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Non-native species litter reduces germination and growth of resident forbs and grasses: allelopathic, osmotic or mechanical effects?

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Abstract Non-native plant species may contain allelopathic substances that might help to out-compete native vegetation. These allelochemicals may be released from live or dead plant tissues and be accumulated in the soil. We tested whether non-native species leaf litter and their leachates reduced seedling establishment and growth of native species. We subjected seeds of six native species to the effect of litter leachates of three of the most important invasive plants in Europe and to mannitol solutions with similar osmotic potential in germination chamber experiments. Additionally, we measured the effect of the same litter on emergence and growth of the native species in an outdoor pot experiment. Litter leachates delayed and reduced germination and affected initial root growth of all native species. The effects of leachates were significantly higher than those of mannitol, indicating the action of toxic, most probably allelochemical substances. Emergence of seedlings in pots was also reduced, but total biomass per pot was not affected and biomass per seedling increased. Allelochemicals may affect germination and early stages of seedling recruitment. However, these negative effects seem to cease shortly after germination, when other mechanisms such as competition may be more important. Consequently, litter-borne allelochemicals are unlikely to drive the invasion of the studied non-native species, but they may contribute to maintain mono-dominant stands reinforcing invasion success.

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Introduction

Successful colonization of new species in an existing community depends on a hierarchal series of ecological filters: these are (1) dispersal, determining whether a species reaches a new site, (2) tolerance of environmental (i.e. abiotic) conditions, and (3) interactions with the organisms already present in the new

environment. All these will finally determine the actual success of an introduced species (Lortie et al. 2004). Very few species are capable of effectively overcoming these filters and establishing in a new community.

Once a species has established in a new environment, it needs to grow and reproduce under the local climate and soil conditions and interact with residents. For non-native species, several mechanisms have been proposed to explain successful invasion (e.g. Inderjit et al. 2005), including the increase of competitive abilities (Maron and Vilà 2001; Keane and Crawley 2002), higher resource availability in the invaded community (Davis et al. 2000) or the action of allopathic substances released from the non-native species tissues (Callaway and Ridenour 2004; Bongard 2012). However, probably several not mutually exclusive mechanisms are responsible for the success of a diverse array of invasive species.

In particular the role of allelopathic substances has been studied for many non-native species in their invaded and natural ranges (see Inderjit et al. 2011 for a review). These allelopathic substances may reach the new environment through several pathways. In some cases, allelochemicals are exuded by the root system, reinforcing the invasion success (e.g. Callaway and Aschehoug 2000). These exudates may accumulate in the soil (e.g. Goel et al. 1989; Inderjit et al. 2008; Kaur et al. 2012). In other cases, leachates of dead plant tissues (i.e. litter) of non-native species can affect germination and establishment of native species (e.g. Barney et al. 2005; Bousquet-Mélou et al. 2005). Litter, especially of fast growing, large invasive species, usually accumulates after above ground plants parts die off at the end of the growing season, creating microenvironmental conditions at the soil surface that may either directly affect seed germination (Inderjit et al. 2008), or may impair subsequent native species establishment through the leaching of allelochemicals to the soil.

Litter cover thus can have mechanical and/or chemical effects on seed germination. The latter are characterised by reduction of soil water potential through the leaching of dissolved substances (Barritt and Facelli 2001) or the release of allelochemicals to the soil (Bosy and Reader 1995; Ruprecht et al. 2008). Wardle et al. (1992) showed that the osmotic potential of litter leachates employed in bioassays are partially responsible for reduced germination; thus, not

considering these osmotic effects may lead to an overestimation of allelopathy. Additionally, the effects of allelochemicals may change with the different phenological stages of the plant tissues (Ruprecht et al. 2010; Bonanomi et al. 2011; Baležentienė 2012). Recent studies showed that several litter types have inhibitory effects on germination and this is not related to one or a few compounds, but is due to a combination of several hundreds of low-molecular organic substances that change their toxicity during the decomposition of litter (Wallenstein et al. 2010; Bonanomi et al. 2011). Although chemical effects through secondary compounds may negatively affect seedling germination (Amatangelo et al. 2008; Ruprecht et al. 2008; Hovstad and Ohlson 2009), mechanical effects of litter, i.e. litter cover as a mechanical barrier to seedling emergence (Facelli and Pickett 1991c; Eckstein and Donath 2005), seem to prevail (Facelli and Pickett 1991b; Equihua and Usher 1993; Xiong and Nilsson 1999). In all cases, the effect of litter on establishment of seedling may vary according to litter type and amount (Bonanomi et al. 2006; Donath and Eckstein 2008; Scharfy et al. 2011) and with environmental conditions (Eckstein et al. 2012) and life-cycle stage (Eckstein et al. 2011). Litter amounts of less than 500 g m^{-2} may have positive or neutral effects on seedling emergence, especially under dry conditions (Loydi et al. 2013). Nonetheless, litter of non-native species seems to have stronger effects on seedling establishment than litter of native species (Minchinton et al. 2006; Scharfy et al. 2011; Meisner et al. 2012) and several authors pointed out that litter accumulation may enhance invasion success (Belote and Jones 2009; Farrer and Goldberg 2009; Vaccaro et al. 2009; Eppinga and Molofsky 2013).

Several neophytes invaded Europe during the last century and strongly increased their range since then (Kowarik and Boye 2003). The presence of litter of these species may change the microsite conditions in which native vegetation must establish (Loydi et al. 2014), resulting in very poor or mono-specific communities (Hüls et al. 2007; Thiele et al. 2010). Although allelopathy through litter is certainly not solely responsible for the success of invasive species in the invaded community, it may help to explain their dominance, and also the response of native communities to these new species. There is a need to study possible allelopathic effects of litter to improve our understanding of population dynamics and invasion success (Pergl et al. 2007). However, the possible

interactions between mechanical and chemical (allelopathic or osmotic) effects of non-native litter on seedling establishment of native species have, to our knowledge, not been studied before.

In the present work, we compared the effect of litter of non-native species with the effects of litter of the native community and with a no litter situation. We selected *Heracleum mantegazzianum* Somm. et Lev., *Impatiens glandulifera* Royle and *Lupinus polyphyllus* Lindl. as non-native species. All three are invasive in herbaceous communities and widespread in Central Europe and they are among the most important invasive species in Central Europe (Kowarik and Boye 2003; Lambdon et al. 2008). Their dense and high foliage (>75 cm) effectively shades lower layers of vegetation; thus, the species successfully compete with native species for light. The species are hemicrypthophytes (*Heracleum* and *Lupinus*) or therophytes (*Impatiens*), which built up large amounts of above ground biomass during the growing season (April–October), and consequently create thick litter layers after flowering and die back. The litter usually remains on site until the start of the next growing season (e.g. Dericks 2006), i.e. it is still present during the main germination period. Additionally, these species, or other congeneric species in their invasion range, contain allelochemicals in their tissues that might affect germination of native species. In the present work, we performed a series of controlled experiments, trying to answer the following questions:

1. Do litter leachates of invasive species reduce the germination and early seedling development of native species? Are these effects due to allelopathic substances or simply the result of lowered osmotic potentials?
2. Does the presence of litter of invasive species reduce the emergence and biomass of seedlings of native species in pots under outdoor conditions?

Materials and methods

Study species

Non-native species

Heracleum mantegazzianum (Giant Hogweed) is a native species from the Great-Caucasus region (Otte et al. 2007), successfully introduced in Europe and one of the most invasive species in Germany (Kowarik and Boye 2003). It may change community composition and structure, and forms species-poor or monospecific dense stands (Hüls et al. 2007), reaching the highest cover in tall herb communities and ruderal grasslands (Thiele and Otte 2006). *I. glandulifera* (Himalayan Balsam) is native from the Himalaya and widespread throughout Europe (Pyšek and Prach 1995). It develops in open and semi-shaded sites, preferring wet and nutrients rich habitats (Hulme and Bremner 2005; Tanner and Gange 2013). *Lupinus polyphyllus* (Garden Lupine) is native from western North-America and widespread in Germany, in particular in mountainous regions such as the Rhön in Hesse and northern Bavaria (Otte and Maul 2005). It is commonly found along road verges and in unmanaged or late-mown grasslands where *L. polyphyllus* forms dense stands, reducing species richness and promoting the formation of species-poor communities (Otte and Maul 2005). Hereafter all non-native species will be referred to by their genus.

Leaf litter from all the non-native species was collected from field populations in the state of Hesse, Germany. Litter was collected at the end of the growing season from standing biomass after the first frosts in the area (November 2012). Afterwards, it was air-dried in lab conditions until the beginning of the experiments.

Target native species

The effect of the non-native species was tested on six native perennial hemicryptophytes, belonging to different species types: three grasses (*Agrostis gigantea* Roth, *Alopecurus pratensis* L. and *Phalaris arundinacea* L.) and three forbs (*Achillea ptarmica* L., *Epilobium hirsutum* L. and *Lythrum salicaria* L.), commonly found in communities invaded by the studied non-natives across Central Europe. All native species are small-seeded (<1 mg per seed, see Table S1 in the Supporting Information) and were obtained from a commercial supplier of regional seeds (Rieger-Hoffamnn® GmbH). An initial germination test under control conditions showed that all seeds used had a germination potential of greater than ∼40 % (Table S1). We decided not to sterile wash the employed seeds since surface sterilization of seeds does not ensure sterile conditions during germination chamber experiments (see

Ruprecht et al. [2008]). Moreover, wet conditions during sterile wash (usually performed with NaClO solutions) can trigger seed germination (Baskin and Baskin [2001]), which may alter experimental outcomes.

Germination chamber experiments

A first germination chamber experiment was run at the Division of Landscape Ecology and Landscape Planning at the Justus-Liebig University, Giessen, Germany. It tested the effects of the following factors: litter leachate type (factor levels [k] = 5; distilled water, native grasses, *Heracleum*, *Impatiens* or *Lupinus* litter leachates) and activated carbon (k = 2; treated vs. untreated) on germination of the selected target species (k = 6) from two different species types (k = 2, grass vs. forb). We prepared a total of 360 sterile Petri dishes (9 cm Ø), 60 per target species. Each combination of factors was replicated six times (i.e. *litter leachate type × activated carbon*, total 10 treatments). We randomly assigned Petri dishes to the different treatments and fifty seeds of one of the target species were spread on a layer of filter paper in each Petri dish. At the beginning of the experiment, we watered each Petri dish with 3 mL of each of the treatment solutions. Distilled water served as a control of litter effects, while native grasses litter (originating from a mesic grassland dominated by the grasses *Poa pratensis*, *Agrostis stolonifera*, *Arrhenaterum elatius* and *Dactylis glomerata*) was considered a control of the effects of native community litter leachate of non-invaded sites. Native forbs were not used in our experiment since native congeners of the invasive species either do not exist in Central Europe (*Lupinus*) or they do not usually attain dominance in open habitats such as ruderal sites or grasslands (*Impatiens*, *Heracleum*). Additionally, it has been reported that these native congeneric species may have weaker allelopathic effects than the non-native ones (Vrchotová et al. [2011]). All leachate treatments were prepared by soaking 400 g of dry leaves in 4 L of distilled water for 72 h (following Hovstad and Ohlson [2008]). These amount of leaves correspond to the average litter quantity in the field for medium productive grasslands (400 g m^{-2} Donath et al. [2004]) and the volume of water correspond to the average amount of rain per day during spring (between 3.7 and 4.3 L m^{-2}, Meteorologische Dienstleistungen GmbH at www.wetteronline.de). The same amount of litter and water was used for all the litter treatments to allow comparisons among different leachates. Half of the resulting volume of the leachate was treated with 20 g of activated carbon for 2 h (type AHFI, EHEIM GmbH, Deizisau, Germany) to reduce the abundance of polyphenols and other secondary metabolites (Callaway and Aschehoug [2000]). This was done by soaking the activated carbon in the leachate solutions and stirring it for a two hours period. Afterwards, all solutions (treated or not with activated carbon) were filtered using filter paper until the whole solution passed through the filter (∼6 h). After this, all samples were centrifuged (30 min at 10,000 rpm at 4 °C with a Beckman J2-21 M/E centrifuge) and sterile-filtered with a 0.22 μm Ø bottle top filter (NalgeNunc International Corp.), except for *Lupinus* leachate solutions that clogged the filters and were not filtered. All Petri dishes were randomly distributed in a germination chamber with diurnally fluctuating temperatures (10/20 °C) and 12 h of darkness and 12 h of light. Petri dishes in the germination chamber were sealed in transparent plastic bags in groups of six to avoid excessive water transpiration. We measured osmotic potential with a cryoscopic osmometer (Osmomat, model 030, Gonotec GmbH, Germany) and the concentrations of the most common nutrients in the litter leachate solutions (Table [1]), such as N and C using a continuous flow analysis (Technicon Corporation, Oakland, CA, US), Ca, K, Mg and Na by means of atomic-absorption spectrometry (Varian Inc., mod. SpectrAA 220 FS, Palo Alto, CA, US) and P through a colorimetrical analysis following Gerike and Kurmies([1952]).

A second experiment was set up comprising the same six target species. However, in this experiment we tested the effect of different osmotic potentials on germination. We prepared three different concentrations of mannitol solutions, i.e. 0.13, 0.16 and 0.21 mol L^{-1}, which resulted in the following osmotic potentials: −0.34, −0.45 and −0.56 MPa (Osmomat, model 030, Gonotec GmbH, Germany). These osmotic potentials roughly correspond to the osmotic potentials of the non-native species litter leachates in experiment 1 (see Table [1]). A distilled water treatment was used as control, since grass litter leachate had similar osmotic potential as distilled water (0.04 vs. 0.02 MPa, respectively, Table [1]). The experiment was set up and run as experiment 1 above, i.e. six

Table 1 Characteristics of litter leachates employed in the germination chamber experiment

Litter leachate type	AC	pH	OP (MPa)	Nutrient concentration (mg g litter^{-1})						
				C	Ca	K	Mg	N	Na	P
Distilled water	No	6.6	−0.02	–	–	–	–	–	–	–
	Yes	7.8	−0.01	–	–	–	–	–	–	–
Grass	No	6.9	−0.04	847.5	0.51	3.73	0.37	0.69	0.03	0.94
	Yes	7.1	−0.05	754.6	0.43	3.80	0.35	0.56	0.03	0.86
Impatiens	No	5.4	−0.33	577.2	7.48	10.89	2.00	4.25	0.04	1.61
	Yes	5.5	−0.30	534.7	7.14	10.99	2.12	4.13	0.05	1.56
Lupinus	No	6.5	−0.40	672.9	11.64	6.06	4.48	3.93	0.99	1.19
	Yes	6.6	−0.41	624.2	11.19	5.91	4.17	3.60	0.92	0.98
Heracleum	No	5.8	−0.53	864.9	6.84	30.01	0.80	4.42	0.03	4.11
	Yes	6.6	−0.47	834.1	6.56	30.50	0.78	4.20	0.03	4.00

AC activated carbon, *OP* osmotic potential

replicates per mannitol solution per species, with a total of 144 Petri dishes. However, due to a shortage of seeds, we were forced to obtain new seeds from the same seed supplier of *A. pratensis*. Germination percentage of the new seed batch was considerably higher than the germination in the litter leachate experiment (mean ± SE: 85 ± 2.4 % vs. 36 ± 4.0 %, respectively), but standardization using natural-logarithmic response ratios allows comparisons of these different seed batches (see statistical analysis section).

In both experiments, seeds were counted twice a week for a period of 3 weeks. On each counting date, germinated seeds were counted and removed; root length was measured for a random sub-sample of a maximum of ten seedlings. Seeds were considered germinated when the radicle protruded through the seed coat. Root length was measured using a digital calliper and averaged per Petri dish using all available data (i.e. mean value across all counting dates). During the experiment, fungi (i.e. mould) developed in high abundance. In the middle of the experiment (i.e. day 11), we visually estimated the cover of mould in each Petri dish. Mould cover remained stable afterwards. However, mould presence did not affect germination of seeds ($F_{(1,239)} = 0.08$; $P > 0.75$) and was thus ignored in all subsequent analyses.

With the germination values per date we calculated the mean germination time (MGT) according to Ranal and García de Santana (2006) as the weighted mean of the time to germination, using days since the start of the experiment as time measurement. The following dependent variables were tested in the statistical analyses: percentage germination (PG), MGT and mean root length (RL).

Common garden experiment

At the beginning of spring we started a pot experiment (using 1 L pots, 10 × 10 × 10 cm) under natural outdoor conditions in a common garden area near Giessen (50°32′N, 8°41.3′E, 172 m a.s.l.) to study the effect of the application of litter from the native grasses and the non-native species on emergence of the six native target species. Each treatment was replicated six times. We also prepared pots without litter as controls. The experiment consisted of 180 pots in total. Pots were filled with commercial potting soil (FruhstorferErde®, Type P, Industrie-Erdenwerke Archut GmbH, Lauterbach, Germany) composed of a mixture of peat, clay and humus (pH-$CaCl_2$ 5.7, 188 mg L^{-1} nitrogen, 136 mg L^{-1} P_2O_5 and 206 mg L^{-1} K_2O) and maintained constantly moist throughout the whole experiment. On 1st March 2013, we sowed 50 seeds of one target species in each pot. Afterwards, we applied 4 g of each litter type per pot, which corresponds to the amount used in the litter leachate experiment (400 g m^{-2}). Germination started in early April. Seedlings emerging above the litter layer were counted three times during the study period to monitor mortality, although they were not marked. Since no seedling mortality was observed during the experiment, we assume that percentage of emerged seedling at the end of the experiment represents cumulative emergence. On 5th

June, seedling above-ground biomass was collected, cleaned and dried for 48 h at 70 °C. For the statistical analyses we used percentage emergence (PE), total biomass per pot (PB) and biomass per seedling (SB) as dependent variables. Additionally, we monitored temperature at the soil surface, and under the litter layer, using micro-T iButton® DS1922L temperature loggers (NexSens Tech, Beavercreek, Ohio, USA, http://www.NexSens.com) from 08/04/2013 to 04/06/2013. These were placed in additional pots with three replicates per treatments (N = 15) and recorded temperature at hourly intervals with a resolution of 0.5 °C.

Statistical analysis

We test differences in mean germination time (MGT), percentage emergence (PE), percentage germination (PG), root length (RL), seedling biomass (SB) and total biomass per pot (PB) by calculating natural-logarithmic response ratios (LnRR) to estimate the responses to the litter leachates and activated carbon (litter leachate experiment), mannitol concentration (mannitol solution experiment) and litter type (common garden experiment). LnRR are standardized with the mean of the control treatment of each species (in our experiments, distilled water or no-litter treatments) and therefore well suited to test for differences between the other factors and allow species comparisons. LnRR was calculated as proposed by Goldberg and Scheiner (2001):

$$LnRR = Ln\left(\frac{P_T}{\overline{P_C}}\right),$$

where P_T is the parameter value of the treated sample and $\overline{P_C}$ is the mean value of the control treatment. The effect of litter or leachate was considered significant when the 95 % confidence interval did not overlap with zero (i.e. control and treatments are significantly different). To compare the LnRR among treatments, we used a factorial ANOVA to analyse the effect of activated carbon (litter leachate experiment), litter leachates (litter leachate experiment), mannitol concentration (mannitol solution experiment) or litter presence (common garden experiment) and species type (all experiments). In the final statistical model the factor species was nested within species type (i.e. grasses or forbs) and all factors were considered fixed. Additionally, in all cases, to detect treatment effects at the species level, we performed a separate ANOVA

for each species. In this case, since data were normalized using Box-Cox transformations, we did not employ the LnRR and used all treatments and the control. As a measure for the relative contribution of each factor and their interactions to the total variability in all ANOVA analyses, we used the ratio of the sum of squares of the factor or interaction of interest to the total sum of squares (i.e. for all factors, their interactions and the error). We also directly compared the results obtained in the two separated germination chamber experiments (i.e. litter leachates vs. mannitol solutions) to test whether there was an allelochemical effect beyond the pure osmotic effect of leachates. Since both experiments were performed at different times, this comparison was done through a Monte Carlo permutation analysis using PopTools 3.2.5 (Hood 2010) in Microsoft Excel 2010. We calculated for different parameters (i.e. PG, MGT and RL) the difference (Δ) between the mean LnRR of each litter leachate type and the mean value for the mannitol solutions with the corresponding osmotic potential and used this value as our test statistics (observed Δ LnRR). A random distribution of Δ LnRR was generated by 10 000 permutations of the data with replacement (Manly 2001). The proportion of random results higher (or lower) than the observed Δ LnRR was used to calculate the P value of the test. To analyse changes in temperature at the soil surface we used a repeated-measure ANOVA with linear mixed effect models (LME) using mean daily temperature and daily temperature amplitude (i.e. difference between minimum and maximum daily temperature) as response variables. Litter treatment was considered a fixed effect and time was treated as a within factor (von Ende 1993). In all cases, Tukey tests were performed following ANOVA as posthoc test. All ANOVA analyses were done using Statistica 10.0 (version 10.0, StatSoftInc, Tulsa, OK, US).

Results

Germination chamber experiments

Percentage germination (PG) varied among species and was also affected by litter leachates and species types, but not by the presence of activated carbon (Table 2). Across species, forbs had a higher PG than grasses (mean ± SE: 63.9 ± 2.0 vs. 38.8 ± 2.8 %,

respectively). *Heracleum* litter (40.0 ± 3.8 %) inhibited germination more strongly than *Impatiens* or *Lupinus* litter (45.8 ± 3.7 vs. 55.3 ± 3.7 %, respectively), and these had a stronger effect than native grasses leachate (64.3 ± 3.4 %, Fig. 1a). All leachate treatments significantly reduced germination compared to the distilled water treatment (68.9 ± 3.3 %). However, when analysed by species, grass litter leachate only reduced germination of *L. salicaria*, but not of the other species and *Lupinus* leachate reduced germination of all species but *L. salicaria* (Fig. 2). Mean germination time (MGT) was shorter with native grasses leachate (mean ± SE, 9.5 ± 0.3 days), than with the other leachate types. *Lupinus* leachate showed intermediate MGT (12.0 ± 0.4 days) and this effect was significantly smaller than with *Impatiens* or *Heracleum* leachates (14.6 ± 0.3 vs. 14.3 ± 0.4 days respectively, Fig. 1b). However, MGT was longer in all leachates relative to the distilled water treatment (8.3 ± 0.3 days). Activated carbon had a very small effect on MGT, explaining less than 1 % of the variance in our model. Forb species germinated faster than grass species (MGT: 11.7 ± 0.27 vs. 13.6 ± 0.31 days, respectively). Seedling root length (RL) was higher in the presence of distilled water than in the presence of leachates (Fig. 1c). Among leachate types, *Heracleum*

had a stronger effect on RL, while native grasses leachate had the weakest effect. Although the interaction *species type × litter type* and *litter type × activated carbon* were significant, forbs and grasses followed the same pattern, but grasses had on average longer roots. Activated carbon had no effect on RL, except for native grasses leachate, where leachate treated with activated carbon resulted in longer roots than without activated carbon (2.69 ± 0.28 vs. 2.25 ± 0.23 mm, respectively).

In the mannitol solution experiment, lower osmotic potentials reduced PG and RL, while increasing MGT (Fig. 1d–f). When analysed by species (Figure S1), *A. gigantea* and *E. hirsutum* were not significantly affected by low osmotic potentials ($F_{3,20}$ = 2.90 and $F_{3,20}$ = 1.36, $P > 0.05$, respectively). *A. pratensis* and *L. salicaria* had significantly higher PG with distilled water, while there were no differences in PG among the different osmotic potentials ($F_{3,20}$ = 19.40 and $F_{3,20}$ = 8.22, $P < 0.01$, respectively). In contrast, PG of *A. ptarmica* and *P. arundinacea* decreased with stronger negative osmotic potential ($F_{3,20}$ = 15.17 and $F_{3,20}$ = 10.34, $P < 0.01$, respectively). MGT increased less than 2 days between control treatment (9.11 ± 0.44 days) and the −0.5 MPa treatment (10.72 ± 0.51 days). Monte-Carlo permutation showed

Table 2 Results of ANOVA on the effects of species (S) nested within species type (ST), litter type leachates (LT) and activated carbon (AC) on natural-logarithm response ratio (LnRR) of percentage germination (PG), mean germination time (MGT), and root length (RL)

Source of variation	df	LnRR(PG)			LnRR(MGT)			LnRR(RL)		
		MS	*P*	% exp	MS	*P*	% exp	MS	*P*	% exp
Intercept	1	**80.65**	**<0.001**		**57.0**	**<0.001**		**294.53**	**<0.001**	
S(ST)	4	**9.94**	**<0.001**	37.99	**1.0**	**<0.001**	8.18	**7.48**	**<0.002**	22.34
ST	1	**4.66**	**<0.001**	17.82	**7.8**	**<0.001**	63.45	**11.72**	**<0.003**	35.03
LT	3	**8.11**	**<0.001**	31.01	**3.1**	**<0.001**	25.02	**12.71**	**<0.004**	37.97
AC	1	0.19	0.159	0.73	0.0	0.572	0.02	0.00	0.831	0.00
S(ST)*LT	12	**1.68**	**<0.001**	6.41	**0.1**	**<0.001**	0.44	**0.28**	**<0.001**	0.82
S(ST)*AC	4	0.04	0.782	0.16	0.0	0.630	0.03	0.04	0.302	0.11
ST*LT	3	**1.17**	**<0.001**	4.48	**0.3**	**<0.001**	2.18	**0.75**	**<0.001**	2.24
ST*AC	1	0.12	0.261	0.46	**0.1**	**0.024**	0.28	0.05	0.200	0.15
LT*AC	3	0.07	0.555	0.25	**0.1**	**0.015**	0.19	**0.23**	**<0.001**	0.68
S(ST)*LT*AC	12	0.06	0.797	0.24	0.0	0.165	0.07	**0.13**	**<0.001**	0.40
ST*LT*AC	3	0.02	0.891	0.08	0.0	0.216	0.08	0.06	0.112	0.18
Error	237	0.10		0.37	0.0		0.05	0.03		0.09

df degrees of freedom, *MS* mean square, *P* error probability, *% exp* percentage explained variance

Significant effects ($P < 0.05$) are given in bold

Fig. 1 Mean (±95 % CI) ln response ratio of percent germination (PG, **a**, **d**), mean germination time (MGT, **b**, **e**) and seedling's root length (RL, **c**, **f**) for different litter leachate treatments (*left panels*) and mannitol concentrations (*right panels*). *Letters* indicate significant differences between treatments (TukeyHSD, *P* < 0.05). Effects of leachates or mannitol solutions were considered significant (i.e. different from the controls) when 95 % CI did not overlap with zero. Osmotic potential (MPa) of the leachates is indicated in *brackets*. Leachates types: *GR* grass litter, *HE Heracleum* litter, *IM Impatiens* litter, *LU Lupinus* litter
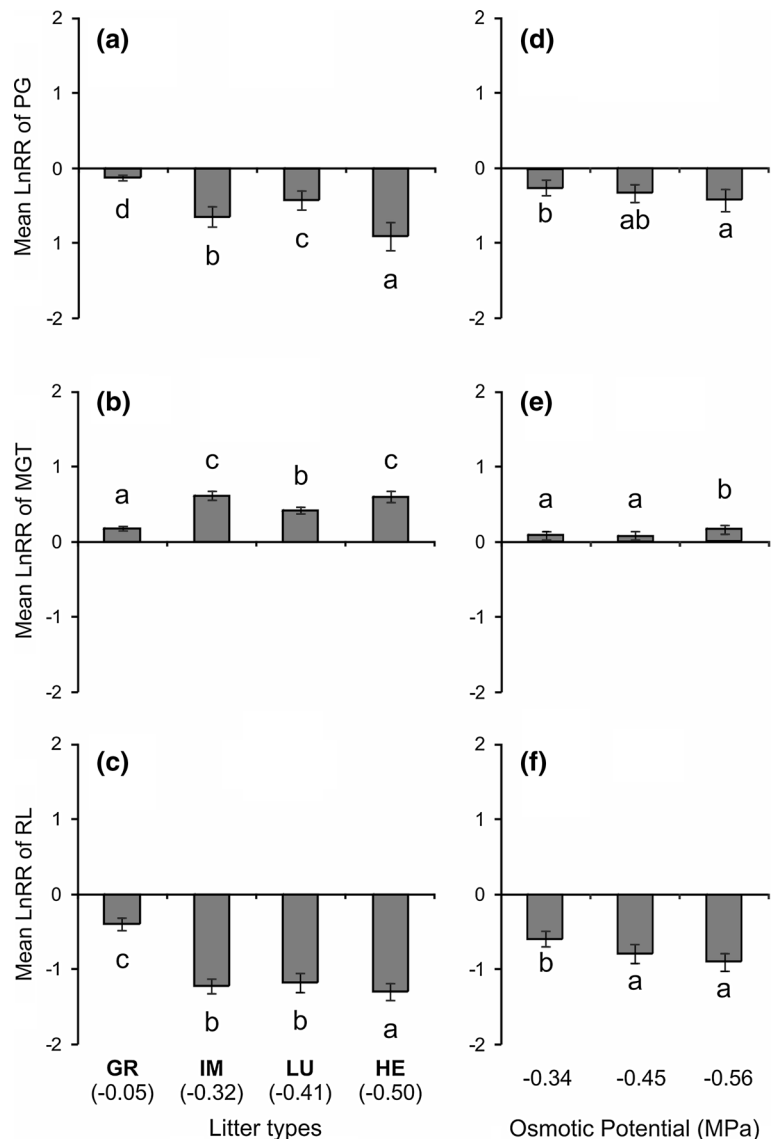
that for *Impatiens* and *Heracleum* the effects of litter leachates on germination percentage of the studied grasses and forbs were more negative than the pure osmotic effect, whereas no significant difference was found for *Lupinus* (*P* > 0.17, Fig. 3). More negative effects of litter leachates were also found for RL compared to mannitol solutions, while MGT was longer in the presence of litter leachates than the pure osmotic potential effect (Fig. 3).

Common garden experiment

Seedling emergence varied between 16.3 ± 2.7 and 77.3 ± 2.8 % among species. Across litter treatments,

A. pratensis and *P. arundinacea* showed higher seedling emergence in pots than in the germination chamber (30.4 vs. 21.3 and 24.3 vs. 17.5 %, respectively). In contrast, the other species showed lower values in the pot experiment, with reductions of ∼40 % for *A. gigantea* and *E. hirsutum* and ∼18 % for *L. salicaria* and *A. ptarmica*.

Across all target species, seedling emergence from beneath grass litter was similar to control pots (55.8 ± 2.4 and 58.4 ± 3.7 %, respectively). In contrast, non-native species litter significantly reduced seedling emergence and this effect was stronger for *Impatiens* and *Heracleum* (31.4 ± 1.7 and 31.4 ± 1.9 %, respectively) than for *Lupinus* (36.9 ± 1.7 %,

Fig. 2 Species germination percentage of seeds treated with different litter leachates. Data are mean ± SE (n = 12). *Different letters* indicate significant differences among means of transformed data for the different litter leachate types (TukeyHSD, $P < 0.05$). *DW* distilled water, *GR* grass litter, *HE Heracleum* litter, *IM Impatiens* litter, *LU Lupinus* litter
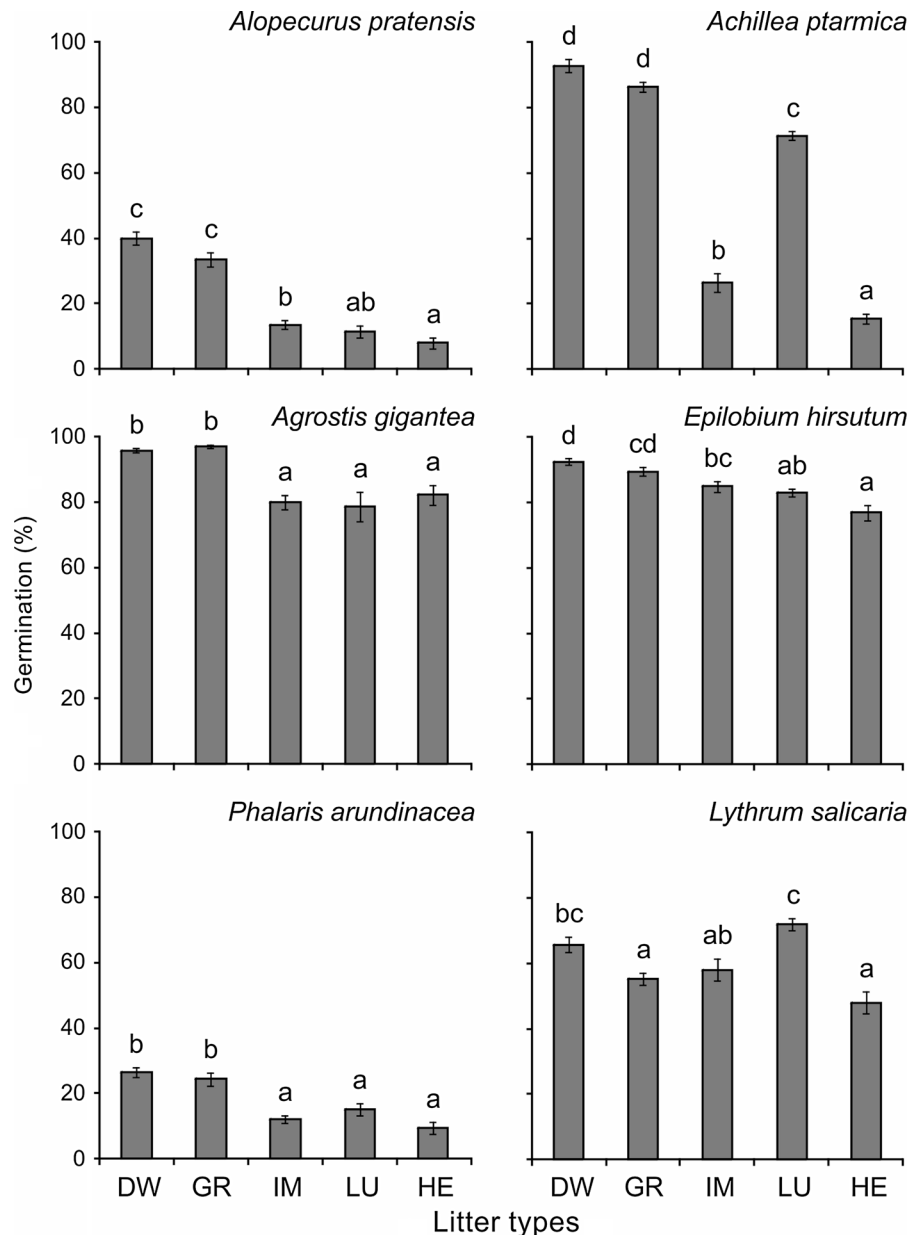
Fig. 4 and Table S3). The same general pattern was found when each species was tested separately (Figure S2), except that *L. salicaria* performed better without litter than with grass litter. Also, *A. pratensis* and *P. arundinacea* showed no reduction of emergence when covered by *Lupinus* litter. For the rest of the species, *Lupinus* litter has the same negative effect as the other non-native litter treatments. With respect to species types, emergence of forb species was more strongly reduced through the presence of litter than grass species ($F_{1,120} = 57.84$, $P < 0.01$). Across target

species, biomass per pot was higher in controls than in the litter treatments (except for *Impatiens* litter) without differences among the different litter treatments (Fig. 4b). However, the presence of grass litter resulted in the lowest biomass per seedling (0.05 ± 0.03 g per seedling). *Lupinus* litter showed similar effects on seedling growth to the control (0.08 ± 0.04 and 0.07 ± 0.03 g per seedling, respectively) while *Heracleum* and *Impatiens* litter resulted in higher biomass values (0.10 ± 0.06 and 0.11 ± 0.07 g per seedling, respectively, Fig. 4c). Forb species biomass (biomass
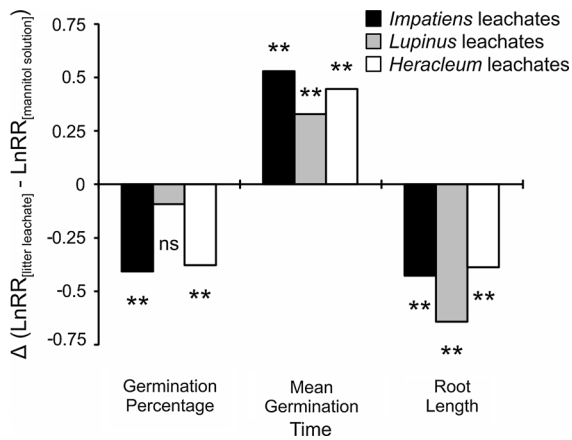
Fig. 3 Difference between the mean LnRR between the litter leachates experiment and the mannitol solution experiment for three studied parameters. Significance of the difference is based in a Monte-Carlo permutation test with 10,000 iterations. **P < 0.01, *ns* non-significant

per pot or biomass per seedling) was reduced more strongly by the presence of a litter layer than grass biomass ($F_{1,120} = 77.13$, $P < 0.01$ and $F_{1,120} = 19.29$, $P < 0.01$, respectively).

Litter presence and the different litter types employed did neither affected mean daily temperature at the soil surface ($F_{4,10} = 0.37$, $P > 0.80$) nor mean temperature amplitude ($F_{4,10} = 1.55$, $P > 0.25$) during the experiment.

Discussion

Non-native species with a high success invading communities change the structure and function as well as species composition of the invaded community (Falinski 1998). Forb species that successfully invade and spread in grasslands increase their contribution to total community biomass. According to the mass ratio hypothesis (cf. Grime 1998) the effects of a plant species on ecosystem functioning is closely related to its relative biomass, but also to its functional group (Scharfy et al. 2011; Longo et al. 2013). Therefore, changes in dominant species composition will have cascading effects on community functioning and dynamics, which may be mediated and reinforced through ecosystem components, such as the composition and mechanical and chemical quality of litter. Although plant litter seems to be one of the major factors controlling grassland diversity (Lamb 2008),
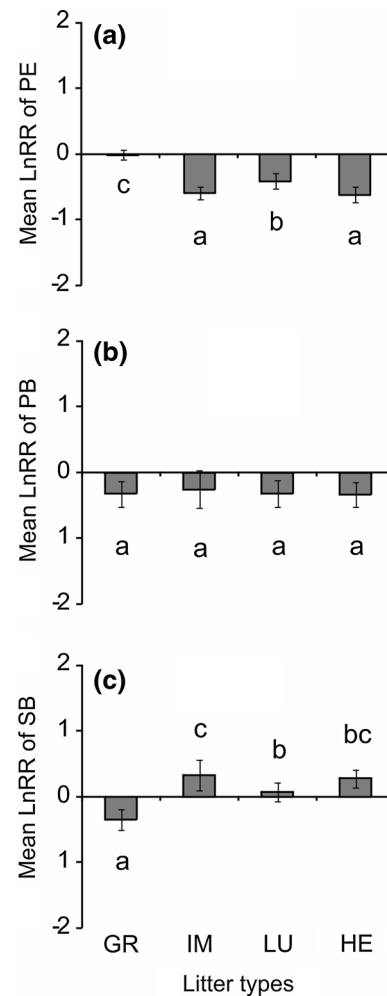

Fig. 4 Mean ($\pm 95\%$ CI) ln response ratio of percent emergence (PE, **a**), pot biomass (PB, **b**) and seedling biomass (SB, **c**) for the different litter treatments. *Letters* indicate significant differences between treatments (TukeyHSD, $P < 0.05$). Effects of litter presence was considered significant (i.e. different from the controls) when 95 % CI did not overlap with zero. Litter types: *GR* grass litter, *HE* Heracleum litter, *IM* Impatiens litter, *LU* Lupinus litter

the effects of changing litter composition and quality have been less thoroughly studied in the literature. Our results confirm that litter of non-native species had stronger effects on the recruitment of native species than the original litter (i.e. dominant native grasses).

Allelopathic, osmotic versus mechanical effects

Although among different litter types the allelopathic effects of forb litter (and especially N-fixing forbs) are usually stronger than grass litter effects (Bonanomi

et al. 2006), the causes for these differences are not yet understood. Allelopathy is widespread (Bonanomi et al. 2006) with several common grass species having allelopathic effects (e.g. Wardle et al. 1992; Chung et al. 2001). Ruprecht et al. (2008) found coumarin in litter leachate of *Stipa pulcherrima* K. Koch, a dominant species of abandoned dry grasslands, and suggested that possible allelopathic effects in combination with a reduction of the osmotic potential (−0.16 MPa) may be responsible for reduced germination and root length of co-occurring forbs and grasses. In contrast, we found that mixed litter of dominant grasses of mesic grassland had low effects on seed germination. This response seems rather general, since only one species showed a negative response to grass litter (*L. salicaria*, see Fig. 2 and S2). Additionally, the grass litter employed in our experiment only slightly reduced the osmotic potential (∼−0.05 MPa). In contrast, in litter leachates of the three common non-native forbs a strong reduction of the osmotic potential (from −0.3 to −0.5 MPa) was associated with a reduced germination of native species. However, the results of our mannitol experiment suggest that lower osmotic potentials are probably not the only reason for the strong inhibitory effect the non-native leachates (Fig. 3). Instead, the comparison of the effects of litter leachates with the pure osmotic effect indicated that also allelochemicals may be involved.

Wallenstein et al. (2010) suggested that litter decomposition may provide several different components with allelopathic effects that can alter the germination patterns of plants. The non-native forbs in our experiment contain specific compounds in their leaves that have known allelopathic effects, such as furanocumarins and other phenols in *Heracleum* (Baležentienė 2012; Moenickes and Thiele 2013), naphthoquinones in *Impatiens* (Lobstein et al. 2001), and quinolizidine alkaloids in *Lupinus* species (Wink 1983; Muzquiz et al. 1994). Litter leachates significantly reduced germination percentages of the native grasses and forbs but also increased germination time (between 3.7 and 6.3 days) and decreased initial root growth. In concert, these changes may negatively affect seedling survival and establishment. Given that germination is usually related to periods with high soil moisture (Fenner and Thompson 2005), missing these favourable periods of time, whether due to delayed germination and/or slow seedling growth, may affect

recruitment as well as population dynamics and survival (e.g. Cook 1980; Weinig 2000). In this context, thick layers of remaining litter in communities dominated by these non-native species (Dericks 2006) or accumulation of different chemicals in the soil (Scharfy et al. 2011) may affect microsite conditions during wet periods and change the chemical environment of seeds. However, the allelochemicals present at the beginning of the litter decomposition process and their negative effects on seedlings may disappear in the course of the decomposition process (Bonanomi et al. 2011). Additionally, secondary metabolites may be deactivated in the soil, which will reduce allelopathic effects under field conditions (Krogmeier and Bremner 1989; Wardle et al. 1998; Del Fabbro et al. 2014). Our results showed that there are very early effects of litter leachates on germination and root elongation of seedlings. However, in pots under outdoor conditions, where seedlings were allowed to growth for several weeks (∼8 weeks), biomass per seedling was higher in the presence of non-native litter treatments. Litter is a major factor affecting seedling emergence, but has very little direct effect on seedling growth later in the life cycle, which is mainly affected by seedling density and the associated intra-specific competition (Violle et al. 2006). Lower intra-specific competition levels in pots with non-native litter and probably also nutrients released through litter decomposition may lead to higher relative growth rates of seedlings and compensate lower germination percentage (Gross 1984; Schmiede et al. 2013). These processes may explain similar total biomass per pot among treatments but higher biomass per seedling in the treatments with litter of non-native species (Fig. 4).

Although we cannot separate mechanical and chemical effects of litter in our pot experiments, there was a strong reduction in overall seedling emergence when litter was present, indicating that mechanical effects of litter also contribute to reduced emergence. However, our results indicate that mechanical effects might not be as strong as suggested before (e.g. Rotundo and Aguiar 2005; Hovstad and Ohlson 2008) at least when dealing with non-native species. Rapid decomposition and shrinking of non-native forb litter resulted in exposed bare substrate (A Loydi, pers obs). However the lack of a subsequent boost in seedling emergence suggests a decisive impact of allelochemicals from non-native litter.

We only found minor effects of activated carbon, promoting root elongation when grass litter leachates were treated. Although the application of activated carbon was done in accordance to other works where it showed positive effects (e.g. Hovstad and Ohlson 2008), these minor effects may reflect ineffectiveness of binding the allelochemicals involved due to high concentration of leachates, especially for the non-native species which have much higher nutrient concentrations (see Table 1).

Effect of the different non-native species

Heracleum had stronger effects than the other non-native species on germination and seedling growth of forbs and grasses. Recently, Wille et al. (2013) found very weak allelopathic effects of *Heracleum* seeds and soil from invaded areas. However, in that paper unpublished data of Thiele and of Dostal et al. are quoted, suggesting lower germination and growth of native species due to allelochemicals from seeds and root exudates (cf. Wille et al. 2013). The leaf litter used in our work clearly had a strong effect on osmotic potential (~ -0.5 MPa) but the results strongly suggest additional allelopathic effects on seed germination, germination time and seedlings root growth. Similarly, *Impatiens* leachates showed a strong negative effect on seed germination, while the osmotic potential of its leachate was lower (~ -0.3 MPa). Although negative effect of *Impatiens* leaf extracts on germination and seedling growth have been shown before (Dericks 2006; Vrchotová et al. 2011; Csiszár et al. 2012), this is the first record of effects on native species occurring in the invaded range of *Impatiens*, thus providing mechanistic evidence for the reported locally negative effects of dominant *Impatiens* stands on native vegetation (e.g. Pyšek and Prach 1995; Hulme and Bremner 2005). Finally, *Lupinus* showed intermediate reductions in osmotic potential (~ -0.4 MPa) that seem to be solely responsible for the reduction in germination percentage (Fig. 3). However, *Lupinus* leachates also reduced root length and increased germination time, although these effects were weaker than those of the other non-natives species studied. This is in contrast to the results of Bonanomi et al. (2006), reporting consistently strong allelopathic effects of members of the same family (i.e. *Fabaceae*), i.e. the genera *Coronilla*, *Medicago* and *Melilotus*. Species of the genus *Lupinus* contain several allelopathic alkaloids, some of which, e.g. sparteine and

gramine (Muzquiz et al. 1994), may affect post-germination stages such as root growth but not germination percentage.

Despite the negative effects found for non-native forbs, we did not test the possible effect of native forb litter on seed germination. Some studies have shown that congeneric native species of *Heracleum*, *Impatiens* or *Lupinus* also may have allelochemicals in their tissue, but these may be weaker than those of the non-native species (Vrchotová et al. 2011) or show a high variability in their effect, depending on the target species (Lehle et al. 1983).

In our work we only found initial negative effects of non-native forbs on germination but not on seedling biomass, indicating that the higher quality with respect to nutrient contents of forb litter as compared to grass litter may benefit native species after germination. Nonetheless, the fact that grasslands are invaded and dominated by these non-native forbs (instead of any native forb species) shows that they are successful competitors and can actually establish and gain dominance in these grasslands. Allelochemicals may not be responsible for the initial spread of non-native species, but their presence in the litter may contribute to reduce the establishment of native species, helping to out-compete perennial grasses, which are known to be good competitors (Yelenik and Levine 2010; Loydi et al. 2012).

Implications for grassland invasion

The introduction of species in new environments has impacts on community species composition, structure and functions (Falinski 1998). In particular, the presence of dense stands of forbs in grassland vegetation changes the structure of the communities, thus affecting native species establishment and survival. Additionally, the dominance of a single species in the community may have feedback effects on the biochemistry of the soil (Wardle et al. 1998). Thus, successful invasion may be due to several mechanisms acting in concert. We showed that the presence of allelochemicals, may strongly affect the germination and the early stages of seedling recruitment of native species. However, these negative chemical effects seem to be lost shortly after, when other mechanisms, such as competition for nutrients or light may be more important. After emergence took place, litter presence may have indirect effect on competition, reducing

competitor biomass (Facelli and Pickett 1991a; Schmiede et al. 2013), or improve microsite conditions (Violle et al. 2006; Hofland-Zijlstra and Berendse 2010), leading to increased seedling growth. However, it is still necessary to study which role allelopathic effects play under field conditions, since a better understanding of the interactions between non-native and native species will allow to predict the success and long-term impact of introduced species (Zarnetske et al. 2013). In many grasslands most of the primary production enters the soil as plant litter (McNaughton et al. 1989) and thus the potential for germination inhibition through toxic substances in this litter may play an important role for composition, diversity and dynamics of invaded ecosystems.

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