Spatial heterogeneity of plant-soil feedback affects root interactions and interspecific competition

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Summary

- Plant-soil feedback is receiving increasing interest as a factor influencing plant competition and species coexistence in grasslands. However, we do not know how spatial distribution of plant-soil feedback affects plant belowground interactions. We examined how spatial heterogeneity of soil biota affects competitive interactions in grassland plant species.

- We performed a pair-wise competition experiment combined with heterogeneous distribution of soil biota using four grassland plant species and their soil biota. Patches were applied as quadrants of ‘own’ and ‘foreign’ soils from all plant species in all pairwise combinations. To evaluate interspecific root responses, species-specific root biomass was quantified using real-time polymerase chain reaction (RT-PCR).

- All plant species suffered negative soil feedback, but strength was species-specific, reflected by decrease in root growth in own compared to foreign soil. Reduction in root growth in own patches by the superior plant competitor provided opportunities for inferior competitors to increase root biomass in these patches. These patterns did not yet cascade into aboveground effects during our experiment.

- We show that root distributions can be determined by spatial heterogeneity of soil biota, affecting plant belowground competitive interactions. Thus, spatial heterogeneity of soil biota may contribute to plant species coexistence in species-rich grasslands.

Key words: soil heterogeneity, plant-soil feedback, root competition, grasslands, soil biota, coexistence
Introduction

Evidence is accumulating that plant-soil feedback interactions (Bever et al., 1997) contribute to plant species coexistence (Bever, 2003) and plant community dynamics in natural vegetation (van der Putten, 2003). Many studies report plant-soil feedback effects that are plant species-specific and negative (Kulmatiski et al., 2008; van der Putten et al., 2013). This means that a particular plant species changes abiotic and/or biotic soil conditions such that establishment and growth of individuals of that species is reduced, while other plant species that are less harmed by the specific soil conditions are favoured (Bever et al., 2012). Therefore, negative plant-soil feedback may promote local plant species richness (Chesson, 2000; Petermann et al., 2008; Mangan et al., 2010b; Mack & Bever, 2014), and on a longer term contribute to succession (van der Putten, 2003; Kardol et al., 2006).

Studies investigating plant-soil feedback have largely focused on homogeneous soil conditions for individual plants in pots (Augspurger & Wilkinson, 2007; Petermann et al., 2008; Mangan et al., 2010b; Hendriks et al., 2013). However, soil nutrients are known to be heterogeneously distributed in soil (Cain et al., 1999; Farley & Fitter, 1999). Similarly, soil biota are distributed heterogeneously as well (Ettema & Wardle, 2002; Bever et al., 2010; Bezemer et al., 2010), for example because each plant species has its own soil microbial community in and around the roots (Mangan et al., 2010b; Philippot et al., 2013). This spatial patterning will lead to spatial variation in plant-soil feedback, so that a diverse plant community, such as species-rich grasslands will consist of a mosaic of different soil microbial and faunal communities (Bezemer et al., 2010). These mosaics of soil biota may cause spatial patterns of (negative) plant-soil feedback within plant communities, and even within different parts of the root system of an individual plant. Little is known about how heterogeneity affects plant-soil feedback and interspecific competition between plant species (but see Brandt et al., 2013; Burns & Brandt, 2014)).

This lack of knowledge contrasts with what we know about plant’s root responses towards soil nutrient patches, affecting competitive outcomes (Robinson, 1996; Fransen et al., 2001; Rajaniemi, 2007; Mommer et al., 2012) and, consequently, the potential to affect species coexistence (Maestre et al., 2005; Wijesinghe et al., 2005; Lundholm, 2009; García-Palacios et al., 2012). It has been shown that plant roots respond to heterogeneous distributions of plant soil feedback (Hendriks et al., 2015). Changes in root distribution induced by soil biota appeared plant species-specific (Hendriks et al., 2013), suggesting that competitive relations may change if plant-soil feedback is distributed heterogeneously in plant communities. Shifts in competitive dominance between plants due to plant-soil feedback have
been demonstrated both under controlled conditions (van der Putten & Peters, 1997) and in
the field (Casper & Castelli, 2007). If these shifts lead to increased opportunity for
competitively inferior plant species to exploit soil patches where competitive pressure is
reduced, spatial heterogeneity of plant-soil feedback may enhance plant species coexistence.

Currently, there is no proof of principle that heterogeneity of plant-soil feedback may
enhance plant co-existence in grasslands. Therefore, the aim of the present study was to test if
heterogeneous distributions of patches with different plant-soil feedback effects, representing
heterogeneity of soil biota and possibly also of nutrients, cause shifts in species-specific root
distribution and competitive relationships among plant species. We examined the following
hierarchical set of hypotheses.

(1) A prerequisite for our present study is that plants grown in monoculture produce
less (root) biomass in soil conditioned by conspecifics (‘own’ soil) than with soil conditioned
by heterospecifics (‘foreign’ soil).

(2) Plants grown in interspecific competition will be at a competitive disadvantage
when confronted only with patches of own soil.

(3) As a consequence of (1), plants grown in monoculture confronted with a
combination of patches of own and foreign soil produce more (root) biomass than when
confronted with own soil only.

(4) In interspecific competition in soils with patches of different soil origins, plants
will produce less root biomass in their own soil patch and increase root biomass in the others;
this alleviates negative plant-soil feedback and reduces the strength of interspecific
competition.

To test these hypotheses, we combined a classic pairwise plant competition
experiment (Wilson & Keddy, 1986) with a plant-soil feedback approach (Brinkman et al.,
2010). Four plant species were grown in all pairwise combinations in heterogeneous soils
containing two patches of conditioned soil and two patches of background soil. The patches
were created from conditioned soils from monocultures of each of these four plant species and
all pairwise combinations of conditioned soil were used (Fig. 1). We used molecular
techniques to quantify plant species specific root biomass in each conditioned soil section
(Mommer et al., 2008).
Materials and Methods

Plant species
We used two grass species, *Anthoxanthum odoratum* L. and *Festuca rubra* L., and two forb species, *Leucanthemum vulgare* L. and *Plantago lanceolata* L. Different degrees of negative plant-soil feedback effects have been demonstrated for these plant species in previous studies (Hendriks et al., 2013; Hendriks et al., 2015). All four are common perennial grassland species in western Europe and mostly occur in traditional hay meadows (van Ruijven & Berendse, 2003).

Seed and pot preparations
Seeds of *A. odoratum*, *F. rubra* and *L. vulgare* were obtained from a seed company (Cruydthoeck, Nijeberkoop, The Netherlands) that collects seeds from wild populations. *Plantago lanceolata* seeds were collected from previous experiments (Mommer et al., 2010). Prior to germination, seeds were surface-sterilized for five hours in a desiccator of 3 l by adding 1.5 ml HCl (37-38 %; v:v) to each of the two beakers with 50 ml sodium hypochlorite (10-15 % chlorine). Subsequently, seeds were germinated on γ-sterilized sand (25 kGy at Synergy Health, Ede, the Netherlands) that was kept moist with sterilized deionized water in small containers (previously sterilized with 70 % EtOH) at 22°C (light conditions 175 µmol PAR m⁻² s⁻¹, day/night regime: 12 h light/12 h dark). Seedlings were transplanted to pots 15 days after germination. This procedure was followed for both phases of the experiment. Pots were sterilized prior to the experiment with a sodium hypochlorite solution (Cl⁻ concentration 0.05 %).

Soil preparations
Like in previous plant-soil feedback studies (Bever et al., 1997; Kulmatiski & Kardol, 2008; Brinkman et al., 2010; Hendriks et al., 2013), we used a conditioning phase, followed by a feedback phase. The main purpose of the conditioning phase was to obtain soils with plant species-specific soil communities of each of the four plant species, which could be used in the feedback phase. In the conditioning phase, on average 25 % (v:v) inoculum of specific soil from 7-year-old monocultures of each of the four plant species from a previous experiment (Mommer et al., 2010) was added to sterilized soils (loamy sand with sandy sand (2:1 v:v)) (Bever et al., 1997; Kulmatiski & Kardol, 2008; Brinkman et al., 2010; Hendriks et al., 2013). The soil of the original plant monocultures was a mixture of loamy sand, sandy sand
and potting soil (Mommer et al., 2010). On these soils, plant monocultures of each of the four species also used in the present experiment were established and grown for seven years, with regular weeding. No nutrients were added to the soil during this period. We used the soil from monocultures from a previous experiment as inoculum, instead of neutral soil, to extend the conditioning phase of the experiment and produce soils with strong plant-soil feedback effects (Hendriks et al., 2013; Hendriks et al., 2015).

In the conditioning phase, 6-8 seedlings were planted in 2 L pots. Pots were watered with deionized water and placed in a climate chamber at 16 h 22°C (day) and 8 h 18°C (night). Light was supplied at 230 µmol PAR m$^{-2}$ s$^{-1}$. Once a week, a random selection of pots was weighed and re-set to initial moisture content by initial weight. After two months, aboveground plant biomass was harvested and soils (including roots that served as inoculum source of soil biota) were cut into ± 4 cm$^3$ pieces and stored in the dark at 4 °C for three months.

For the feedback phase, we created two different types of soil: background soil and patch soil. The background soil was created by mixing sandy soil with $\gamma$-irradiated loamy sandy soil (3:1 v:v). For the patch soils, we mixed sandy soil and $\gamma$-irradiated loamy sandy soil (2:1 v:v), and subsequently added conditioned soil of one of the four plant species (1:1 v:v), creating four different soil types, one for each plant species. Roots of the plants in the conditioning phase were present in the soil of the feedback phase, which is common practice in plant-soil feedback experiments, since microorganism in the roots and rhizosphere serve as inoculum for the soil biota community. Concentrations of extractable N and P, as a proxy for differences in all nutrient concentrations, were measured in all soils (see below).

**Experimental setup**

Pots of 15 cm diameter (top) x 15 cm (2.4 L) were split into four compartments (quadrants) using an iron frame and filled with designated soils (Fig. 1). After filling of each quadrant with soil, the iron frame was removed; hence, no physical boundaries between the quadrants were present during the experiment.

Two opposite quadrants (Q1 and Q3, the ‘plant’ quadrants) were filled with background soil, so that plants could establish in the pot before being confronted with conditioned soils. Moreover, plants are expected to have a lower chance to establish in own patches than in soil of other plant species due to negative plant-soil feedback. If own soil would have been present in the plant quadrants, the plant growth would have been hampered immediately, as demonstrated in Van der Putten & Peters (1997) and Hendriks et al. (2013).
Having background soil, rather than own or mixed soil in the home quadrant also decreased variation in size among plant species, which would also have affected competition. The compartments in between the ‘plant’ quadrants (Q2 and Q4) were filled with conditioned soil of one of the four plant species (Fig. 1) in different combinations. All ten possible soil combinations of soil types (four ‘mono soils’ and six ‘mixed soils’) were used and patch soil types were randomly assigned to quadrants (Q2 and Q4).

In each ‘plant’ quadrant (Q1 and Q3), a 15-day-old seedling of either *A. odoratum*, *F. rubra*, *L. vulgare* or *P. lanceolata* was placed (Fig. 1), allowing for all ten possible plantings (again, four intraspecific ‘plant monocultures’ and six interspecific ‘mixed plant communities’). Plants were randomly assigned to plant quadrants (Q1 and Q3). The plant species under investigation will be referred to as the target species, the other one will be referred to as competitor species. Since roots grow rapidly and can reach all parts of a pot in a matter of weeks, it is likely that roots competed also in the ‘plant’ quadrants (Q1/Q3). However, our hypotheses addressed the effect of root competition in conditioned soils of the vacant patches (Q2/Q4).

So, the experiment consisted of 10 plantings x 10 soil combinations, resulting in 100 different treatments. Each treatment was replicated six times. The entire experiment was equally divided into two blocks over time with a two-week delay between blocks. Each block contained three replicates of each treatment. Plants were grown for seven weeks in a climatized greenhouse in September/October 2013 in the greenhouse facility of Radboud University (Nijmegen, the Netherlands). During the day (8.00 am – 8.30 pm), temperature was on average 22.4 °C, during the night (8.30 pm – 8.00 am), temperature was on average 18 °C. Light levels varied between 20 – 470 µmol PAR m$^{-2}$ s$^{-1}$. The watering procedure was similar to the conditioning phase.

*Nutrient concentrations of the soils*

As we used four different plant species in the conditioning phase, nutrient content of the patch soil types may have differed (Kardol *et al.*, 2006; Brinkman *et al.*, 2010). Therefore, we analyzed the concentrations of available nutrients (NO$_3^-$, NH$_4^+$, and PO$_4^{3-}$) in these four patch soil types and in the background soil separately. Differences between the four patch soil types were absent or small (significant only for NO$_3^-$). We tested for correlations between the fraction of total root biomass per pot and the fraction of available NO$_3^-$ per patch by calculating Pearson’s r. For NO$_3^-$, the correlation was weak and negative ($r = -0.16$). Therefore, it is unlikely that the difference in NO$_3^-$ affected root distributions rather than
differences in plant-soil feedback. Nutrient analyses and results are given in the Supplementary Information (Table S1 and Supplementary Methods).

Harvest
At plant harvest, shoots were clipped at soil level. Soil cores (4 cm diameter) were taken in the middle of each of the four quadrants (30% of quadrant volume) in order to avoid edge effects. Roots from the patch quadrants (Q2 and Q4) were carefully washed by the authors and experienced technicians and helpers who could recognize and remove old root fragments that originated from the conditioning phase, using 0.5 mm sieves. The remaining roots were dried between tissues and weighed fresh. Two subsample of ± 50 mg fresh weight were taken for molecular analysis to determine species-specific root abundance (Mommer et al., 2008) and frozen in liquid nitrogen before storing at -80 °C. The remainder of the roots was reweighed for fresh weight. The unwashed samples from the plant quadrants (Q1 and Q3) were stored at 4 °C no longer than two weeks and washed after all Q2 and Q4 samples for molecular analysis were washed. Shoot and root material was dried at 65 °C to constant weight and weighed. We calculated a fresh:dry weight ratio for roots.

Molecular analyses
To estimate the proportion of each of the plant species in the mixed root samples in Q2 and Q4, we applied the RT-PCR method of Mommer et al. (2008). DNA was extracted using a DNeasy 96 Plant Mini Kit following the manufacturer’s protocol (Qiagen, Venlo, the Netherlands); and DNA concentrations were measured using a Qubit Fluorimeter (Invitrogen© through Life Technologies, Carlsbad, CA, United States of America). Each plant species was separately amplified from each extract using RT-PCR using primer pairs described in (Mommer et al., 2008), with the exception of P. lanceolata where a different primer pair (“Pl3”) was used (5’-GAGAAAGCAGTAGAAACCACAGT-3’, 5’-GATCGAGATCTCTCTCCTCAGATACTCACCACC-3’). RT-PCR reactions were performed with HOT FIREPol Eva Green (Solis BioDyne, Tartu, Estonia) qPCR Mix Plus with an addition of 0.94 µM MgCl₂, a primer concentration of 120 nM for F. rubra, L. vulgare and P. lanceolata and 60 nM for A. odoratum, and 4 ng genomic DNA for P. lanceolata or 1 ng genomic DNA for the other species, in a reaction volume of 20 µl. The RT-PCR program was as follows: 15 min at 95 °C; then 45 cycles of 20 s at 95 °C, 30 s at 62 °C and 15 s at 72 °C; and finally a melting curve analysis of 5 s per cycle with an increase of 0.5 °C per cycle, starting at 70 °C and
RT-PCR analyses were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-rad Laboratories, Hercules, California, USA).

In order to test the robustness of the RT-PCR estimates of abundances of roots, reference curves were produced (Fig. S1). Twenty six standards were made using pooled monocultural roots originating from all soil types. Ten of these standards contained equal proportions of all plant species (25% each) and in the remainder, individual plant abundances were between 5-80%. The four best fitting standards with equal proportion of all plant species (based on smallest summed discrepancy between measured and actual presence) were used as reference standards on all 96 well qPCR plates in which samples were run. The correlations between actual species proportion (from hand-made mixed samples from monoculture roots) and estimated species proportions in the reference curves appeared linear, with $R^2$ values of 0.89, 0.90, 0.95 and 0.85 for *A. odoratum*, *F. rubra*, *L. vulgare* and *P. lanceolata* respectively (see Fig. S1).

Root biomasses per species per conditioned quadrant were determined based on fresh weight of the root sample multiplied by the fraction of the plant species in that sample as determined by RT-PCR, and transformed to dry weight using the fresh:dry weight ratio of each plant species on each soil type (as in Mommer *et al.*, 2010).

### Statistics

All statistical analyses were performed in R (R-Core-Team, 2014), using the nlme (Pinheiro, 2011), car and agricolae packages. All plant biomass data were analyzed with linear mixed effects ANOVA (type III sums of squares). The random part of the models consisted of block, with pot nested within block and quadrant within pot when applicable, to account for dependence of measurements of both plant individuals in one pot. In addition to full-model analyses, we split all analyses for individual plant species or plant mixed communities, when relevant. All plant biomass data were square-root transformed to meet assumptions of ANOVA, unless mentioned otherwise.

To assess plant-soil feedback for all plant species (hypothesis 1), we first analyzed monoculture root biomass (two individuals per pot) of all four plant species on the four mono soils (*A*=*A. odoratum*, *F=* *F. rubra*, *L=* *L. vulgare*, *P=* *P. lanceolata*). This analysis was also run with soil types defined as own or foreign (Petermann *et al.*, 2008; Hendriks *et al.*, 2013).

In order to evaluate hypothesis 3, we analyzed plant monoculture root biomass (two individuals per pot) on *all mono and mixed* soils to assess release from plant-soil feedback as
expected from hypothesis 1. Soil combinations were defined as ‘own-own’, ‘foreign-own’ or ‘foreign-foreign’.

Subsequently, we analyzed individual plant responses, for root as well as for shoot biomass. To check whether differences in root mass and distribution patterns occurred based on interspecific competition (hypotheses 2 and 4), root biomass of individual plants was analyzed using the following fixed factors: target species, competitor species, target soil (soil type in target quadrant, the quadrant from which the root biomass was analyzed), and opposite soil (soil type in opposite quadrant). This analysis was also performed with the soil factors defined own or foreign.

We tested whether, in a mixed plant community on mixed soils, both plant species of a competing pair respond differently to the soils in the conditioned quadrants within a pot. We did this with a separate ANOVA analysis for each mixed plant community on every mixed soil separately (target species x target soil, two levels each per analysis). An uneven root distribution over conditioned soils (indicated by a target species x target soil interaction) might indicate root mass re-distribution below ground as hypothesized under 4. A different distribution could indicate differential effects of different plant-soil feedback on root growth (Hendriks et al., 2015).

Finally, we analyzed individual shoot biomass using target species, competitor species and soil combination as fixed factors, the latter being merged from target and opposite soil (ten levels).
Results

Interspecific and intraspecific competition on mono soils

Root biomass in plant monocultures differed among plant species (Species effect: $F_{3,79}=30.99$, $P<0.001$, Table S2A,B, Fig. 2), and depended on type of mono soil (i.e. two quadrants with same conditioned soil) (Plant species x Mono soil type $F_{9,79}=7.74$, $P<0.001$, Table S2A, Fig. 2). Overall, plant species produced less biomass in own than foreign mono soils, but negative plant-soil feedback was not apparent in all four species, as indicated by the significant interaction between plant species and soil type (Plant species x Mono soil type (own vs foreign): $F_{9,87}=12.58$, $P<0.001$, Table S2B, Fig.2). The plant species with the highest biomass production ($P. lanceolata$) also had the strongest negative plant-soil feedback: a 54% reduced root biomass in own compared to foreign mono soil. *Festuca rubra* had 38% reduced biomass in own compared to foreign mono soil, while the plant-soil feedback effects were not significant for *A. odoratum* and *L. vulgare* (8% and 13% reduction, respectively in own compared to foreign mono soil).

In interspecific competition in mixed plant communities on mono soil (i.e. two quadrants with the same conditioned soil) *P. lanceolata, F. rubra* and *L. vulgare*, but not *A. odoratum* produced less root biomass in own compared to foreign mono soil (Table S3C, target x soil (own vs foreign): $F_{3,1245}=26.5$, $P<0.001$; Fig. S2). Concomitantly, the interspecific competitor species produced more root biomass in these two patches of own soil of *P. lanceolata, F. rubra* and *L. vulgare* than in two patches of soil foreign to both plant species (Fig. S2, Table S3C: target x competitor x soil (own vs foreign), $F_{9,1245}=2.90$, $P = 0.002$). Hence, on own soil, all species except *A. odoratum* produced less root biomass against an interspecific competitor.

Interspecific and intraspecific competition in mixed soils

In plant monocultures in pots where at least one of the two quadrants was filled with foreign soil, three plant species were released from negative plant-soil feedback as total root biomass increased from own-own, to foreign-own, to foreign-foreign soil (Species x Soil combination: $F_{6,227}=12.92$, $P<0.001$ with soil type defines as own-own, foreign-own, or foreign-foreign; Fig. S3). *Festuca rubra* and *P. lanceolata* both showed a gradual decrease of root biomass from foreign-foreign, to foreign-own, and again to own-own soil, but the total reduction was stronger for *P. lanceolata* than for *F. rubra* (54% and 38%, respectively). Root biomass of *L. vulgare* was 18% higher in soils with two foreign patches compared to situations where own soil was present in one (foreign-own) or two quadrants (own-own).
odoratum was unresponsive to ‘own’ soils as it produced similar amounts of root biomass in foreign-foreign, foreign-own and own-own soil (Fig. S3).

The outcome of belowground interspecific competition on mixed soils (i.e. two quadrants with different patches of conditioned soil) depended significantly on the combination of soils in the quadrants (Fig. 3). For example, P. lanceolata was the strongest competitor (Table S3B, target: F\textsubscript{3,1245}=322, P<0.001), producing high root biomass in foreign patches, irrespective of competitor species. Interestingly, in pots with quadrants with own and foreign soil, P. lanceolata root biomass in the own quadrant was significantly lower than in the foreign quadrant. This negative effect of own soil biota on root biomass of P. lanceolata allowed an increase of root biomass of the neighbouring plant species in the patch with P. lanceolata soil (which was thus a foreign patch for this neighbouring plant species). This shift in root distribution was particularly significant in cases where the foreign patch for P. lanceolata was the own soil of the neighbouring plant species (Fig. 3c, e, f; highlighted treatments, all P < 0.01). These shifts occurred only in mixed plant communities with P. lanceolata and not among the three other plant species (Fig. 3a, b, d).

Plant mixtures: aboveground biomass in competition

Monoculture shoot biomass of F. rubra and P. lanceolata growing in mono soils was (marginally) significantly reduced in own compared to foreign soil (Species x Soil type (own vs foreign): F\textsubscript{3,87}=2.447, P=0.069), but the shoot biomass of other two plant species was not affected by mono soil type (Fig. S4). Therefore, aboveground responses were similar to belowground responses with regard to effects of negative plant-soil feedback in plant monocultures on mono soils (Fig 2). Also aboveground, F. rubra, P. lanceolata, and to a lesser degree L. vulgare showed release from negative plant-soil feedback in monoculture when one or two quadrants contained foreign instead of own soil (Fig. S4; Planting x Soil combination, defined as own-own, foreign-own, or foreign-foreign: F\textsubscript{6,227}=3.88, P=0.001). Shoot biomass in interspecific competition was not affected by soil combination (Table S3A, Soil combination: F\textsubscript{9,586}=0.69, P=0.717, Fig. 4 and S4), neither by mono soils nor the mixed soils. The effect of soil combination (10 pairwise combinations) on shoot biomass differed, depending on plant species: soil combination affected shoot biomass of P. lanceolata (F\textsubscript{9,199}=2.20, P=0.024) while it did not affect shoot biomass of the grasses (A. odoratum: F\textsubscript{3,196}=0.64, P=0.762 and F. rubra F\textsubscript{9,198}=1.58, P=0.122, respectively) and of L. vulgare (F\textsubscript{9,197}=1.76, P=0.078).
We found a clear competitive hierarchy since the effect of competitor on shoot biomass (Table S3A, Competitor: \(F_{3,447}=3.11, P=0.026\); Figure 4 and S4) did not differ between target plant species (Target x Competitor: \(F_{9,447}=0.35, P=0.957\)), or on different soil combinations (Target x Competitor x soil combination: \(F_{81,447}=0.48, P=0.999\)). Independent of soil combinations, shoot biomass was always larger when plant species were competing with *F. rubra* than with *L. vulgare* and *A. odoratum*. Additionally, shoot biomass was always lowest when competing with *P. lanceolata*. This indicated a clear hierarchy of competitive effect strengths of the competitors, with the largest plant species also being the strongest competitor: *P. lanceolata* $\rightarrow$ *A. odoratum* & *L. vulgare* $\rightarrow$ *F. rubra*. 
Discussion

Until now, effects of plant-soil feedback on competition have been studied in soils that were uniformly conditioned by only one of the competing plant species (van der Putten & Peters, 1997; Casper & Castelli, 2007; Kulmatiski et al., 2008; Petermann et al., 2008; van der Putten et al., 2013). In such cases, plants exposed to negative soil feedback are replaced by other species leading to directional or cyclic succession (van der Putten & Peters, 1997; Casper & Castelli, 2007; Bever et al., 2012). Our study provides evidence that spatial heterogeneity of plant-soil feedback may lead to different belowground plant-plant interactions, potentially affecting community dynamics. When the strongest competitor is confronted with own soil (i.e. conspecifically conditioned soil), root growth of this plant species is inhibited, giving the roots of the inferior competitor an advantage in that specific patch. Under heterogeneous conditions, negative plant-soil feedback thus creates competitive opportunities for inferior species, with roots able to escape from other soil patches where they lose competition. Soils heterogeneous in the abundance of species-specific soil biota seem the rule in species-rich plant communities (Ettema & Wardle, 2002; Bever et al., 2010; Bezemer et al., 2010). If the responses that we observed hold in the field, this heterogeneity may contribute to plant community dynamics and species coexistence.

Differential plant-soil feedback patches within the rooting zone affect plant interactions belowground

Negative plant-soil feedback was strongest for *P. lanceolata*. This plant species was the only one grown from seeds collected from a previous experiment with the same soil. Possibly, the seed provenance might have affected the degree of plant-soil feedback, for example by adaptation of *P. lanceolata* to its soil biota by selection, or by selection effects of the plants on the soil biota. There are only very few studies on plant adaptation to soil biota (Lankau, 2011; Schweitzer et al., 2014; terHorst et al., 2014), so that it is not well predictable which of these two options might provide a better explanation. Moreover, strong negative plant-soil feedback of *P. lanceolata* in our study was consistent with other plant-soil feedback studies, which did not necessarily started from such locally collected plant seeds (Petermann et al., 2008; Harrison & Bardgett, 2010; Hendriks et al., 2015). *Plantago lanceolata* was also the plant species with the largest amount of total biomass, giving it a significant advantage in belowground competition over the other three plant species (Cannell et al., 1984; Bartelheimer et al., 2008). Interestingly, the distribution of soil biota within the rooting zone of *P. lanceolata* lead to changes in root distribution over patches in mixed soils: reduced root
growth of the strongest competitor \((P. \textit{lanceolata})\) in own soil compared to foreign patches, allowing opportunities for its competitors in the avoided patches. This increased opportunity for root growth even resulted in the inferior competitor \((L. \textit{vulgare})\) surpassing the dominant competitor in terms of root mass in that patch. Belowground competition is an important part of plant competition (Wilson, 1988; Mariotte \textit{et al.}, 2012; Kiær \textit{et al.}, 2013). In due course, the strong negative plant-soil feedback and reduced competitiveness of the potentially strongest competitor also may enhance the aboveground competitive abilities of subordinate competitors (Mariotte \textit{et al.}, 2012).

In the present study, soil conditioning by different plant species was used as a proxy for changing relative abundance of soil biota. Identifying the soil biota was outside the scope of this study, since the aim was to test whether heterogeneity of plant-soil feedback within a plant’s rooting zone changes belowground interactions and competitive performance. Negative plant-soil feedback effects can be induced by fungal pathogens (Raaijmakers \textit{et al.}, 2009) and nematodes (van Ruijven \textit{et al.}, 2003; de Deyn \textit{et al.}, 2004), whereas little is known about bacterial diseases that may cause plant-soil feedback (Mendes \textit{et al.}, 2013). Negative plant-soil feedback effects may be alleviated by beneficial soil biota such as arbuscular mycorrhizal fungi (van der Heijden \textit{et al.}, 1998; Bever, 2002; Helgason \textit{et al.}, 2002; Mangan \textit{et al.}, 2010a). The soil biota involved in plant-soil feedback can also affect patch dynamics by their persistence in the soil in the absence of host plants. For example, when fungi with highly persistent spores are causing negative feedback it may take several years for fungal densities to decrease and new seedlings can establish (Van der Putten \textit{et al.}, 2001). When short-living nematodes are the main cause for negative plant-soil feedback, establishment of seedlings may potentially occur much faster (Van der Putten \textit{et al.}, 2001; van der Putten, 2003). Thus, to understand temporal patch-dynamics in natural plant communities, identifying soil biota that cause negative plant-soil feedback will be relevant in future studies.

There are two major alternative mechanistic explanations for plant-soil feedback effects in our study. Detrimental effects of soil on roots might also have been caused by autotoxic compounds. Such effects might be due to chemicals exuded by the dead root tissues, or by microbial breakdown products (Mazzoleni \textit{et al.}, 2015). Generally, non-biotic autotoxicity is very difficult to be demonstrate. Alternatively what is considered as autotoxicity might still be a result of microbial degradation of dead plant tissues (Bais \textit{et al.}, 2006). Another possibility is that the plant-soil feedback effects are due to nutrient limitation (other than N and P) caused during the conditioning phase. For example, Bezemer \textit{et al.} (2006) proposed that in specific cases potassium might have been a limiting factor, However,
in our approach, the inoculation of 25% conditioned soil with sterilized background soil will have largely avoided such strong effects of nutrient limitation.

**Belowground vs aboveground competitive responses**

In monoculture soils (i.e. two patches of the same conditioned soil), negative effects of soil biota on belowground plant biomass were reflected in growth reductions aboveground. In mixed soil (i.e. patches containing different types of conditioned soil), however, soil biota also affected root distribution, but this did not yet cascade into aboveground effects, leaving the aboveground competitive hierarchy outcome unaffected at least for the duration of the experiment. One likely reason is that two unsuitable ‘own’ soil patches in monoculture soils, where on average 46% of the roots were located, induced stronger aboveground responses than one ‘own’ soil patch in mixed soils. Another explanation is that plastic root responses to soil biota have buffered aboveground biomass responses. The negative effects of soil microbes might be compensated for by increased nutrient uptake rates in other quadrants in mixed soil combinations, as shown for these plant species in (Hendriks et al., 2015), or by a change in allocation leading to different root-shoot ratios (Mommer et al., 2010; Padilla et al., 2013). Additionally, the set-up of the present study contained ‘plant’ quadrants of neutral (unconditioned; Q1 and Q3) soil which might have been additional ‘escapes’ for plant roots, in both monoculture soil combinations as well as mixed soil combinations.

Compared to aboveground, belowground plant competition is less likely to cause competitive exclusion due to size symmetry (Weiner & Thomas, 1986; Hautier et al., 2009; Lamb et al., 2009) and thus might have less immediate effects on plant community structure. Moreover, Price et al. (2012) suggested that belowground rooting patterns can temporarily be neutralized by competitive interactions aboveground (Price et al., 2012). However, also the opposite has been demonstrated as detrimental effects of plant-soil feedback on belowground plant structures appeared to have a lag phase of 3 months before affecting aboveground competitive ability (van der Putten & Peters, 1997). Future studies will have to reveal on what spatial and temporal scales plant-soil feedbacks operate and drive plant competitive interactions below and aboveground in natural grasslands.

**Consequences for species coexistence in natural ecosystems**

Until now, plant-soil feedback studies have not considered the interactive effects of different soil legacies within the root system of a single plant. Plants occupy different patches in vegetation at different timescales. Roots, however, expand further horizontally than shoots
(Pecháčková et al., 2004; Hiiesalu et al., 2012), and will explore soil patches with different strengths of plant-soil feedback (Hendriks et al., 2015). Price et al. (2012) found that both biotic and abiotic heterogeneity positively affected plant species coexistence belowground. Our results suggest that plants do not need to perish due to their own soil enemies, but can escape on small spatial scales to better resorts in terms of plant-soil feedback. Bezemer et al. (2010) showed that entire soil food webs can differ in small-scale soil patches under individual plants, investigating semi-natural grasslands of the same type as where our plant species originate from. Small-scale patches of soil biota are thus likely to occur naturally in these grassland systems.

The plant species used in the present study may occur in the field as rather isolated individuals, as well as larger patches of individuals of the same species. In the absence of information on soil biotic patchiness in the field, we have chosen grids of single sizes that may be either too small or too large to represent all natural conditions. However, our approach provides a proof of concept and subsequent studies may be needed in order to determine how grid size may influence plant-plant interactions through spatial variation in plant-soil feedback. It also remains to be investigated how these small-scale root-soil and root-root interactions will affect the shifting mosaics on small spatial scales induced by soil-borne pathogens (Olff et al., 2000; Bonanomi et al., 2005; Bever et al., 2012; Mack & Bever, 2014).

In the field, negative plant-soil feedback might also be diluted when plant roots grow intermingled. Recent studies that showed the role of plant-soil feedback suppression as a possible cause of overyielding in species-diverse plant communities (Maron et al., 2011; Schnitzer et al., 2011) proposed that the main cause was a dilution of soil pathogens. Here, we show that this effect might as well be due to patchy distribution of negative plant-soil feedback effects. There are relatively few field studies where plant-soil feedback effects have been studied in relation to plant competition (Casper & Castelli, 2007). More of these types of studies are needed, to investigate how root responses allow plants to escape from their own plant-soil feedback, and how heterogeneity in soil biota changes competitive balances and contribute to plant species coexistence in the field.

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References:


Supplementary Information

Supplementary Methods: Description of soil nutrient (NO\textsubscript{3}\textsuperscript{-}, NH\textsubscript{4}\textsuperscript{+} and PO\textsubscript{4}\textsuperscript{3-}) extraction and analysis and statistical analysis of the nutrient concentrations.

Table S1: Soil nutrient concentrations (NO\textsubscript{3}\textsuperscript{-}, NH\textsubscript{4}\textsuperscript{+} and PO\textsubscript{4}\textsuperscript{3-}) in µmol per kg dry soil in all soils.

Table S2: Results of linear mixed-effects ANOVA (type III sums of squares) of plant monoculture community root biomass (in 30 % of soil volume) in g dry weight per pot.

Table S3: Results of linear mixed-effects ANOVA (type III sums of squares) of individual shoot biomass (A) and root biomass (B and C) on all soils.

Figure S1. Reference curves of estimated plant species proportion (y-axis) against actual species proportion in sample (x-axis) for all four plant species in the mixed root samples, used for estimating species proportions after RT-PCR analysis.

Figure S2. Species-specific root mass per soil quadrant (Q2 and Q4) in mixed plant communities on mono soils.

Figure S3. Plant monoculture community root biomass (in 30 % of soil volume) in g dry weight in all soil combinations.

Figure S4. Species-specific shoot biomass in g per individual in monoculture communities in all soil combinations.
Figure 1. Experimental design. Four plant species (*A. odoratum, F. rubra, L. vulgare* and *P. lanceolata*) were planted in interspecific and intraspecific competition. Plants were planted in quadrants with ‘neutral’ background soil (Q1 and Q3), the patches in between (Q2 and Q4) contained conditioned soil (soil origin: A, F, L, P). This resulted in a full-factorial design in which we combined ten plant community compositions with ten soil combinations.

Abbreviations and symbols: Ao=*A. odoratum*, Fr=*F. rubra*, Lv=*L. vulgare*, Pl=*P. lanceolata*. A= soil of *A. odoratum*, F=soil of *F. rubra*, L=soil of *L. vulgare* or P=soil of *P. lanceolata*. 
Figure 2. Plant monoculture community root biomass (g dry weight in 30% of total soil volume) in four different mono soils (A, F, L, P). Each panel (a-d) represents a different plant monoculture. Shaded bars show biomass in own-own soil. Abbreviations used: A=soil of *A. odoratum*, F=soil of *F. rubra*, L=soil of *L. vulgare*, P=soil of *P. lanceolata*. Values are means, error bars depict + 1 SE. Different letters above bars indicate significant differences as determined by Tukey’s HSD test.
Figure 3. Species-specific root mass per soil quadrant (Q2 and Q4) in mixed plant communities (a) Ao-Fr (b) Ao-Lv (c) Ao-Pl (d) Fr-Lv (e) Fr-P. (f) Lv-Pl on all mixed soils. Mixed soils are separated by the soil used in each conditioned soil quadrant (Q2 or Q4). Purple bars = *A. odoratum*; red bars = *F. rubra*; green bars = *L. vulgare*; blue bars = *P. lanceolata*. Abbreviations used: Ao = *A. odoratum*, Fr = *F. rubra*, Lv = *L. vulgare*, Pl = *P. lanceolata* and A=soil of *A. odoratum*, F=soil of *F. rubra*, L=soil of *L. vulgare*, P=soil of *P. lanceolata*. Values are means, error bars depict ± 1 SE. Dense shading indicates roots growing in own soil. Highlighted (boxed) sets of bars indicate competition of a plant species pair on both their own soils. Asterisks indicate a statistically significant plant species x soil interaction within a mixed soil (tested for each pairwise combination of plants and soils separately, two species and two soils per analysis), hence, a different distribution of root mass over the quadrants; § 0.10 < P < 0.05, * 0.05 < P < 0.01, ** 0.01 < P < 0.001, *** 0.001 < P < 0.
Figure 4. Species-specific shoot biomass in g per individual in mixed plant communities on all soil combinations. (a) Ao-Fr (b) Ao-Lv (c) Ao-Pl (d) Fr-Lv (e) Fr-P. (f) Lv-Pl. Purple bars = *A. odoratum*; red bars = *F. rubra*; green bars = *L. vulgare*; blue bars = *P. lanceolata*. Abbreviations used: Ao = *A. odoratum*, Fr = *F. rubra*, Lv = *L. vulgare*, Pl = *P. lanceolata* and A = soil of *A. odoratum*, F = soil of *F. rubra*, L = soil of *L. vulgare*, P = soil of *P. lanceolata*. Values are means, error bars depict + 1 SE. Dense shading indicates a plant growing on own-own soil; wide shading indicates a plant on foreign-own soil.
Experimental design. Four plant species (A. odoratum, F. rubra, L. vulgare and P. lanceolata) were planted in interspecific and intraspecific competition. Plants were planted in quadrants with 'neutral' background soil (Q1 and Q3), the patches in between (Q2 and Q4) contained conditioned soil (soil origin: A, F, L, P). This resulted in a full-factorial design in which we combined ten plant community compositions with ten soil combinations. Abbreviations and symbols: Ao=A. odoratum, Fr=F. rubra, Lv=L. vulgare, Pl=P. lanceolata.

A= soil of A. odoratum, F=soil of F. rubra, L=soil of L. vulgare or P=soil of P. lanceolata.
Plant monoculture community root biomass (g dry weight in 30% of total soil volume) in four different mono soils (A, F, L, P). Each panel (a-d) represents a different plant monoculture. Shaded bars show biomass in own-own soil. Abbreviations used: A=soil of A. odoratum, F=soil of F. rubra, L=soil of L. vulgare, P=soil of P. lanceolata. Values are means, error bars depict + 1 SE. Different letters above bars indicate significant differences as determined by Tukey’s HSD test.

174x132mm (96 x 96 DPI)
Species-specific root mass per soil quadrant (Q2 and Q4) in mixed plant communities (a) Ao-Fr (b) Ao-Lv (c) Ao-Pl (d) Fr-Lv (e) Fr-P. (f) Lv-Pl on all mixed soils. Mixed soils are separated by the soil used in each conditioned soil quadrant (Q2 or Q4). Purple bars = A. odoratum; red bars = F. rubra; green bars = L. vulgare; blue bars = P. lanceolata. Abbreviations used: Ao= A. odoratum, Fr= F. rubra, Lv= L. vulgare, Pl= P. lanceolata and A=soil of A. odoratum, F=soil of F. rubra, L=soil of L. vulgare, P=soil of P. lanceolata. Values are means, error bars depict + 1 SE. Dense shading indicates roots growing in own soil. Highlighted (boxed) sets of bars indicate competition of a plant species pair on both their own soils. Asterisks indicate a statistically significant plant species x soil interaction within a mixed soil (tested for each pairwise combination of plants and soils separately, two species and two soils per analysis), hence, a different distribution of root mass over the quadrants; § 0.10 < P < 0.05, * 0.05 < P < 0.01, ** 0.01 < P < 0.001, *** 0.001 < P < 0.

277x198mm (96 x 96 DPI)
Species-specific shoot biomass in g per individual in mixed plant communities on all soil combinations. (a) Ao-Fr (b) Ao-Lv (c) Ao-Pl (d) Fr-Lv (e) Fr-P. (f) Lv-Pl. Purple bars = A. odoratum; red bars = F. rubra; green bars = L. vulgare; blue bars = P. lanceolata. Abbreviations used: Ao = A. odoratum, Fr = F. rubra, Lv = L. vulgare, Pl = P. lanceolata and A = soil of A. odoratum, F = soil of F. rubra, L = soil of L. vulgare, P = soil of P. lanceolata. Values are means, error bars depict + 1 SE. Dense shading indicates a plant growing on own-own soil; wide shading indicates a plant on foreign-own soil.