Functional groups affect seedling survival both through a negative soil feedback and changes in abiotic conditions

Funkční skupiny ovlivňují přežívání semenáčků prostřednictvím negativní půdní zpětné vazby i změnami abiotických podmínek

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Seedling establishment, growth and survival are influenced by the competition from neighbouring plants for resources and their effect on the environment, including plant–soil feedback. Do species-level mechanisms operate at the functional group level? We used a long-term removal experiment in a temperate grassland to study seedling survival and growth of two forb species and two species of grass in plots with either, only forb, only grass or mixture of grass and forb plants. The seedlings were followed for 16 months and environmental factors measured. All species survived best and grew larger in plots with plants of the same functional group. The largest differences in seedling survival between grass and forb plots were recorded in winter. We therefore carried out an additional experiment that focused on the effects of above-ground biomass and plant–soil feedback on overwintering of seedlings. Removal of above-ground plant biomass at the beginning of winter increased seedling survival of all four species in grass plots, but only of the forb species in forb plots. Negative plant–soil feedback was detected only for forbs during the winter experiment. At a finer scale in the main experiment, an increasing abundance of conspecific neighbours (of the same functional group) had a negative effect on seedling survival, but in some cases had a positive effect on seedling size. The environmental conditions measured were generally more favourable for seedling establishment in the forb plots (higher PhAR transmission and R/FR ratio in early spring, lower summer soil-surface temperatures, higher soil moisture throughout the season, higher long-term nitrate flow), but in the grass plots seedling establishment was probably enhanced by a higher spatial heterogeneity. Our results indicate that seedlings of both functional groups thrive better among plants of the other functional group, although reasons differ for the two functional groups compared: negative plant–soil feedback effect on the forb functional group combined with a negative effect of biomass and the high spatial heterogeneity in the grass plots.

Keywords: Anthoxanthum odoratum, Centaurea jacea, forb, grass, grassland, Plantago lanceolata, plant–soil feedback, Poa angustifolia, seedling survival

Introduction

For the successful establishment of a plant in a community, its germination ability, seedling survival and growth can be crucial, although many grassland species rely to a large extent on clonal growth and the ability to regrow from underground clonal organs or buds (Klimešová & Klimeš 2007). Factors that influence germination and seedling survival and growth are both abiotic (light amount and quality, temperature, moisture and nutrient availability, mechanical barriers) and biotic (mycorrhizal fungi, herbivores and pathogens, allelochemicals of neighbouring plants) (Baskin & Baskin 2014). Importantly, plants that are already established in a community can influence the emergence and
growth of their neighbours, not only by direct competition (exploitation of common resources) or facilitation (e.g. a common mycelial network, Taylor 2006), but also through the effects of their litter (shading, mechanical barriers, composition of leachate; Loydi et al. 2013) or via the accumulation of pathogens (e.g. Bever 2003, van der Putten et al. 2013).

The effect of litter on emerging seedlings can be both positive and negative, depending on the amount of litter and its composition (Quested & Eriksson 2006, McLaren & Turkington 2010a, Ruprecht et al. 2010), and on the environmental conditions at the locality (latitude, altitude and exposure, soil properties, moisture regime). A meta-analysis of the results of 46 independent studies on the effect of litter on seedling establishment in grasslands (Loydi et al. 2013) revealed that litter had a positive effect on seedling recruitment when water is limiting or there are low to medium amounts of litter. The physical effects of litter on plant establishment are generally stronger than the chemical effects (Hovstad & Ohlson 2008). McLaren and Turkington demonstrate that plant functional group identity influences litter decomposition through both changes in the decomposition microenvironment and changes in litter quality (McLaren & Turkington 2011).

The concept of plant–soil feedback outlined by Bever (Bever 1994, Bever et al. 1997) has improved our understanding of the mechanisms of intra- and interspecific interactions and vegetation dynamics (de Kroon et al. 2012, van der Putten et al. 2013). Van der Putten et al. (2013) distinguishes between conspecific (direct) plant–soil feedback, when a plant species influences itself through the modification of the soil environment, and heterospecific (indirect) plant–soil feedback, when such modification of the soil environment by one species affects the growth of another species. Plants influence the surrounding soil not only by releasing or absorbing chemicals, but also via the microorganisms that grow in, on, or around their roots. The direction (negative – neutral – positive) of the conspecific and heterospecific plant–soil feedback effects of a species determine to a great extent its relationship with other species of plants and consequently, its position in the plant community. Whereas negative conspecific plant–soil feedback effects promote species diversity, positive effects promote species dominance (van der Putten et al. 2013). Although some results indicate that plant–soil feedback effects operate not only at the species level, but also at the functional group level (Symstad 2000), and that inclusion of competition into plant–soil feedback experiments can change the outcomes (Casper & Castelli 2007, Petermann et al. 2008), most experiments performed so far have focused on the plant–soil feedback at the level of individual species under glasshouse conditions. Results from such controlled plant-soil feedback indoor experiments have to be complemented and verified under field conditions (van der Putten et al. 2013) and generalized at the level of species sharing similar functional traits. Currently, however, there are no such studies.

Grasses and dicotyledonous herbaceous plants (called ‘forbs’ in this paper), two basic functional components of grassland communities, differ not only in their functional traits (e.g. Craine et al. 2001, Šmilauerová & Šmilauer 2007, 2010), but also in how they affect their environment and each other (Symstad 2000, Symstad & Tilman 2001, Pokorny et al. 2005, Quested & Eriksson 2006, Cahill et al. 2008, McLaren & Turkington 2010a, b, 2011, Šmilauer & Šmilauerová 2013). Both the chemical properties of soils and soil microbial communities can differ significantly in grass- and forb-species monocultures, although their relative effect on chemical vs microbiological properties depends also on
the soil (Bezemer et al. 2006). Davies et al. (2007) found that removal of grass increases
the soil nitrate concentration more than the removal of any other group of plants, but their
short-term removal experiment could not distinguish between an increase in soil N
caused by the decay of roots and rhizomes of dead plants and soil microorganisms linked
to them, or a less effective use of nutrients by a plant community that consists of fewer
functional groups of plants.

Several experiments that focus on the community properties that affect community
invasibility indicate that introduced species are most strongly inhibited in plant communities
that already contain species of the same functional group (Fargione et al. 2003,
Dukes 2010). Nevertheless, a meta-analysis of the experimental evidence that functional
similarity of the invaders and community members reduces community invasibility only
found evidence for this in synthetic assemblage experiments, and mainly for forbs (Price
&Pärtel 2013).

Our long-term research into the role of plant functional groups (mycorrhizal forbs and
grasses) in a grassland community involved the removal of plants by hand-weeding from
experimental plots, which began more than 10 years ago in a traditionally managed cen-
tral-European grassland. We already have evidence for the complementarity of both
functional groups when comparing peak biomass in functional-group monocultures and
mixtures (Šmilauer & Šmilauerová 2013). Species composition and diversity of the forb
group are affected by the presence of grasses, but not vice versa.

We also noticed visible differences in the amount of litter and durability in different
functional group plots (unpublished results), which might have a crucial effect on nutrient
availability and microclimate (temperature and light conditions, soil moisture), with both
groups of factors playing an important role during germination, seedling survival and
growth. In a short-term experiment that focused on mycorrhizal colonization, the survival
of the seedlings of grass and forb plants depended on the plant functional groups present in
the plot, suggesting some degree of negative feedback at the functional group level (unpub-
lished results). Based on all these findings, ours and those published by others, we used our
removal plots for the experiments presented here that focus on the effects of the functional
group composition of a grassland community on seedling survival and growth.

In addition, to compare the survival of cohorts of spring and autumnal seedlings and
evaluate the effect of above-ground plant biomass and plant–soil feedback on seedling
overwintering we complemented the main experiment running for 16 months with
a shorter, six months long experiment. Our experiments addressed three research hypoth-
eses: (i) Seedlings survive better in a community of species of the other functional group;
better abiotic conditions (light, soil moisture and temperature, nutrients) in forb plots will
be favourable for seedling growth, but grass plots might offer escape from the negative
plant-soil feedback effects that are more pronounced in the forb plots. (ii) The above-
ground biomass of the surrounding plant community plays an important role in seedling
survival during the winter; slowly degrading grass biomass will have a negative effect on
survival of seedlings of both functional groups, while the effect of quickly degradable
forb biomass will be either negligible or positive. (iii) The negative plant-soil feedback
effect operates also at the functional group level; seedlings are more likely to survive in
plots occupied by the other functional group, possibly in the gaps resulting from the
removal of above-ground biomass.
Material and methods

Site and experimental design

The study site is located near Zvíkov village, 10 km east of České Budějovice, Czech Republic (48°59’20’’N, 14°36’28’’E, 500 m a.s.l.). The soil is a cambisol that has developed on paragneiss bedrock. The concentrations of macronutrients available in the soil are low (dry weight per 100 g of soil from the A horizon): 0.22 mg NH₄⁺, 0.06 mg NO₃⁻ and 0.38 mg of inorganic extractable phosphates. The vegetation is oligotrophic and is traditionally managed as a meadow (mown once in early June, with no grazing and no application of fertilizer at least for the past 25 years) on a shallow valley slope, with a high species richness (~85 species within the experimental plots) and great diversity in composition across the site. In the area where the experiment was located the following six species were dominant: Holcus lanatus, Plantago lanceolata, Sanguisorba officinalis, Anthoxanthum odoratum, Alopecurus pratensis and Poa pratensis, and accounted for approximately half of the above-ground biomass. The nomenclature of the species of plants used in this paper follows Kubát et al. (2002).

The field experiment reported in this study was established within the plots of a long-term field experiment, which is described in the remaining paragraphs of this section. As a part of this long-term experiment, we compared the roles that the grasses and the forbs play as hosts of arbuscular mycorrhizal fungi and therefore, the experimental design recognizes three main groups of vascular plants: mycorrhizal forbs, mycorrhizal graminoids (all of the species of grass in the area studied), and non-mycorrhizal species of plants (four sedge and six forb species). The non-mycorrhizal species (which make up between 6 and 10% of the above-ground biomass) were removed from most of the plots (see below).

The design of the experiment is based on 10 completely randomized blocks, arranged in two rows and five columns, running in parallel over a local gradient of soil moisture and nutrient availability. The composition of the vegetation was manipulated by removing plants, which has many advantages over the synthetic assembly approach and complements its results (Díaz et al. 2003). Each of the blocks contained the following four 1 × 1 m treatment plots: reference plots (henceforth called Ref plots) with intact vegetation, from which no species were removed; mixture plots (F+G plots), where the two focal species groups (mycorrhizal forbs and grasses) were retained and the non-mycorrhizal species removed, and the forb and grass plots, which each retained only one of the two groups (F plots and G plots). The Ref plots allowed us to separate the potential effect of weeding (biomass removal as well as soil and community structure disturbance) by comparing them with the F+G plots, and the removal of non-mycorrhizal species from the F+G plots ensured that the absence of one functional group in F or G plots was not confounded by the effect of disturbance. The extent of the soil disturbance was considerably greater in the F and G plots during the initial preparation of the plots more than 10 years ago, but maintenance subsequently imposed similar levels of disturbance on all three manipulated types of plots.

The initial removal of non-target species was done between 2001 and 2003 (due to logistical constraints), with a subsequent and continual maintenance of the experimental treatments. All plots (except the Ref plots) were weeded three to four times each year after their establishment, with the soil disturbance kept to a minimum as much as possible. In addition, the soil in each weeded plot is cut at the borders to a depth of 15 cm to
sever rhizomes or roots growing into the plot from the surrounding vegetation, in early spring and autumn of each year. The initial, transient changes were observed during the first two to three years after the plots were established (Šmilauer & Šmilauerová 2013). Table of vegetation records for individual plots in the year 2011 (with estimates of species cover) is available in the Electronic Appendix 1.

**Main field experiment**

Ten, one-week old seedlings of four species (two forbs: *Plantago lanceolata* and *Centaurea jacea*; and two grasses: *Poa pratensis* and *Anthoxanthum odoratum*, hereafter mentioned only by generic names) were transplanted into each of the 40 plots at the beginning of April 2011. In total, 1600 seedlings were transplanted. In each plot, seedlings were arranged in 10 groups of four, each containing one seedling of each species, and each individual was tagged with an untied plastic ring.

We recorded seedling survival weekly (during the first month), biweekly (during the second month) and then once a month until the end of growing season (early November 2011), and then again from April to June 2012. The surrounding vegetation was clipped carefully approximately 5 cm above the ground during mowing in June 2011; the mowing of plots was delayed in 2012 until the end of seedling sampling.

We also measured all living seedlings in situ at the end of July 2011, and recorded the number of leaves and their dimensions (length and maximum width) to calculate total leaf size (approximated by the sum of leaf length × width for each seedling). We collected all surviving individuals during July 2012, which were transported using a portable refrigerator to the laboratory where they were stored at 5 °C before further processing. The number of inflorescences (for *Plantago*) was noted. Leaves were spread onto a glass plate and scanned with an Epson Perfection 4990 photo scanner (resolution 300 dpi) for subsequent calculation of the leaf area. The leaves were then dried (48 h, 80 °C) and weighed.

**Overwintering experiment**

To obtain more detailed information about seedling survival during winter and to compare the fate of spring and autumnal cohorts of seedlings, we planted a new set of seedlings of the same four species in late autumn (1 November 2012) in plots F and G. We removed the above-ground plant biomass from a part of each plot (approx. 25 × 50 cm) outside the central 50 × 50 cm square. Half of the seedlings were planted within this cleared area, and the other half among the standing vegetation. Seedling survival was recorded the following spring (23 April 2013). This supplementary experiment therefore also provides information about the effect of standing biomass (the amount of which differed between plots F and G, particularly during the winter and early spring) and the effect of the soil (conditioned in the long term by the present functional group) on seedling survival.

**Supplementary field measurements**

Temperature and light conditions were measured on sunny days at different times during the years 2011 and 2012. We used a M4016 data logger (Fiedler-Mágr, Elektronika pro ekologii, České Budějovice, Czech Republic) for temperature measurements and two photometer units (SPh 116, the Optical Development Unit of the Academy of Sciences of
the Czech Republic, Turnov, Czech Republic; Skálová et al. 1999) for measurements of light conditions (intensities of PhAR, red and far-red radiation). The temperature was measured at the centre of each 1 × 1 m plot of four experimental blocks (sensors were placed on the soil surface). Measurements were recorded in early summer (June), mid-summer (August) and spring (April).

Light conditions were measured in parallel by two independent units: the sensor of the first unit was positioned 2 cm above the soil surface in the centre of each group of four seedlings, whereas the sensor of the second reference unit was located 1.5 m above the soil surface (to measure light levels above the canopy). The measured light conditions were then recalculated as the percentage light intensity of a particular wave-length measured at a particular place inside the canopy relative to the reference value above the canopy measured at the same time. These measurements were recorded in June 2011, and we also measured the light conditions at the plot centre, inside a tussock and in a gap between tussocks in all experimental plots on a mid-summer sunny day (July).

Soil moisture was measured using a HH2 Moisture Meter equipped with a SM200 sensor (Delta-T Devices Ltd., United Kingdom) at five locations inside the central 0.5 × 0.5 m part of each experimental plot on a spring day (April), and on a summer day (July) after a period of hot dry weather.

Abundance of plants close (within a radius of 10 cm) to each group of four seedlings was recorded using a semi-quantitative scale at the beginning of May 2012 (0: absent, 1: one adult individual or several seedlings, 2: at least two adult individuals covering less than half of the monitored area; 3: a large tussock or many individuals covering more than half of the monitored area).

Soil nutrient availability was measured using two different methods: (i) soil from two soil depths (0–10 cm, 10–20 cm) was sampled at each plot on 7 June 2011, and was immediately transported in a portable refrigerator box to the laboratory for measurement of N-NH₄ and N-NO₃ (KCl extraction and flow injection analysis), P-PO₄ (according to Mehlich 1984), and the total N and C:N ratio (using an NC 2100 Soil Analyzer, Carlo Erba, Italy). (ii) To measure the long-term in situ plant-available nitrate and ammonium fluxes, capsules with ion exchange resins were inserted horizontally at two soil depths (5 and 15 cm) at each experimental plot and were exchanged six times during the two-year period (from October 2009 to October 2011). We used plastic rings (40 mm in diameter, 10 mm in height), both sides of which were covered with a polyamide net (Uhelon 160T, Silk&Progress, Czech Republic), filled with mixed-bed cation-anion resin Purolite C100E and Purolite A520E (1:1, Purolite, Bala Cynwyd, USA) soaked in 5 M NaCl and rinsed in deionized water. After the assay period, resin from the capsules was processed using the method of Tahovská et al. (2010) and the concentration of N-NH₄ and N-NO₃ was determined using a flow injection analyzer (FIAstar 5012, Foss Tecator, Sweden) and expressed as mg·m⁻²·day⁻¹.

Rate of litter decomposition was estimated using the litter-bag method. Dead plant material of graminoid (grasses and sedges) and forb species was sorted from the above-ground biomass collected during October 2010 at the study site (outside the experimental plots), dried at room temperature and cut into 2 cm pieces. Litter bags (size 8 × 9 cm, made from polyamide technical cloth with a 1.1 mm mesh size) were filled with 2 g of graminoid material, or 2 g of forb material, or with a mixture of both (1.2 g of graminoid material and 0.8 g of forb material – based on the relative proportions of these two plant
groups in the dead plant material at the study site), were sealed with polyester thread and labelled. Two litter bags of each type were placed on the soil surface in each F, G and Ref plot, and were attached to the ground with wooden toothpicks in late November 2010 (the weather was rainy after litter-bag installation and winter weather with snow and frost started a week later). The litter bags were collected after four and seven months, transported to the laboratory, cleaned of any soil and extraneous plant material or animals, dried (3 days at 60°C) and weighed. The P content was determined after mineralization, by a photometric method using an ammonium molybdate reaction, and C and N contents were determined using an HCN analyser (NC 2100 Soil Analyser; Carlo Erba, Italy) in the laboratory of the Institute of Botany, The Czech Academy of Sciences.

**Statistical analysis**

The survival of planted seedlings was compared based on Cox proportional-hazard mixed effect models, using the `coxme` package in the R program, version 3.0.1 (R Core Team 2013). Two random effects corresponding to the variation at the plot and quadruple spatial levels were included. Chosen fixed effects were evaluated using the likelihood-ratio test (LRT) approach. In addition to examining the treatment effects on seedling survival over the whole time period of the experiment, we also studied the differences in survival within narrower, ecologically meaningful periods: (i) first spring period (April to May 2011), (ii) the summer period (June to August 2011), (iii) the over-wintering period (September 2011 to March 2012), (iv) the second spring period (April to May 2012). When evaluating seedling response within these narrower periods, seedlings that died before the period started were ignored and the status of all seedlings that survived to the end of the period was set as ‘censored’ (i.e. continuing their life for an unknown, unobserved time span).

The effects of species identity, experimental treatments and additional measured predictors upon seedling performance descriptors (leaf count, leaf area, biomass and number of flowering stems) were evaluated using linear mixed-effect models (LMM), using the `lme4` package in the R program. Plot and planting quadruple identity were again used as random effects. Performance descriptors were log-transformed to achieve homogeneity of variance. When testing the effects of neighbourhood on seedling survival, we used a generalized mixed-effect model (GLMM), assuming binomial (Bernoulli) distribution for the seedling state (dead or alive) at the time closest to the recording date of the surrounding community. To quantify the size of such effects, we used the odds change statistic, defined as $100 \times (\exp(bj) - 1)$, where bj is the estimated regression coefficient, and is the percentage increase or decrease in the odds ratio $(p/(1-p))$ (see Fox 2008) with a unit change of a predictor. The similarly defined relative change statistic was also used to summarise the size of the effects of neighbourhood upon seedling total leaf size, which is its percentage increase or decrease for each unit change of the predictor.

Differences in the light environment and surface temperature among the plots were evaluated using LMM, with the plot identity used as a random effect and sampling date as a fixed-effect covariate. In the case of monitoring soil moisture, where we had just one value per plot at each sampling date, the LMM used only the block identity as a random effect and sampling date as a covariate. Light intensity and its ratios, as well as soil moisture estimates were also log-transformed. When testing the differences in nutrient availability
among the plots, sampling depth (horizon) was used as a covariate and experimental block as a random effect. In this model, we tested not only the main effect of treatment, but also its interaction with sampling depth. To express the size of the examined effect, we used the marginal $R^2_{\text{GLMM}}$ of Nakagawa & Schielzeth (2013).

To compare the rates of decomposition of the different types of litter on differently treated plots, we used the decay rate $k$ of the Olson (1963) exponential decay model as the response variable. To examine our specific hypotheses concerning the differences between litter type and how its rate of decomposition is affected by the environment provided by the different functional groups, we used planned comparisons based on specific linear contrasts (comparing the decomposition rate between samples that have the forb or grass litter matched by the functional group at the exposure plot, with an average for all other combinations), using the multcomp package in the R program (Bretz et al. 2010). Custom linear contrasts were also used to compare seedling total leaf size in plots with plants of complementary functional group with the average of other three types of plots.

### Results

#### Seedling survival

Of the 400 seedlings of each species planted, 147 *Anthoxanthum*, 140 *Centaurea*, 61 *Plantago* and 49 *Poa* plants survived to the end of the main experiment. Survival differed significantly among the four species ($\chi^2 = 149.8, P < 0.001$), although in post hoc comparisons, neither *Plantago* and *Poa*, or *Centaurea* and *Anthoxanthum* differed significantly from one another.

When all seedlings were compared ignoring species identity, no significant difference in seedling survival due to plot treatment was detected ($\chi^2 = 6.52, P = 0.089$). However, the ability of our model to explain seedling survival was substantially improved by allowing for a different effect of plot treatment for different seedling species (effect of adding an interaction term: $\chi^2_{12} = 134.24, P < 0.001$). The effect of plot treatment on species survival was different for different species and had no substantial component that was shared by all the species studied. We therefore subsequently fitted survival models of plot-type effects for individual seedling species.

The survival of all species differed significantly in plots with the different treatments ($\chi^2$ and $P$ values for individual species were 30.9 and $< 10^{-4}$ for *Anthoxanthum*, 16.07 and 0.0011 for *Poa*, 16.79 and $< 0.001$ for *Centaurea*, and 12.24 and 0.007 for *Plantago*, respectively).

*Anthoxanthum* had the highest survival probability in the forb plots (Fig. 1A). When comparing the F and G plots, *Anthoxanthum* had a 67% lower risk of death in the F plots ($\chi^2 = 15.84, P < 0.001$). This trend was most pronounced (78% lower risk, $\chi^2 = 12.83, P = 0.004$) during the third (overwintering) period.

*Poa* also had the highest survival probability in the F plots (Fig. 1B). When comparing only F and G plots, *Poa* had a 54% lower risk of death in the F plots ($\chi^2 = 9.6, P = 0.002$). The highest difference (62%, $\chi^2 = 10.87, P = 0.004$) in *Poa* survival between these two treatments was during the first two months of the experiment.
Centaurea had the highest survival probability in the G plots (Fig. 1C). When comparing only F and G plots, its risk of death was 45% lower in the G plots ($\chi^2 = 14.28, P < 0.001$). This effect was most pronounced during the third (overwintering) period, when the risk of death was 89% lower in the G plots ($\chi^2 = 18.67, P < 10^{-4}$).

Plantago also had the highest survival probability in the G plots (Fig. 1D), where seedlings had a 46% lower risk of death in a pairwise comparison with the F plots ($\chi^2 = 6.83, P = 0.009$). The difference in Plantago survival between these two types of plot was again greatest ($\chi^2 = 6.88, P = 0.035$) during the third (overwintering) period, when the probability of death was 70% lower in the G plots.

Fig. 1. – Survival curves of the four species of plants planted in the experimental plots of the main field experiment. The horizontal axis shows the days since planting and an additional scale shows the months of 2011/2012.

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Seedling overwintering: effect of above-ground biomass

In our short-term winter field experiment, seedling survival differed among species ($\chi^2 = 27.81, P < 0.001$), with the risk of death increasing in the order *Anthoxanthum* < *Poa* < *Centaurea* < *Plantago*. As in the main experiment, grass survived better in the F plots and forbs in the G plots ($\chi^2 = 62.0, P < 0.001$). Biomass removal had a generally positive effect on seedling survival ($\chi^2 = 36.0, P < 0.001$), although the size of this effect depended on plot type ($\chi^2 = 21.25, P < 0.001$) and to a lesser extent ($\chi^2 = 7.00, P = 0.072$) on seedling species identity (Table 1). Specifically, biomass removal had a positive effect on seedling survival of all species in the G plots, but only on the survival of forb species in the F plots. *Poa* was the only species affected negatively by biomass removal in the F plots, whereas *Anthoxanthum* did not respond at all to biomass removal in the F plots.

When evaluating seedling survival in the parts of the plots from which the above-ground biomass was removed, the survival of grass species did not differ between plots ($\chi^2 = 0.44, n.s.$ for *Anthoxanthum*, and $\chi^2 = 1.097, n.s.$ for *Poa*), but the survival of the forb seedlings was significantly better in the G plots ($\chi^2 = 10.963, P < 0.001$ for *Centaurea*, and $\chi^2 = 7.748, P < 0.010$ for *Plantago*).

Table 1. – Seedling survival in plots differing in functional group composition and their dependence on the above-ground biomass removal in the overwintering experiment. Effects of experimental treatment (plot type, biomass removal) and their interaction are indicated by $\chi^2$ values and significance (**P < 0.001, n.s. – non significant result). F and G are the plots with species of particular functional group (forbs or grasses).

<table>
<thead>
<tr>
<th>Plot type</th>
<th>Short-term treatment</th>
<th>Anthoxanthum</th>
<th>Poa</th>
<th>Centaurea</th>
<th>Plantago</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>Biomass removed</td>
<td>74%</td>
<td>40%</td>
<td>32%</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td>Biomass intact</td>
<td>70%</td>
<td>60%</td>
<td>12%</td>
<td>16%</td>
</tr>
<tr>
<td>G</td>
<td>Biomass removed</td>
<td>68%</td>
<td>52%</td>
<td>68%</td>
<td>60%</td>
</tr>
<tr>
<td></td>
<td>Biomass intact</td>
<td>16%</td>
<td>20%</td>
<td>40%</td>
<td>34%</td>
</tr>
<tr>
<td>Effect of plot type</td>
<td>7.764***</td>
<td>2.286 n.s.</td>
<td>12.122***</td>
<td>9.534***</td>
<td></td>
</tr>
<tr>
<td>Effect of biomass removal</td>
<td>21.229***</td>
<td>0.836 n.s.</td>
<td>15.005***</td>
<td>7.534***</td>
<td></td>
</tr>
<tr>
<td>Interaction of plot type × biomass removal</td>
<td>13.484***</td>
<td>19.930***</td>
<td>0.008 n.s.</td>
<td>0.853 n.s.</td>
<td></td>
</tr>
</tbody>
</table>

Growth of surviving seedlings

Seedling size (number of leaves, and length and maximum width of each leaf) was first recorded three months after planting and no plant was flowering at that time. The total leaf size of each species was highest in the plots with only the complementary functional group’s vegetation (Fig. 2, $z = 6.74$ for *Anthoxanthum*, $z = 3.32$ for *Poa*, $z = 7.01$ for *Centaurea*, and $z = 5.50$ for *Plantago*, $P < 0.001$ for all species). Models that used the number of leaves as the response variable provided similar results.

Using the measurements recorded at the end of the experiment, we found significant interactions between species identity and plot type for both leaf area and leaf dry weight ($\chi^2 = 63.143, P < 0.001$; $\chi^2 = 62.792, P < 0.001$, respectively). Because the results for both measured characteristics were similar, we present here only those for leaf area (Fig. 3).
Table 2. Effect of the neighbourhood plant composition on seedling survival. Only the plots with both functional groups (F+G and Ref plots) were included in the analysis. Arrows represent, respectively, decrease or increase in seedling survival with an increasing abundance of conspecific individuals or with an increasing proportion of species of the same functional group in the neighbourhood of the seedlings; the arrows are followed by the odds change statistic indicating the estimated size of the effect. In the second row of each inner cell there are $\chi^2$ statistic values and significance estimates from a likelihood-ratio test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

<table>
<thead>
<tr>
<th>Species</th>
<th>Effect of increasing abundance of conspecific neighbours</th>
<th>Effect of increasing proportion of same-group neighbours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthoxanthum</td>
<td>$-15.4%$</td>
<td>$-4.6%$</td>
</tr>
<tr>
<td></td>
<td>$7.0^{**}$</td>
<td>$6.4%$</td>
</tr>
<tr>
<td>Poa</td>
<td>$-22.2%$</td>
<td>$-32.1%$</td>
</tr>
<tr>
<td></td>
<td>$4.7^{*}$</td>
<td>$5.0%$</td>
</tr>
<tr>
<td>Centaurea</td>
<td>$-117.8%$</td>
<td>$-20.9%$</td>
</tr>
<tr>
<td></td>
<td>$17.7^{***}$</td>
<td>$7.2^{**}$</td>
</tr>
<tr>
<td>Plantago</td>
<td>$-32.6%$</td>
<td>$+11.7%$</td>
</tr>
<tr>
<td></td>
<td>$10.1^{**}$</td>
<td>$8.7^{**}$</td>
</tr>
</tbody>
</table>

Fig. 2. Mean total leaf size ± 1 SE of three-month-old seedlings of the four species of plants planted in plots with different combinations of functional groups: forbs (F), grasses (G), both forbs and grasses (F+G), and intact vegetation (Ref). Letters above the bars identify the types of plots that were significantly different in multiple comparisons at $P < 0.05$. 
Anthoxanthum and Centaurea had a significantly greater leaf area in plots with only the complementary functional group, than in the other three plot types (P < 0.001 for all six comparisons). The leaf area of Poa was significantly larger in F plots, compared with the F+G plots (P = 0.033) and substantially (4.5-times), yet not significantly (P = 0.084) larger in forb plots, compared with the Ref plots. Difference between F and G plots was not significant.

Plantago seedlings also had larger leaves in the G plots than in the F+G (P = 0.016) or Ref (P = 0.018) plots, but the difference between the G and F plots was not significant. The number of Plantago flowering stems did not differ significantly between treatments.

**Effect of neighbouring plants**

Seedling survival rate decreased with an increasing number of conspecific plants in the close vicinity for all target species (Table 2). This trend remained identical, even if only
the functional group of neighbours (forb vs graminoid) was taken into account (see the third column of Table 2) for all but one target species, namely *Plantago*. The survival of *Plantago* seedlings increased with an increasing proportion of forbs in the surrounding vegetation.

The effects of the abundance of conspecifics or the proportion of matching functional groups in the vicinity of the seedling on its size (measured by total leaf size) are summarized in Table 3. Because the effects of conspecific neighbours differed, depending on the types of plots included in the analyses (F+G and Ref plots vs. F+G, Ref plus the plots with the matching functional group), we show results for both sets of plot types.

The effects of conspecific neighbours on the leaf size (Table 3) of forb (*Plantago* and *Centaurea*) seedlings differed; for *Plantago*, the total leaf size increased with an increase in the abundance of conspecifics and of the forb group in the near neighbourhood, whereas the total leaf size of *Centaurea* decreased. Seedlings of both species of grass (*Poa* and *Anthoxanthum*) had a larger total leaf size when they were surrounded by conspecifics in vegetation that consisted of both functional groups (in F+G and Ref plots), but not when the single functional group plots (the G plots here) were included in the analyses, and when the proportion of grasses in the surrounding vegetation was considered. The total leaf size of *Anthoxanthum* seedlings even decreased with an increase in the abundance of conspecifics after the inclusion of the G plots in the analysis.

### Effect of functional groups on environmental conditions

The main summary statistics of the abiotic environmental parameters measured can be found in Electronic Appendix 2. We found that the light conditions differed between plots with plants from different functional groups ($\chi^2 = 11.8$, $P = 0.008$, $R^2_{\text{GLMM}} = 0.104$) and that these differences varied with season ($\chi^2 = 34.3$, $P < 0.001$, $R^2_{\text{GLMM}} = 0.136$). In April, the F plots had a significantly higher PhAR transmission than the G plots ($z = 4.013$, ***$P < 0.001$, n.s. ~ a non-significant result).
P < 0.001, 25.9-times) and the Ref plots (z = 3.854, P < 0.001, 22.7-times). At the same time, the F plots had about a 3.8-times higher R/FR ratio than the G plots (z = 2.67, P = 0.038). Later in the season, neither the amount of incident PhAR radiation nor the R/FR ratio differed significantly among plots with different treatments. When the light conditions inside tussocks and in gaps (measured in early June) were compared, we found that the PhAR heterogeneity was much larger in the G plots (on average 7.9-times larger) than in any other type of plot (F+G: t36 = 3.46, P = 0.008, F: t36 = 4.42, P < 0.001, Ref: t36 = 4.19, P < 0.001), whereas only the difference for R/FR ratio between the G and Ref plots was significant (t36 = 3.11, P = 0.018, three-fold higher in G).

Soil-surface temperature and soil moisture differed among treatments ($\chi^2 = 10.95$, P = 0.012; and $\chi^2 = 13.39$, P = 0.004, respectively), but when examining the results for individual sampling dates separately, only the measurements recorded in August differed ($\chi^2 = 16.5$, P < 0.001, R$_{GLMM}^2 = 0.210$; April: $\chi^2 = 2.2$, n.s., June: $\chi^2 = 3.0$, n.s.). In August (when the Ref plots were not measured), the soil-surface temperature was higher in the G plots than in the F+G plots (by 6.6° C, z = 4.72, P < 0.001) and the F plots (by 5.0° C, z = 3.53; P = 0.001). Soil moisture was higher in the F plots than in the F+G plots (16.6% higher, z = 3.34, P = 0.005) and the G plots (by 12.8% larger, z = 2.64, P = 0.042) throughout the season.

We found no differences in soil nutrients (available P, total N, NH$_4$-N, NO$_3$-N, C/N ratio) among plots of different types, based on the results of the single sample, but we found significant differences among the plot types in long-term nitrate flow measured using the exchange resins ($\chi^2 = 8.07$, P = 0.045, R$_{GLMM}^2 = 0.110$). Nitrate flow was highest in the F plots (on average 74% higher than in the other treatments), but it was significantly higher there only when compared with the Ref plots in post-hoc comparisons (z = 2.78, P = 0.028).

In the litter-bag experiment, we found that litter type had a great effect on the rate of decomposition ($R^2_{GLMM} = 0.488, \chi^2 = 63.1, P < 0.001$), but the effect of plot type (present functional groups) and the interaction between plot and litter types were also significant and relatively important (plot type: $R^2_{GLMM} = 0.088, \chi^2 = 16.6, P < 0.001$; litter type: $R^2_{GLMM} = 0.050, \chi^2 = 15.6, P = 0.004$). Overall, the rate of decomposition differed between all three types of litter (at P < 0.005) and was most rapid for forb litter (2.72-times faster than for graminoid litter and 1.77-times more rapid than for mixed litter). The rate of decomposition was also fastest in the Ref plots (1.38-times higher than that in the F or G plots, z = 4.48, P < 0.001, and z = 2.69, P = 0.020, with no significant difference between F and G plot types). Post-hoc comparisons further revealed that the rate of decomposition of forb litter was lowest in G plots, when compared to F plots (z = 3.88, P = 0.002) and Ref plots (z = 4.11, P < 0.001).

The character of the changes in the chemical composition of decaying material also differed depending on the litter composition. On average, forb litter lost during the winter months 42.2±2.3% (mean±SE) of the initial amount of P, 11.6±1.7% N and 38.4±0.9% C, whereas graminoid litter lost 44.7±1.3% P, 38% N and 25±0.5% C. During the following spring, changes in the P amount were negligible for both functional groups, but they differed considerably in losses of both N and C (forb litter lost 33.9±3.6% N and 42.4±2.4% C, whereas the amount of N in the graminoid litter did not change, and the amount of C decreased by a further 22.9±0.7%). The significance of the litter-identity
effect was confirmed for the decrease in N in winter \( (\chi^2 = 67.9, P < 0.001) \), and for the decrease in C in winter \( (\chi^2 = 65.02, P < 0.001) \), as well as during the whole exposure period \( (\chi^2 = 65.7, P < 0.001) \). Changes in the C/N ratio were also affected by litter identity both in winter \( (\chi^2 = 121.56, P < 0.001) \), when this ratio decreased in the forb litter but increased in the graminoid litter, and during the whole exposure period \( (\chi^2 = 111.31, P < 0.001) \), when the C/N ratio decreased more rapidly in forb litter than in graminoid litter. We found no effects of plot type on the rate of C/N/P leaching.

**Discussion**

Field experiments provide more reliable information about the processes taking place under natural conditions, but at the expense of greater variability (stochasticity) in the results, when compared with laboratory or glasshouse experiments. Greater background variation consequently requires a large number of replicates, which must be offset, for a feasible experimental design, by limiting the coverage of the research questions. In the case of our experiments, we were forced to limit each of the two plant functional groups studied to two species and this might be rightly seen as a serious limitation of this study. This is, however, mitigated to some extent by the fact that the species selected are reasonably important in the grassland community studied. Even more assuring for the representativeness of our results is that both of the species in each of the two functional groups responded in a consistent way (with the extent of their response possibly differing, but not the direction of the difference).

On the other hand, an important strength of the evidence provided by this study comes from the fact that the manipulated plant communities in individual plots (F, G, F+G, and Ref plots) are part of the original natural composition, modified by hand-weeding the undesired species, so that neither the species identity nor the age structure within each functional group is artificial, as is inevitably the case for commonly used sowing of experimental plots in other field experiments. The effect of soil conditioning (from the perspective of the soil feedback study) is therefore representative and of long-term nature.

**Seedling survival and growth**

All the species studied were more successful (had a higher survival rate and a larger total leaf area and dry weight per seedling) in plots occupied only by plants of the other functional group (although some trends were not significant, due to great variability in the measurements; mean leaf size for *Poa*, see Fig. 2, and total leaf dry weight for *Poa* and *Plantago*, see Fig. 3). This trend was consistent not only across all four species in the main 1.5 year experiment but also when the cohorts of spring and autumn seedlings were followed (see Fig. 4). In a short-term removal experiment, Symstad (2000) also found that the presence of C3 graminoids in a grassland community had a negative effect on the seedling survival of C3 and C4 graminoids, but not on forbs and legumes, and the results of other studies (Fargione et al. 2003, Turnbull et al. 2005, Mwangi et al. 2007, Roscher et al. 2009) indicate that introduced species are most strongly suppressed in plant communities that already contain species of the same functional group.

However, a meta-analysis performed by Price & Pärtel (2013) supports such conclusions only partially: communities containing functionally similar resident species reduce
invader colonization and the performance of forb invaders, but not of grasses (although when one study was excluded from their analysis, the overall effect for grass performance was significant and supported limiting similarity). Moreover, support of the limiting similarity rule was only detected in synthetic assemblage experiments and not in functional group removal experiments under more natural conditions. The outcome of these experiments also depends on soil properties, as found by Bezemer (Bezemer et al. 2006) for a plant–soil feedback experiment in two grasslands; one on a sandy and the other on chalky soil.

In this study, a remarkable difference between forb and grass species survival was apparent at the very beginning of the season (see Fig. 1) when grasses had a significantly greater risk of death in the G plots than in the F plots, whereas the survival of forb species did not differ in these two types of plots. Differences in forb survival in G and F plots

Fig. 4. – Survival (mean and 95% confidence intervals) of spring and autumn cohorts of seedlings of the four species of plants grown in forb-only (F) or grass-only (G) plots. Seedlings were planted into intact vegetation or into subplots with all above-ground biomass removed (-biom). This graph compares the results of the main (spring cohort) and the overwintering (autumnal cohort) experiments. To compare the trends, survival rates over the same time spans are shown (first 175 days, which is the duration of the overwintering experiment). Letters indicate the results of multiple comparisons of differences in survival rate, for each possible pair of treatments (for a given species, i.e. subplot) sharing a letter if the survival rates do not differ significantly.
became evident later in the growing season. We found the light conditions (both PhAR and R/FR ratio) in the F plots was favourable in spring, probably due to the lack of dead plant biomass, as it decomposed during the winter. Effect of biomass presence/absence on seedling performance can change during a year from negative to positive (e.g. competition for light vs wetter microhabitats during hot weather), depending both on environmental factors and seedling age (see Fibich et al. 2013, Kelemen et al. 2015). It seems that competition plays an important role in the final outcome of plant–soil feedback experiments (Petermann et al. 2008). We suppose that the differences in light conditions determine the differential survival of grass and forb seedlings in G and F plots in early spring, as the lack of competition for light decreases the effect of negative plant–soil feedback in forb plots.

Seedling overwintering

The largest differences in seedling survival in the grass and forb plots in the main experiment were recorded during winter for three of the four species studied. These two types of plots, with only one functional group present, differ visibly in the amount of plant biomass (alive, standing dead, as well as litter) after each winter: the soil surface in the forb plots is almost completely bare. Indeed, we confirmed previous findings (e.g. Facelli & Pickett 1991, Cornelissen 1996) that the rate of decomposition of forb litter was faster than that of grass litter or a mixture of both (but the opposite ranking was found by McLaren & Turkington 2010b), although decomposition of litter in litter bags can differ from freely decaying plant material.

The importance of gaps in the vegetation for seedling recruitment of grassland species was confirmed previously (e.g. Kotorová & Lepš 1999, Fibich et al. 2013), although it was studied mostly during the vegetation season. Kelemen et al. (2015) stress the importance of an individual’s life stage for the outcome of the effect of gaps in the vegetation on its performance, but the effect of seedling age is confounded by the development of the vegetation during a season in their experiment. The removal of above-ground plant biomass at the beginning of winter in our experiment increased seedling survival and decreased differences between all target species in the G plots, but the same removal in the F plots only enhanced survival of forb species. The above-ground biomass of grasses that persists in large amounts into the next season can play an important negative role in seedling overwintering, independently of seedling identity, whereas the forb biomass disappears almost completely during winter and its effect on seedling survival can be more divergent (e.g. the negative response of Poa to forb biomass removal). Comparing seedling survival only in gaps where the above-ground biomass was removed, the survival rate of the grass species was similar in plots that were influenced (presumably through soil) in the long term by either functional group, whereas negative plant–soil feedback was evident for both forb species. There are two possible explanations for the differential effect of forb biomass on grass and forb seedlings: (i) grass resistance and forb intolerance of an allelopathic effect of decomposing forb biomass or to forb-specific pathogens, or (ii) a greater intolerance of forb species of shading. The second explanation is supported by Kull & Aan (1997), who report that forb species are inferior competitors under low-light conditions, but this is not very probable in our case, as the seedlings of forbs survived better under persistent grass above-ground biomass than under rapidly decomposing forb
biomass, and comparably well under grass biomass and in the gaps after the removal of forb biomass (see Fig. 4).

Our findings indicate that the negative effect of resident grasses on the overwintering of their “own” seedlings is mainly due to an effect of their above-ground biomass, whereas for forbs, some soil-borne effects (e.g. specific pathogens surviving in the soil and remaining in the roots of forb plants in plots where the above-ground biomass was removed), specifically suppress the forb seedlings. These differences cannot be detected in standard glasshouse experiments carried out under artificial conditions (temperature, light and soil moisture) and the absence of surrounding vegetation, due to the potential interaction between competition and plant–soil feedback (Casper & Castelli 2007, Petermann et al. 2008). Our results corroborate the findings of several previous studies (Quested & Eriksson 2006, Hovstad & Ohlson 2008, Ruprecht et al. 2010, Loydi et al. 2013) that the effect of litter on seed germination and seedling survival depend on the species (and functional type), litter composition and environmental conditions in the experimental plots.

**Effect of neighbouring plants**

The negative effects across functional groups are apparent not only when comparing the fate of seedlings in plots in which the vegetation consisted of only a single functional group, but also in plots with plants of both functional groups. In these cases the increasing abundance of conspecific neighbours, and for most species, also of neighbours of their own functional group, had a negative effect on seedling survival. But in most cases, increasing abundance of conspecific neighbours had a positive effect on seedling size (total leaf area). This apparent contradiction might result from the fact that only the surviving plants could be used to measure size parameters. Plants that suffered among conspecifics (e.g. due to specific pathogens thriving in a dense community of conspecific hosts or those from the same functional group) died, and only individuals that were resistant or that encountered specific beneficial mutualistic microorganisms (possibly supported by other plants of the same species or functional group) could survive and grew better than those without such a specific advantage.

Semchenko et al. (2013) report significant differences between grass and forb survival rates when growing close to conspecifics: grasses grew larger in competition with conspecifics, whereas forbs grew better with heterospecifics. These relationships were correlated with the spatial aggregation of species (graminoids had a higher degree of conspecific aggregation than forbs). We can confirm these results for three out of our four species (both grasses and Centaurea) for seedling size, but not for their survival, because all our species were suppressed more by their conspecific neighbours. Moreover, for grasses the relationship between seedling size and conspecifics abundance depends on which type of plots is included into the analysis: a significant positive correlation found for seedlings growing in mixed vegetation (F+G and Ref. plots) was cancelled by the inclusion of grass-only plots into the analysis. Light conditions in the G plots were much more heterogeneous than in any other plot type, so an increasing abundance of conspecifics (as well as any other grasses) will in consequence have a larger impact to seedling light benefit in G plots than in plots with mixed functional groups and consequently more homogenous light conditions.
Effect of functional groups on environmental conditions

Multiple environmental characteristics differed between plots with different established functional groups. In early spring, the F plots with almost a bare soil surface experienced the highest photosynthetically active radiation (PhAR) and red/far red light ratio, providing favourable conditions for seed germination and high rates of photosynthesis. Differences in the light conditions between experimental plots decreased later in the season, reflecting rapid above-ground biomass production in all plots. Soil moisture was highest in the F plots throughout the year and this can play an important role in seedling survival, mainly during the period of summer drought. Unlike forbs, regrowth in grasses is delayed when the weather is hot and dry after mowing, which results in the soil surface being more or less bare. Plots with vegetation consisting only of grass had the highest soil temperatures during late summer, making them inhospitable for seedlings.

The G plots in our experiment, with patches of tussock grasses (although not all the grass species have a tussock growth form), had the highest spatial variability in PhAR intensity of all the plots. This result illustrates the homogenising effect of forb species, which often exploit space among the grass tussocks in multi-functional-group vegetation.

We found, similarly to Symstad (2000), no difference in soil nutrient availability between plots with different treatments, based on a single soil-sample, but detected a higher flow of nitrates in the F plots, when measured over a long time period using exchange resins. This latter finding matches the results of Davies et al. (2007), who report an increase in nitrate concentration in the upper soil layer after grass removal, and also our finding that the forb litter decomposes more quickly and with a larger N leaching than graminoid litter.

In addition to the differences in rate of litter decomposition, graminoid and forb litter differed also in its chemical composition dynamics during decomposition, as the graminoid litter lost most of its N during winter, whereas N leaching from forb litter increased throughout the following spring. Forb litter lost a larger proportion of C (80% in comparison with the 47% loss from graminoid litter), which is the most important litter trait that influences the soil microbial community (Fanin et al. 2014).

Our study also provides a novel result, the different types of litter (forb, graminoid and a mixture of the two) decomposed most rapidly in a complex plant community (i.e. the community containing both grasses and forbs, including mycorrhizal and non-mycorrhizal species of plants, as in the Ref plots in our experiment). Graminoid to forb biomass ratio and the total biomass (measured in mid-June) do not differ between Ref and F+G plots; the only differences recorded were the absence of non-mycorrhizal species and the mild soil disturbances due to regular weeding in the F+G plots.

Conclusions

The results of this study summarized above corroborate to a great extent all three hypotheses; only the plant–soil feedback effect was not confirmed for the grass functional group. Our findings provide strong and complementary evidence that the seedlings of both functional groups thrived better when surrounded by plants from the other functional group and that the negative plant–soil feedback occurs not only at the conspecific level, but also at the level of functional group, namely forbs. In addition, different mechanisms regulating
seedling establishment were recorded for each functional group. Grasses generate quite inhospitable conditions, lower light transmittance and lower R/FR ratio in spring, higher soil surface temperature in summer, lower soil moisture throughout the year, but create a more spatially heterogeneous environment with sunny gaps. Forbs, on the other hand, provide much more suitable abiotic conditions for seedling establishment, but the strong plant–soil feedback effect within this group inhibits its own seedlings. We believe that these and other findings of this study will contribute to continuing discussion about the species diversity of grassland communities, and the role of plant–soil feedback in determining community structure.

See www.preslia.cz for Electronic Appendices 1–2

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