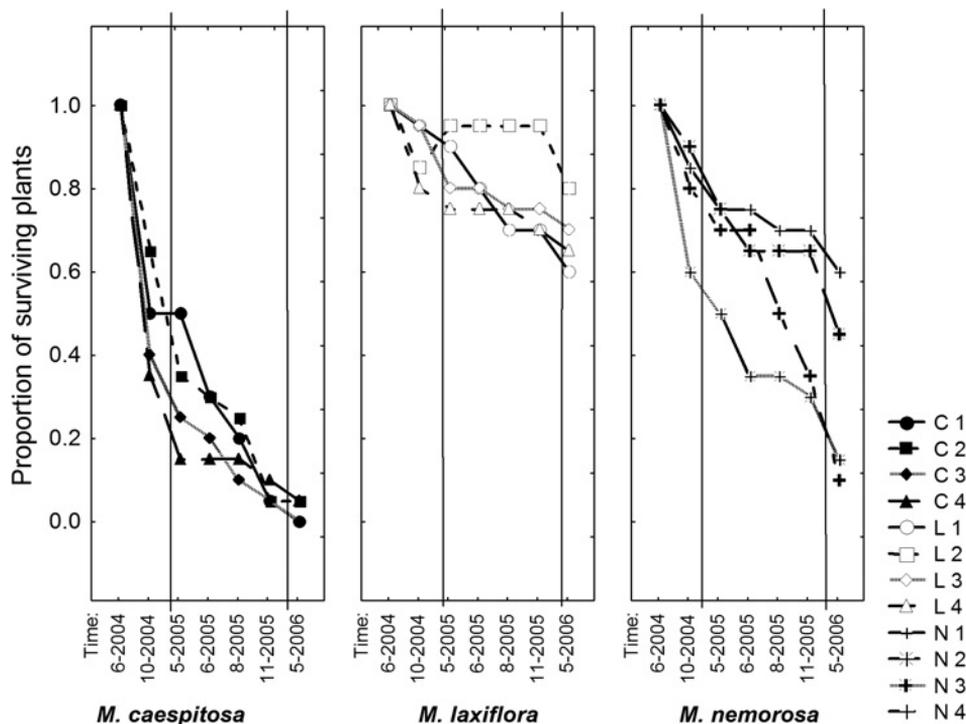


Fig. 2. – The proportions of plants from twelve populations of three *Myosotis* species that survived in all the treatments. Population is a random factor nested in species. The differences in dynamics among species and among populations within species are both highly significant (the interaction Species \times Time $F_{12, 54} = 9.48$; $P < 0.001$, Population (Species) \times Time, $F_{54, 1152} = 2.05$; $P < 0.001$). Abbreviations of species: C – *M. caespitosa*, L – *M. palustris* subsp. *laxiflora*, N – *M. nemorosa*, with population numbers according Table 2.

$F_{1, 202} = 12.01$; $P < 0.001$). There were differences among populations ($F_{9, 202} = 6.03$; $P < 0.001$). In the second season, plants of all species were again taller in the treatments without competition ($F_{1, 126} = 14.60$; $P < 0.001$, Fig. 5). Difference in the height of the species changed, because *M. caespitosa* plants were the smallest in comparison with both *M. palustris* subsp. *laxiflora* and *M. nemorosa* (Tukey post-hoc comparison, $F_{2, 13.46} = 9.88$; $P < 0.002$ for overall ANOVA). This difference between seasons was the result of all clones having slightly taller shoots in the soil than the sand mixture in this year ($F_{1, 126} = 3.92$; $P < 0.05$) and species reacting with various intensity to competition (interaction of species and competition: $F_{2, 126} = 4.30$; $P < 0.016$). The most pronounced was the reaction of *M. caespitosa* plants all of which died in the competitive treatment. Also, the *M. nemorosa* plants were taller in the without-competition treatment. Significant among population differences and the interaction between substrate and competition treatments disappeared in the second year.

Significant differences in the number of flowers were observed only between the competition treatments, where flowering frequency was greater in all species in the treatment without competition ($F_{1, 114} = 14.55$; $P < 0.001$, in June 2005).

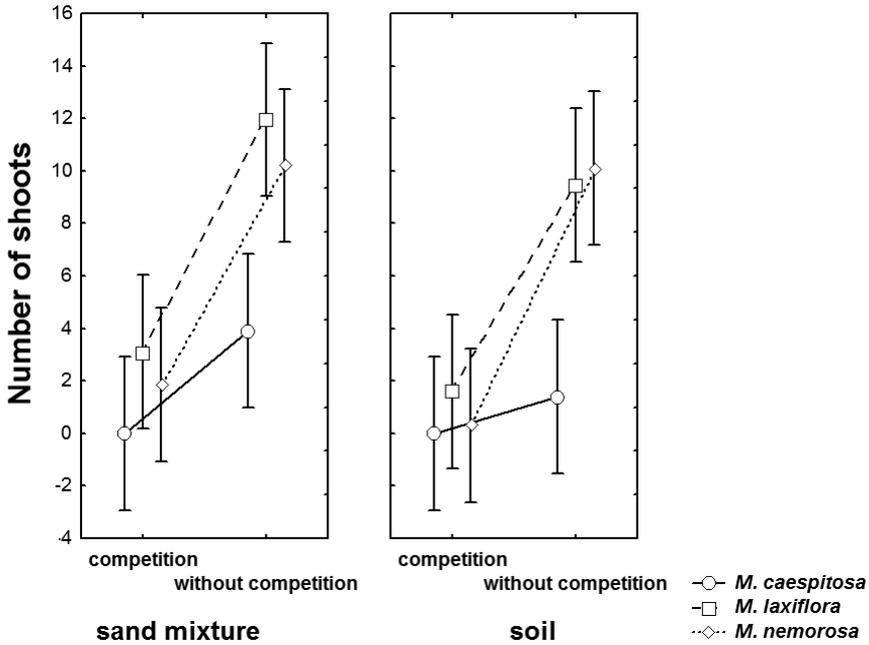


Fig. 3. – Number of shoots recorded for each of the three *Myosotis* species in the different competitive and substrate treatments in August 2005. Vertical bars denote 95% confidence intervals, LS (= least square) means. All plants are included and the dead plants recorded as having zero shoots.

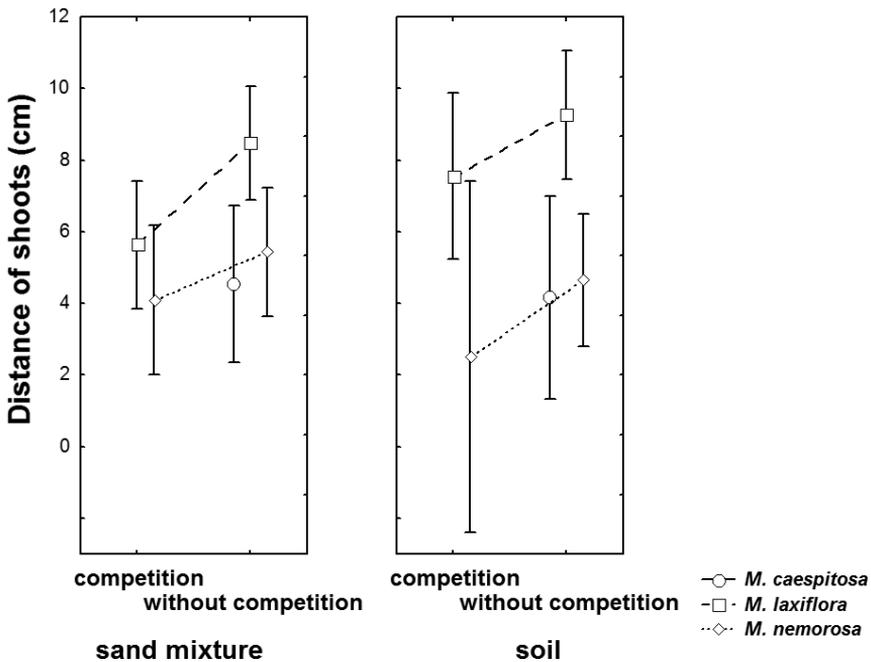
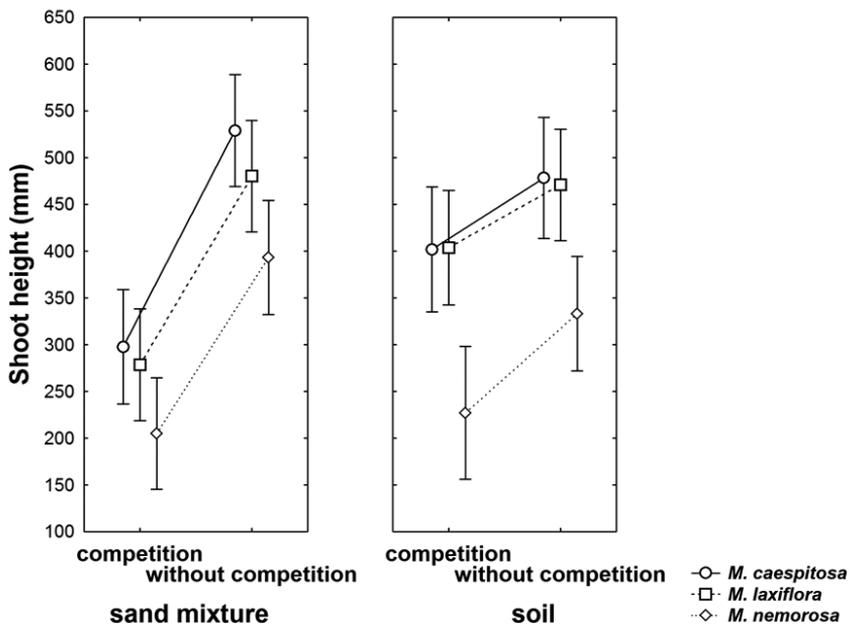


Fig. 4. – Shoot distance recorded for each of the three *Myosotis* species in the different competitive and substrate treatments in June 2005. Only plants that survived are included. For *M. caespitosa* no plants survived in the competition treatment and so the average for this treatment is missing. Vertical bars denote 95% confidence intervals, LS (= least square) means.

August 2004



May 2005

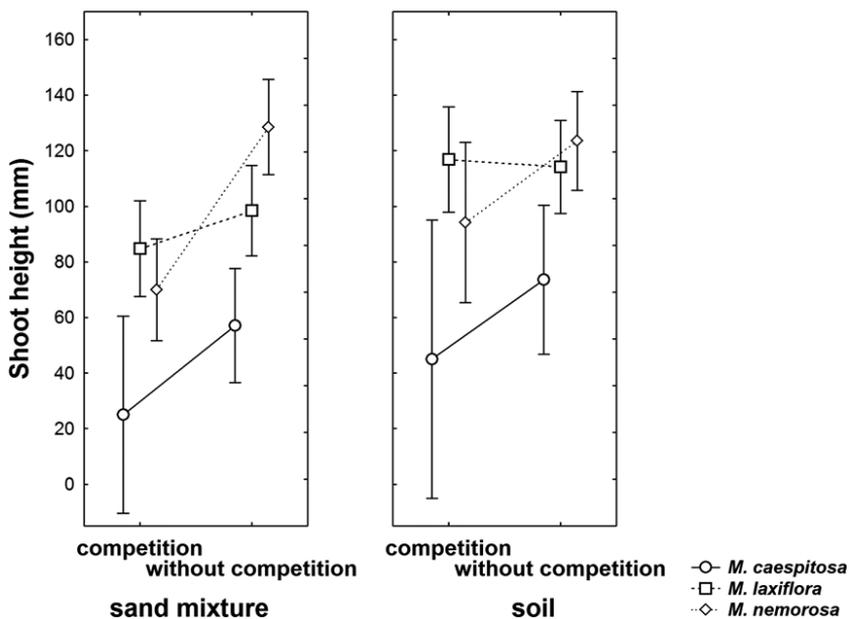


Fig. 5. – Shoot height recorded for each of the three *Myosotis* species in the different competitive and substrate treatments in August 2004 and May 2005. Only plants that survived are included. Vertical bars denote 95% confidence intervals, LS Means.

Flowering had no effect on the formation of new shoots. From autumn 2004, flowering individuals were bigger than non-flowering ones, but the flowering had no effect on the change in the number of shoots the next spring ($P = 0.56$ for the flowering by time interaction in the repeated measures ANOVA). This highly non-significant interaction was consistently obtained, when species was used as the second factor in the repeated measures ANOVA, or when the manipulations (competition, substrate) were included for some species (in some groups, all individuals were flowering, and so the complete ANOVA with all factors including flowering could not be carried out). This suggests that vigorous individuals were able to flower, but that the flowering had no negative effect on subsequent shoot formation.

The first seedlings in the pots were recorded in autumn of the first season (2004), showing that the seeds of (at least some of the species) are able to germinate immediately after being shed. Nevertheless, there was an obvious peak in seedling germination in spring (May 2005 and May 2006). Germination was poor during the vegetative seasons. A second pronounced, but lower peak in germination was observed in autumn (October 2004 and November 2005). This temporal pattern in germination is the same as that observed in the field. Seedlings were more abundant in the sand-mixture treatment. A higher number of seedlings was found in the without-competition treatment until May 2005, after which there were no differences, probably due to the dying off of the *Holcus* plants.

Discussion

Our results only partially confirmed the published life histories of the individual species based on non-manipulative field observations; the discrepancies are discussed below. Further, we demonstrated a large variability among the populations within each species; however, despite this large variability, there were significant differences among the species (tested against the variability among populations within species) both in survival and growth characteristics, and also in their response to competition.

Characteristics of individual species

Myosotis caespitosa had the shortest life-span of the three species and is usually considered to be an annual (Grime et al. 1981, Štěpánková 2000), non-clonal annual to biennial without clonal potential (Klimešová & Klimeš 1998) or biennial semirosette hemicyptophyte (Klotz et al. 2002). However, results of our long-term field experiment (E. Koutecká & J. Lepš, unpublished) suggest that this species is able to survive several seasons and form daughter rosettes or stolons in permanent habitats, at least for three seasons. But permanent habitats are not natural and typical for this species. It is then a question whether the short life span of most individuals of the species is a consequence of the inability of the species to survive the winter, or just a consequence of the fact that the habitats vanish after one or two years. Our results show that *M. caespitosa* is the weakest competitor in comparison with the other two species, although all three *Myosotis* species are considered to be CSR-strategists (Klotz et al. 2002). The low survival of this species, especially in the competitive treatment, was consistent for all four populations studied. In the field, *M. caespitosa* is confined to temporary and changeable habitats (i.e. exposed pond bottoms experiencing high levels of disturbance such as periodic flooding). After more

than one year, ponds are either flooded again or become overgrown by stronger competitors than *M. caespitosa*. In both cases, the species is able to grow there for only a very limited time. Consequently, it is expected to be most dependent on a seed bank with long-term dormant seeds (e.g. Ritland 1983, Venable & Brown 1988, Grime 2001) and not on vegetative propagation. Clonal spread in a temporary habitat is probably a way of producing more daughter rosettes and stolons and thus more flowers and seeds within one season. Dependence on, and the ability for, generative propagation is demonstrated by its abundance in the seed bank and its fast and high germination rate, which often reached 100% in favourable conditions (Grime et al. 1981, Koutecká & Lepš 2009).

Myosotis palustris subsp. *laxiflora* exhibited the best survival in comparison with the congeners in our pot experiment. The individual plants mainly produced long below-ground rhizomes but also some stolons. The species had longer shoots than the other two species, particularly in the without-competition treatment. In fact, this is the only species for which the shoots grew round the borders of the pots, which restricted them. Our field observations indicate that this species is dominant along streams and rivers, but only in the initial stages of succession, when competition from other vegetation is low. This situation frequently occurs after flooding, when the vegetation is washed away from banks. *Myosotis palustris* subsp. *laxiflora* typically occupies plots after temporary flooding: with its strong clonal potential it is able to vegetatively colonize gaps by spreading by means of stolons and rhizomes or fragments of shoots that are broken off by flood water. The severed parts are scoured by the streams, resulting in long distance spreading. This species is able to grow even at waterlogged sites (Grime et al. 1987, Lenssen et al. 1998, 2003, Štěpánková 2000, Klotz et al. 2002). A relatively good germination rate (40–60% in our experiments, Koutecká & Lepš 2009) suggests that this species is able to reproduce both generatively and by clonal propagation. Its stolons root at the nodes from axillary buds, therefore they are able to gain a footing and survive after disconnection from the mother plant or to regenerate from a very small fragment. The strategy of this species is to colonize and spread in vegetation-free habitats, because, despite its clonal potential, this species is a weak competitor and gradually outcompeted in dense vegetation.

The highest variability among populations within the species of the congeners was recorded in *M. nemorosa* for all of the characteristics measured, including survival. The high inter-population variability of this species was also observed in our germination study with the same three *Myosotis* species (Koutecká & Lepš 2009). *Myosotis nemorosa* occupies sites with a wide spectrum of conditions, from drying soils, typical wet meadows of *Molinion* or *Violion caninae* in the phytosociological classification, but also ditches, wet swamps and bogs. Therefore, the high among-population variability recorded in our study may be due to a high genotypic variability associated with the wide spectrum of habitats this species occupies. Interestingly, published records on the life history and clonality of this species differ (Table 1). Our previous study on *M. nemorosa* (Chaloupecká & Lepš 2004) indicates that individual shoots are monocarpic and die after flowering at the end of the season, which is typical of a short-lived plant. Usually, the same clone forms rhizomes, which give rise to another shoot in the next season, which might look like a new individual. This species spreads by rhizomes, but only over short distances of up to 10 cm per year. Spreading is influenced by microsite conditions; it can survive in gaps, but only in wet years (Chaloupecká & Lepš 2004). In our pot experiment, *M. nemorosa* plants produced mainly rhizomes (below-ground shoots). Stolons were also observed, but they did not root at the nodes and spread only a short distance from the

mother shoot. *Myosotis nemorosa* is able to grow in quite dense vegetation (e.g. species rich wet meadow). Clonal spread by rhizomes should help *M. nemorosa* to persist in an area rather than spread a great distance from the mother shoot.

An experimental study at one of the localities that was a source of the *M. nemorosa* seed used in our experiment (Ohrazení oligotrophic wet meadow, Špačková et al. 1998, Špačková & Lepš 2004) demonstrated a high variability in *M. nemorosa* seedling establishment among years, most probably because of year-to-year variation in moisture conditions. In addition, generative reproduction in the meadow community is dependent mainly on gaps, which are suitable, but often rare, microsites for seedling establishment. Therefore, perenniality and clonal propagation of established plants are very important for this species persistence.

Whereas no trade-off was found between flowering and investment in clonal growth in the relatively long-lived *M. nemorosa* (Chaloupecká & Lepš 2004), we expected that this might be the case in the short-lived *M. caespitosa*, but no evidence of such a trade-off was found in any of the species.

Intraspecific variability

The variability among populations of individual species was high for all the characteristics (always highly significant differences among populations) and was most pronounced in *M. nemorosa*. Similarly, high and significant variability among populations of *Rumex palustris* was found by Chen et al. (2009). This suggests that any interspecific differences should be demonstrated always against the variability among populations, not against variation of individuals (and particularly not against variation of individuals of a single population of each species). This does not seem to be a rule in comparative studies, because most of them test the differences between species against the variability among individuals, not among populations.

Seedling germination

The influence of substrate on seedling establishment was consistent with the establishment of seedlings better in the sand mixture. In our experiment, there were two pronounced peaks in the germination of seedlings; a high one in spring (May, in the second and third season) and a low one in autumn (October – November). In the field, germination of *Myosotis* also peaks in spring and autumn (E. Koutecká & J. Lepš, personal observation for *M. nemorosa*).

Effect of experimental treatments

In our experiment, survival and almost all growth characteristics (number and height of shoots, distance of shoot tips, flowering) were negatively affected by competition; this accords well with the preference of the species studied for initial successional stages. As expected, the negative effect was most pronounced for *M. caespitosa*, a species typical of the youngest successional stages and temporary habitats.

Better growth in non-competitive environments and microsites (e.g. gaps in vegetation) is a very general phenomenon, confirmed by many pot and field studies, for various species: e.g. for *Trifolium repens* by Mehrhoff & Turkington (1996) or three grass species, *Schizachyrium scoparium*, *Poa pratensis* and *Agropyron repens*, by Wilson & Tilman (1991). The latter

experiment demonstrates that this occurs regardless of nutrient level, and whether total or only below-ground competition is considered. As in our experiment, the competition effect was the most pronounced, both on survival, seedling establishment and all growth characteristics, no matter on which substrate they established (both vegetatively and generatively). In a comparative study of three closely related fern species (Rünk et al. 2004), all the species were also negatively affected by competition. In their case, the competition effect was related to local abundance – the less abundant species was affected most. Also, the most abundant species exhibited the fastest post-emergence vegetative growth (Rünk & Zobel 2009).

Nutrient conditions are expected to influence the character of competition. In our experiment, the competitor *Holcus lanatus* was cut regularly during the experiment, therefore below-ground competition was permanently strong and above-ground changeable. When the two types of substrates used in the experiment were compared, they differed in nutrients, but also in their texture, which influence e.g. desiccation, surface temperature, aeration and sustenance of plant roots. Surprisingly, the substrate with the higher nutrient content did not have a marked positive effect. When significant differences were found, the survival of experimental plants, shoot production and seedling establishment were all better in the sand mixture with a low nutrient content. Shoot height was the only characteristic influenced positively by the nutrient-rich soil substrate. Here, the substrate also modified the effect of competition; the competition effect was more pronounced in the sand mixture than in the soil treatment. Probably, the sand mixture is more suitable for growth of plants and penetration of shoots than soil, because of better air and moisture conditions.

The little sensitivity to the soil substrate corresponds well with the behaviour of this species in the field. All three *Myosotis* species grow on a wide range of substrates, including various sandy or marshy soils, in their typical habitats (E. Koutecká & J. Lepš, personal observation). The main restriction in the field is competition from other plant species.

Differences among species and habitat preferences

Related species usually differ in some of their characteristics, but the adaptive significance of the differences is not always clear. For example, Dorman et al. (2009) found differences between related species of *Iris*, but conclude that there is no evidence of adaptation to local conditions (adaptation means adaptation of the species, because each species was represented by a single population). In contrast, the responses of various *Mimulus* species to drought (Wu et al. 2010) correspond well with the conditions prevailing in their habitats.

The differences recorded for the species used in our experiment correspond well with their habitat preferences. The habitats of *M. nemorosa* suffer little disturbance and this species is subject to strong competition from a matrix of species. Producing short rhizomes is an adaptation to this situation. The habitats of the two other species are frequently disturbed, but in different ways: those of *M. caespitosa* are usually flooded by stagnant water for a long time, whereas those of *M. palustris* subsp. *laxiflora* are usually flooded by a torrent of water for a short time. Clonal shoots and mother plants of *M. caespitosa* are killed by prolonged submersion in stagnant water and so it is better for this species to invest in seeds; the long above-ground stolons of *M. palustris* subsp. *laxiflora* are severed by torrential floods and because of their excellent ability to regenerate can be dispersed over long distances downstream. This suggests that there is not a simple measure of disturbance intensity and that the type of disturbance determines the type of clonal adaptation.

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Souhrn

Tato experimentální práce se zabývá odezvou tří blízce příbuzných druhů pomněnek ze skupiny *Myosotis palustris* na konkurenci a typ substrátu. Během tříletého květináčového pokusu bylo sledováno přežívání, klonální růst a některé další charakteristiky jednotlivých druhů. Pokusné rostliny byly z více populací, proto mohly být navíc sledovány nejen rozdíly mezi druhy, ale bylo zároveň možné porovnat i mezipopulační variabilitu v rámci druhů. Chování pokusných druhů dobře odpovídalo jejich preferencím pro jednotlivé typy stanovišť. V řadě případů se ukázalo, že rostliny žijí déle, než je uváděno v literatuře. Všechny tři druhy *Myosotis* se shodně ukázaly jako konkurenčně slabé, negativní vliv kompetice byl velmi silný. Pro růst a přežívání všech druhů se jako vhodnější jeví živinami chudší písčité směs; podobně i pro klíčení semenáčků, které probíhalo ve dvou vlnách: na jaře (květen) a na podzim (říjen–listopad). V živinami bohatší půdě byly však rostliny vyšší. Byly zaznamenány i rozdíly ve vegetativním růstu mezi jednotlivými druhy rodu *Myosotis*, které odpovídají jejich ekologickému chování v krajině. Druh *M. caespitosa* je považován za krátkověký, omezený na dočasná stanoviště typu obnažených rybníčních den. V našem květináčovém pokusu tento druh reagoval na konkurenci nejcitlivěji a do konce druhé sezóny v konkurenčních zásadách všechny rostliny zahynuly. V květináčích bez konkurence však několik rostlin přežilo i do třetí sezóny a překvapivě úspěšně se klonálně rozrůstaly pomocí deefinných růžic či krátkých stolonů. Druh *M. palustris* subsp. *laxiflora* v našem pokusu přežíval nejlépe a zároveň se i nejlépe klonálně rozrůstal pomocí vysokého počtu vytvářených rhizomů i stolonů, schopných prorůst plochu i obvod celého květináče. Tento druh tím potvrdil svůj velký potenciál k rychlé vegetativní kolonizaci narušených a dosud neobsazených stanovišť. Druh *M. nemorosa* byl druhý nejúspěšnější v přežívání i v klonálním růstu, kdy rostliny vytvářely buď velmi krátké nekořenující stolonu či krátké rhizomy. Jako významná se v naší práci ukázala mezipopulační variabilita, která se lišila mezi druhy. Zatímco odezvy populací *M. caespitosa* byly velmi podobné, mezi populacemi *M. nemorosa* byla variabilita ve všech sledovaných charakteristikách největší, což zřejmě souvisí s lokální adaptací tohoto druhu k podmínkám na velmi různých typech stanovišť.

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