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Abstract Plant-soil microbial interactions have moved into focus as an important mechanism for understanding plant coexistence and composition of communities. Both arbuscular mycorrhizal (AM) as well as other root endophytic fungi co-occur in plant roots, and therefore have the potential to influence relative abundances of plant species in local assemblages. However, no study has experimentally examined how these key root endosymbiont groups might interact and affect plant community composition. Here, using an assemblage of five plant species in mesocosms in a fully factorial experiment, we added an assemblage of AM fungi and/or a mixture of root endophytic fungal isolates, all obtained from the same grassland field site. The results demonstrate that the AM fungi and root endophytes interact to affect plant community composition by changing relative species abundance, and consequently aboveground productivity. Our study highlights the need to explicitly consider interactions of root-inhabiting fungal groups in studies of plant assemblages.

Keywords Soil microbes · Endophytic fungi · Parasites · Arbuscular mycorrhiza · Community composition

Introduction
Understanding drivers of plant community composition is a central endeavor in community ecology. Plants interact with many groups of organisms, including pollinators, herbivores, symbionts and pathogens. In particular interactions among the above- and belowground component of ecosystems have moved into focus (e.g., Klironomos 2002; Wardle et al. 2004; Bardgett and Wardle 2010; Bever et al. 2010). Incorporating such interactions with current resource-competition based concepts of plant communities may decisively contribute to explaining plant coexistence (Bever et al. 2010).

Arbuscular mycorrhizal (AM) fungi together with root endophytic fungi are common root symbionts with the potential to impact the plant community, but only in very few cases have their interactions or interactions with other organism groups been considered, as recently discussed by Klironomos et al. (2011). Specifically, we are not aware of any experimental study in which the combined effects of root endophytic and AM fungi on plant communities have been examined. This is even more surprising given that at the level of the individual plant, several studies have demonstrated that AM fungi can protect plants from effects of root-colonizing fungi that are directly parasitic. For example, Newsham et al. (1995) using Fusarium sp. and Glomus intraradices, showed a protective effect of AM fungi in an annual plant. Wehner et al. (2011), in a greenhouse study using different plant species individually showed that such protective effects could also occur when the symbiotic microorganisms are added as assemblages rather than individual isolates.
At the plant community level, there are several studies employing “filtrates” of microbial communities that exclude by size AM fungi, but which include saprobes, parasites and others. For example, Schnitzer et al. (2011) demonstrated profound effects of such microbial filtrates on plant communities and productivity; more so in their particular study than the otherwise much better-documented effects of AM fungi (e.g., van der Heijden et al. 2008). In the plant-soil feedback literature there is also widespread appreciation of root parasites as agents of negative feedback, experimentally represented as microbial filtrates (e.g., Klironomos 2002), but also working with defined fungal parasite isolates (e.g., Mills and Bever 1998; Klironomos 2002).

However, we currently have no information about if and how these groups of soil biota, AM fungi and endophytes, interact to affect plant–plant interactions and ultimately plant community composition. Here we used endophytic fungi isolated from roots of plants used in our experiment. Endophytic fungi are a highly diverse group of root inhabitants (Vandenkoolhuyse et al. 2002) which can give rise to a wide range of host effects, i.e., they are root-colonizing fungi with different degrees of virulence or even positive effects (Schulz and Boyle 2005). In the classification system of Rodriguez et al. (2009), we are referring to class 4 endophytes, that is root-colonizing nonclavicipitaceae fungi with horizontal transmission. The isolates we selected are from species known from an agricultural setting (but hence not necessarily in our plants) to be capable of parasitic interactions.

Owing to the lack of specific inhibitors of AM fungi or root endophytic fungi we carried out a mesocosm experiment, in which we added these components in a fully factorial manner. Our central hypothesis was that AM fungi will reduce any negative impacts of root endophytic fungi on plant biomass, and that these two organism groups interact in their influence on either plant community composition or relative abundances or both.

Materials and methods

Study site and plants

Our study site was a fertile meadow in Berlin (Germany; 52°27′13″N, 13°18′5″E); the grassland is mown twice a year. The site is part of the Tanacetoo-Arrhenatheretum Fischer ex Ellmauer (in Ellenberg 1996) with Arrhenatherum elatius as characteristic species, and is rich in Fabaceae. It is dominated by different grass and Trifolium species. We selected four perennial plant species, Leucanthemum vulgare Lam. (Asteraceae), Trifolium pratense L. (Fabaceae), Plantago lanceolata L. (Plantaginaceae), and Arrhenatherum elatius (L.) Beauv. Ex J. Presl et C. Presl (Poaceae) and one biennial plant species Daucus carota L. (Apiaceae), which are typical of this grassland community and known to be colonized by AM fungi, for inclusion in mesocosms.

Isolation of root endophytes and inoculum preparation

Plants (five individuals per species) were excavated from the site in October 2010, and roots were used for isolation of root-inhabiting fungi. Roots were processed right after harvest. For this, roots were washed with tap water and cut into 1-cm-long pieces, then rinsed with sterile water. Roots were surface sterilized by immersion in a 10% sodium hypochloride solution (1 min) and subsequently rinsed off in sterile water. Root pieces were then placed on malachite green agar media selective for Fusarium species (Castellá et al. 1997), common root endophytic fungi, at 25 °C for 1 week. Upon observation of fungal growth emanating from the cut ends of root pieces, these fungi were picked and transferred to potato dextrose agar. Fungal cultures were transferred until they appeared free of contaminants, and were then cultured additionally with streptomycin (100 p.p.m.) to eliminate potential bacterial contaminants. Isolates were then keyed out to genus (Domsch et al. 2007), and only those of the 150 isolates were retained that belonged to genera that are known to contain root pathogens and endophytes. The most commonly observed genera across all five plant species were Fusarium, Alternaria, Colletotrichum and Cylindrocarpon (data not shown). For identification we sequenced the internal transcribed spacer (ITS) region of six isolates from different genera and plant species, using the primer pair ITS 1F/ITS 4 (Gardes and Bruns 1993; White et al. 1990) and compared the obtained sequence data to reference sequences in the National Center for Biotechnology Information GenBank (see Table 1: fungal species and closest matches). We selected these fungi based on their ability (1) to sporulate readily, and (2) to be present on at least one isolate from each of the five plant species. We could therefore standardize the fungal inoculum, and be reasonably assured of successful inoculation. For preparation of spore suspensions for inoculation we transferred actively growing mycelium onto water agar plates to obtain large numbers of spores. After 3 weeks of growth in water agar, spores were separated from hyphae by gently shaking the plate with sterile water, and a spore suspension containing 3,240 spores/500 µL was prepared (540 spores of each isolate; determined by direct counting with a hemocytometer).

Preparation of AM fungal inoculum

AM fungal inoculum was prepared from the same grassland. Three soil cores (550 g each) were extracted from the
site, dispersed in water and mixed. The material was passed through a series of sieves (500, 220 and 38 μm), and the fraction collecting on a 38-μm sieve was centrifuged in 60% sucrose solution at 4 °C for 2 min. The spore material was washed with water and subsequently surface sterilized for 20 min in a 2% chloramin T solution with streptomycin (400 μg L^-1) (Giovannetti et al. 1999). Spore numbers were counted under a dissecting scope, and a suspension containing 400 AM fungal spores mL^-1 was prepared in sterile water for use as inoculum. This is a much smaller number of spores than for the endophyte inoculation, but AM fungal spores are very large and thus not directly comparable. Seeds of D. carota, A. elatius and P. lanceolata were collected from the grassland site itself, and for L. vulgare and T. pratense seeds came from a commercial supplier (Albert Treppens & Co Samen, Berlin).

### Greenhouse experiment

The greenhouse experiment consisted of two factors (AM fungi and root endophytic fungi, with the levels present and absent, respectively), which were crossed factorially to yield four treatment combinations with ten replicates each, for a total of 40 mesocosms. Treatments were randomly assigned to experimental units, and positions in the greenhouse were re-randomized every 2 weeks to minimize positional effects. An experimental unit consisted of a 3-L pot (19 cm height, 14 cm diameter), filled with a soil originating from near the grassland site [an Albic Luvisol (Rillig et al. 2010)]. The soil was steamed at 80 °C for 4 h each time on 2 consecutive days, and mixed with 30% play sand for improved drainage. Each experimental unit received ten plants, two of each of the five species, planted in a pre-determined regular pattern. Each of the ten replicates of the four treatment combinations was planted with one of ten positional designs, which were treated as blocks in initial statistical analyses. Since block effects were never significant ($P > 0.70$), these were subsequently dropped from all models (see “Results”). Seeds were germinated on sterile glass beads for 1 week and then transferred to pots. For AM fungal inoculation, the root of every seedling received 1 mL of the spore suspension upon transplanting; roots in the non-AM fungal treatments received 1 mL of autoclaved water instead. After another 3 weeks, we added the spore suspension to every pot belonging to the endophyte treatments. We gave AM fungi a head start, since we wanted to provide AM fungi priority in colonizing the root, which may be important for protection of the root against potentially parasitic fungi (Borowicz 2001). For the endophyte treatment, we carefully exposed parts of the roots and added 500 μL of the spore suspension to every plant. Non-endophyte treatments received 500 μL of autoclaved water instead, also exposing roots in the same way.

### Harvests and analyses

After 54 days of growth we cut aboveground biomass, simulating mowing in the meadow system. We removed plant material 5 cm above the soil surface, and dried (40 °C to constant weight) and weighed it separately for each species. After a further 54 days we conducted the final destructive harvest. We separated shoots from roots, carefully washing the latter to obtain belowground biomass. Roots could not be separated into species, since they were thoroughly intertwined. We determined dry weight after drying at 40 °C. To demonstrate effectiveness of inoculation, we measured root colonization (on a per experimental unit basis) after staining with ink and vinegar (Vierheilig et al. 1998) using the magnified intersections method (McGonigle et al. 1990) on half of the samples. We observed a minimum of 100 root intersects at 200× magnification noting the presence of two groups of fungi: AM fungi and all other fungal structures (Rillig et al. 1999); the latter could not be further distinguished into classes.
Data analysis

All analyses were conducted with R version 2.13.2 (R Development Core Team 2011) and the vegan package (Oksanen et al. 2011). Root-colonization data for AM fungi and other endophytic fungi were arcsine-square root transformed and tested by two-way ANOVA of the factors AM fungal and endophyte inoculation and treatment pairs compared using the Tukey honestly significant difference test in the case of a significant interaction effect. Treatment effects on shoot and root biomass were tested in the same model structure. Since root-colonization data and root and shoot biomass data were taken from the same replicates, we ran multivariate (MANOVA) before taking into consideration the results of individual ANOVAs (the Pillai trace statistic was used in MANOVA: see below). To analyze changes in plant community composition we used MANOVA on standardized (z-scores) biomass data of plant species, testing the null hypothesis by means of the Pillai trace statistic, which accounts for the variance between groups and is robust to violation of multivariate normality and homogeneity of the variance–covariance matrix (Quinn and Keough 2002). MANOVA is based on the Mahalanobis distance, which equals Euclidean distance when the covariance matrix is an identity matrix, and takes into account the correlations between variables. Thus, it is generally not recommended to apply this distance to species by sample matrices; Euclidean-like distances are poor and even inappropriate measurements of community dissimilarity because they do not account for joint absences (Legendre and Legendre 1998). Also, the fact that species by sample matrices are characterized by the presence of many zeros implies that the violation of multivariate normality can be serious in these matrices, to the point that MANOVA results cannot be trusted. However, under specific circumstances, these drawbacks do not affect the analysis of species by sample matrices. In our case, there were no zeros in the matrix and the biomass of each plant species is approximately normally distributed (data not shown). Therefore, we expect to observe shifts in the relative proportions of species. These conditions make MANOVA and Mahalanobis distance not only appropriate but even advantageous in terms of statistical power. In order to visualize multivariate patterns we applied principal component analysis (PCA) to the correlation matrix of the species by sample matrix, which is equivalent to a PCA of the variance–covariance matrix of z-scores.

Results

Root colonization

MANOVA on root-colonization data showed that the two tested main effects and their interaction were highly significant (AM fungi main effect, Pillai = 0.88, F = 52.8. P << 0.01; endophytes main effect, Pillai = 0.84, F = 37.9. P << 0.01; interaction, Pillai = 0.54, F = 8.7. P = 0.003; refer to Table 2 for the df). Indeed, root colonization by AM fungi was not detected in the non-inoculated treatments, was highest in the AM fungi-only treatment, and significantly decreased in the combined treatment (Fig. 1a). There was background root colonization by non-AM fungi in all treatment combinations, including the control, but non-AM fungal colonization was higher in the two treatments which received the endophytic fungi spore suspension (Fig. 1a), suggesting that the endophytic fungi added indeed colonized the roots. Also, the addition of AMF fungi led to a significant decrease in the colonization of background endophytic fungi. Root colonization in the endophyte treatments was morphologically dissimilar from the “background” fungal colonization (the latter being represented mostly by spores, whereas in the endophyte treatments, septated hyphae were present).

Overall effect on above and belowground biomass

Aboveground biomass results of the first cutting showed few effects (data not shown), and we thus focus our presentation on the final harvest. MANOVA on root and shoot biomass showed that only the interaction term was marginally significant (interaction: Pillai = 0.17, F = 3.6.

Table 2 Results of ANOVAs on root colonization by arbuscular mycorrhizal (AM) fungi and endophytes and shoot and root biomass

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root colonization by AM fungi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM fungi</td>
<td>1</td>
<td>69.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endophytes</td>
<td>1</td>
<td>10.79</td>
<td>0.005</td>
</tr>
<tr>
<td>AM fungi × endophytes</td>
<td>1</td>
<td>10.79</td>
<td>0.005</td>
</tr>
<tr>
<td>Residuals</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root colonization by endophytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM fungi</td>
<td>1</td>
<td>15.89</td>
<td>0.001</td>
</tr>
<tr>
<td>Endophytes</td>
<td>1</td>
<td>50.89</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AM fungi × endophytes</td>
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<td>3.19</td>
<td>0.09</td>
</tr>
<tr>
<td>Residuals</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoot biomass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM fungi</td>
<td>1</td>
<td>1.66</td>
<td>0.206</td>
</tr>
<tr>
<td>Endophytes</td>
<td>1</td>
<td>4.12</td>
<td>0.049</td>
</tr>
<tr>
<td>AM fungi × endophytes</td>
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<td>0.41</td>
<td>0.528</td>
</tr>
<tr>
<td>Residuals</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root biomass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM fungi</td>
<td>1</td>
<td>4.22</td>
<td>0.047</td>
</tr>
<tr>
<td>Endophytes</td>
<td>1</td>
<td>4.18</td>
<td>0.048</td>
</tr>
<tr>
<td>AM fungi × endophytes</td>
<td>1</td>
<td>4.14</td>
<td>0.049</td>
</tr>
<tr>
<td>Residuals</td>
<td>36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
P = 0.04; refer to Table 2 for the df). However, individual ANOVAs showed that on average, shoot biomass decreased when endophytes or endophytes and AM fungi were present (Fig. 1b) but the interaction effect was not statistically significant (Table 2). Only the main negative effect of endophytes was statistically significant. Root biomass significantly decreased when both AM fungi and endophytes were present (Fig. 1b).

Plant species-level data and community responses

At harvest, the largest plants were *T. pratense*, the shoot biomass of which ranged from an average of 9–16 g and *A. elatius* and *P. lanceolata*, with average shoot biomass values between 1 and 2 g (Table 3). The other two species (*D. carota* and *L. vulgare*) had biomasses lower than 1 g. Thus, the community was basically dominated by *T. pratense*. However, when taking into account this large difference in absolute biomass by standardizing the data, MANOVA showed that the interaction between AM fungi and endophytic fungi significantly altered the multivariate distribution of plant biomass (Table 4). A PCA of the same data (Fig. 2; Table 5) clarified these overall patterns, showing that shifts in plant relative abundances are more pronounced when both AM fungi and endophytes are present. This change in relative abundances was mostly due to an increase in *L. vulgare* with respect to *T. pratense*, which caused the PC1 scores of the AM fungi-endophytes treatment to shift towards negative values (Fig. 2). Along the second PCA axis, there was a negative correlation between *D. carota*, *A. elatius* and *L. vulgare*, respectively, and *P. lanceolata* (Table 5; compare plant eigenvector coefficients of PC2): when AM fungi and endophytes are present, *D. carota*, *A. elatius* and *L. vulgare* were relatively less abundant than *P. lanceolata* but the pattern reversed when only AM fungi were applied. Interestingly, there is a strong and significantly positive linear correlation (r = 0.91, p < 0.001) between the first community axis (PC1) and overall shoot biomass (Fig. 3). Since PC1 describes the shift in relative abundances between *L. vulgare* and *T. pratense*, this figure shows that as *L. vulgare* increases and *T. pratense* decreases due to the combined effect of AM fungi and endophytes, overall plant shoot biomass decreases.

**Discussion**

We were able to show, for the first time, that two major groups of root-inhabiting biota, AM fungi and endophytic fungi, interact with each other to influence plant biomass. The interaction between AM fungi and endophytes was significant, indicating that the combination of these two groups of fungi can have a greater effect on plant growth than either group alone. This interaction may be due to the fact that AM fungi and endophytes have different mechanisms for nutrient acquisition and carbon allocation, which may lead to changes in plant biomass when they are present together. Overall, these findings suggest that understanding the interactions between AM fungi and endophytes is crucial for understanding plant growth and community dynamics in ecosystems.
fungi interacted to influence plant community structure in terms of shifts in relative species abundances. However, we found no support for the hypothesis that AM fungi would reduce any negative effects of endophytic fungi on biomass. The shifts we observed in relative abundances correlated with an overall shift in total community root and shoot biomass. This change in biomass mostly depended on the fact that the individual mass of one of the smallest species (*L. vulgare*) increased relative to that of the largest one (*T. pratense*). Our experimental mesocosms were eventually dominated by *T. pratense*, a legume capable of nitrogen fixation (Huss-Danell et al. 2007). As AM fungi and endophytes were added, this plant remained dominant, but slightly decreased to the advantage of *L. vulgare*. Thus, *T. pratense* is driving overall plant biomass but as the abundance of this species changes to the advantage of other plant species via the interaction between AM fungi and endophytes, overall productivity is affected.

The nature of the interaction of root endophytic fungi with the root can be positive, neutral or negative (Schulz and Boyle 2005). Positive interactions are possible either through direct growth-promoting effects or through mineralization activities of soil-borne hyphae of these fungi. Likewise, negative effects are possible through nutrient immobilization in the soil or via direct parasitic interactions with the root. We selected our endophyte isolates so that it is more likely that they would be weak parasites, because these species are all known from an agricultural context to be able to cause disease. However, this is substantially less well studied in natural ecosystems, and thus we are not fully confident that endophytes behaved parasitically here. We did not observe any lesions in the roots, for example, suggesting that the endophytes were not predominantly active as necrotrophic parasites, but that they were growing asymptotically in the root tissues.

AM fungi, which were permitted to colonize the roots first (i.e., a priority effect), apparently reduced the amount

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**Table 4** Results of multivariate ANOVA for community biomass

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>Pillai trace</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM fungi</td>
<td>1</td>
<td>0.28</td>
<td>2.44</td>
<td>0.055</td>
</tr>
<tr>
<td>Endophytes</td>
<td>1</td>
<td>0.40</td>
<td>4.26</td>
<td>0.004</td>
</tr>
<tr>
<td>AM fungi × endophytes</td>
<td>1</td>
<td>0.32</td>
<td>3.08</td>
<td>0.022</td>
</tr>
<tr>
<td>Residuals</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5** Eigen-vectors for the principal component analysis shown in Fig. 1

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. vulgare</em></td>
<td>−1.28</td>
<td>0.40</td>
<td>0.12</td>
</tr>
<tr>
<td><em>D. carota</em></td>
<td>0.15</td>
<td>0.97</td>
<td>−1.28</td>
</tr>
<tr>
<td><em>T. pratense</em></td>
<td>1.18</td>
<td>0.01</td>
<td>−0.33</td>
</tr>
<tr>
<td><em>A. elatius</em></td>
<td>0.92</td>
<td>0.84</td>
<td>0.88</td>
</tr>
<tr>
<td><em>P. lanceolata</em></td>
<td>0.31</td>
<td>−1.40</td>
<td>−0.30</td>
</tr>
</tbody>
</table>

Coefficients of first three principal components (PCs) are reported.

---

**Fig. 2** Ordination graph based on principal component analysis of the species biomass correlation matrix (i.e., standardized biomass data) from the four microbial treatments. Ellipses around multivariate centroids describe 95% confidence intervals (outer) and SEs (inner), respectively. In practice, the 95% interval include 95% of the replicated samples (not plotted) for the relevant treatment. **PC1** First principal component, **PC2** second principal component.

**Fig. 3** Aboveground biomass in relation to PC1. Low scores along PC1 indicate *Leucanthemum vulgare* is relatively more abundant than the dominant species *Trifolium pratense*.
of non-AM fungal root colonization, which likely included the endophytic fungi added in our study. This effect is consistent with a recent meta-analysis showing that AM fungi can suppress parasitic/pathogenic fungi in roots of many plant species (Veresoglou and Rillig 2012); the studies included in the meta-analysis were typically on specific pathosystems and individual plants. In addition, Wearn et al. (2012) recently documented a negative correlation between the abundance of AM fungi and root-colonizing endophytes in an observational field study in a grassland. These patterns and observations, including our results, are suggestive of competition between these fungi, for example for resources such as root carbon or mineral nutrients in the soil; alternatively, interference competition may be involved.

In conclusion, for the first time we have shown that key soil organism groups such as AM fungi and endophytic fungi can interact to affect plant community and productivity. Given the generally poor status of knowledge about root endophytic fungi this highlights the need to study ecological traits and functions of these enigmatic fungi in greater detail. In the future, it will also be crucial to unravel the biological mechanisms through which different groups of soil microbes interact to determine plant community assembly and ecosystem functions, and under what environmental settings (e.g., site fertility).

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References


