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Root biomass and exudates link plant diversity with soil bacterial and fungal biomass

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Plant diversity has been shown to determine the composition and functioning of soil biota. Although root-derived organic inputs are discussed as the main drivers of soil communities, experimental evidence is scarce. While there is some evidence that higher root biomass at high plant diversity increases substrate availability for soil biota, several studies have speculated that the quantity and diversity of root inputs into the soil, i.e. through root exudates, drive plant diversity effects on soil biota. Here we used a microcosm experiment to study the role of plant species richness on the biomass of soil bacteria and fungi as well as fungal-to-bacterial ratio *via* root biomass and root exudates. Plant diversity significantly increased shoot biomass, root biomass, the amount of root exudates, bacterial biomass, and fungal biomass. Fungal biomass increased most with increasing plant diversity resulting in a significant shift in the fungal-to-bacterial biomass ratio at high plant diversity. Fungal biomass increased significantly with plant diversity-induced increases in root biomass and the amount of root exudates. These results suggest that plant diversity enhances soil microbial biomass, particularly soil fungi, by increasing root-derived organic inputs.

Plant diversity is a significant driver of multiple ecosystem functions, such as plant biomass production¹, and carbon sequestration². Recent studies in controlled experiments indicate that soil microorganisms are closely tied to plant diversity effects on multiple ecosystem functions^{3–6}. Accordingly, the functional composition of soil microbial communities changes with plant diversity, e.g., by increasing the relative biomass of fungi over bacteria^{3,7} and altering soil enzyme activities^{3,7}. Notably, evidence from natural ecosystems for significant relationships between plant diversity and microbial community composition and functions is mixed^{8,9}, partly because both plant diversity and soil microorganisms are influenced by a plethora of abiotic and biotic determinants^{10,11}. This is why we refer to the results of controlled plant diversity experiments in the following.

Standing root biomass is known to increase with plant diversity^{12–14}, and enhanced root biomass is likely to increase the resource availability for soil microbial communities¹⁵ *via* altered root turnover and/or root exudation. While root turnover has been shown to decline with plant diversity^{16,17}, knowledge of effects of plant diversity on root exudation is scarce (but see e.g., ref. 18). In fact, several studies have speculated that the quantity and diversity of root inputs into the soil, i.e. through root exudates, drive plant diversity effects on soil biota. The quantity and quality of root exudates released into the soil is context-dependent, e.g., differ with plant species, functional dissimilarity among plant species¹⁰, plant age¹⁹, and soil conditions²⁰. Root exudates have been shown to play a significant role in numerous ecosystem functions, such as nutrient availability²¹ and cycling²². The biological activity of root exudates might vary with both the structure of the compounds and their local concentration in the rhizosphere²³, while synergistic effects with other root exudates might alter this activity, subsequent effects on soil microbial communities, as well as interactions among plants and rhizosphere microbial communities²¹. Thus, root exudates are likely to shift with changing biotic and abiotic conditions along the plant diversity gradient^{3,24}.

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Variable	1 species	3 species	6 species	df	fit	F	p
Shoot biomass	2.48 ± 0.86	2.53 ± 0.81	3.05 ± 0.89	1, 90	l	4.10	0.046
Root biomass	4.57 ± 1.71	5.40 ± 2.34	7.19 ± 2.30	1, 90	l	17.98	<0.001
Shannon diversity roots	0.00 ± 0.00	0.86 ± 0.18	1.35 ± 0.14	1, 88	l	699.5	<0.001
Root complementarity effect	nc	-0.65 ± 1.15	-0.39 ± 0.66	1, 32	c	1.80	0.187
Root selection effect	nc	1.82 ± 3.50	3.30 ± 2.62	1, 32	c	0.56	0.458
Root net biodiversity effect	nc	1.18 ± 2.96	2.92 ± 2.76	1, 32	c	3.11	0.086
Exudate richness	2.55 ± 1.46	2.50 ± 1.42	3.50 ± 2.17	2, 51	c	1.58	0.215
Exudate amount (log10)	1.05 ± 0.51	1.00 ± 0.54	1.50 ± 0.41	2, 51	c	3.29	0.045
Bacterial biomass	22.70 ± 6.54	21.58 ± 5.84	25.70 ± 3.84	2, 85	c	3.78	0.027
Fungal biomass	3.52 ± 2.44	3.64 ± 2.24	5.08 ± 2.24	1, 86	l	9.39	0.003
Fungi: bacteria	0.16 ± 0.08	0.17 ± 0.08	0.20 ± 0.09	1, 84	l	4.46	0.038

Table 1. GLM table of effects of plant diversity (1, 3, and 6 plant species per microcosm) on plant shoot and root biomass [g microcosm⁻¹], Shannon diversity of root biomass, root complementarity effect [g microcosm⁻¹], root selection effect [g microcosm⁻¹], root net biodiversity effect [g microcosm⁻¹], exudate richness [# of compounds], exudate amount [μM], soil bacterial biomass [nmol g⁻¹ soil dry weight], fungal biomass [nmol g⁻¹ soil dry weight], and ratio between fungal and bacterial biomass. Given are means ± standard deviation, degrees of freedom (df), fit of the factor plant species richness, and F- and p-values. Nc: not possible to calculate because monocultures are used as reference for additive partitioning calculations; c: categorical factor; l: linear variable; significant plant species richness effects ($p < 0.05$) are given in bold.

Evidence for root exudate-mediated effects of plant diversity on soil microbial communities stems from indirect approaches, such as ascribing unexplained variance in linear regression models²⁵ or structural equation models²⁶ to root exudates. Recently, using a ¹³C labelling approach, Lange *et al.*⁶ provided experimental evidence for the significant role of root exudates in fuelling soil microorganisms. They showed that plant diversity increases the transfer of ¹³C from plants to soil microbial fatty acids. Moreover, Sauheitl *et al.*²⁷ found that the composition, but not the amount, of amino acids changed along a plant diversity gradient. However, it remains unclear if soil microbial communities depend on plant diversity-driven variations in root biomass and composition²⁸, root diversity²⁹, quantity and diversity of root exudates^{6,26}, or their combination.

Here we used a microcosm experiment to investigate effects of plant diversity on the biomass of soil bacteria and fungi. We hypothesized that plant diversity effects are mediated by changes in root biomass^{25,28,30} and the diversity and quantity of root exudates^{6,25,29}. Species-specific root biomass was determined *via* quantitative PCR, and the diversity and quantity of root exudates were assessed *via* high pressure liquid chromatography (HPLC).

Results

Plant biomass. Shoot and root biomass increased significantly with increasing plant species richness (Table 1; Figs 1 and 2). However, belowground net biodiversity effect (NE), complementarity effect (CE), and selection effect (SE) did not differ significantly between plant mixtures with 3 and 6 species (Table 1).

Root exudates. In root exudates, we identified a total of 15 plant-derived compounds, i.e., fumaric acid, chlorogenic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid, 4-hydroxybenzaldehyde, vanillin, polydatin, p-coumaric acid, *t*-2-methoxycinnamic acid, phenylacetic acid, coumarin, a flavone-type compound, 2 quercetin glycoside derivatives, and a benzoate ester. Chlorogenic acid is a root exudate marker of *P. pratense*, polydatin of *R. acetosa*, and coumarin of *A. odoratum*. Most frequent compounds were used for quantification of root exudates as they were detected in many samples, i.e., fumaric acid (48 of 54 samples), vanillic acid (38/54), p-coumaric acid (14/54), and 4-hydroxybenzoic acid (9/54). The amounts of these four compounds were summed up and used as index of total root exudate amount. This index of the total amount of root exudates represented the amount of all 15 compounds very well ($r = 0.63$, $p < 0.001$).

Root exudate diversity and the amount of root exudates were significantly positively correlated (Fig. 2). The amount of root exudates increased significantly with increasing plant diversity, while exudate diversity did not (Table 1; Figs 1 and 2). While root exudate diversity only tended to increase with root biomass and Shannon diversity of roots (which strongly depended on the experimental treatment plant species richness of necessity), the amount of root exudates was significantly positively correlated with the Shannon diversity of roots, but not with root biomass (Fig. 2).

Bacterial and fungal biomass. The biomass of bacteria and fungi as well as the ratio between fungi and bacteria increased significantly with increasing plant species richness (Table 1; Fig. 1). The biomass of bacteria and fungi were significantly positively correlated (Fig. 2). Bacterial biomass tended to increase with increasing root biomass, while the biomass of fungi increased significantly with increasing root biomass. Further, fungal biomass (Fig. 2) and the ratio between fungi and bacteria ($r = 0.32$, $p = 0.02$) increased significantly with increasing amount of root exudates. Fungal biomass (Fig. 2) and the ratio between fungi and bacteria ($r = 0.25$, $p = 0.057$) also tended to increase with exudate diversity.

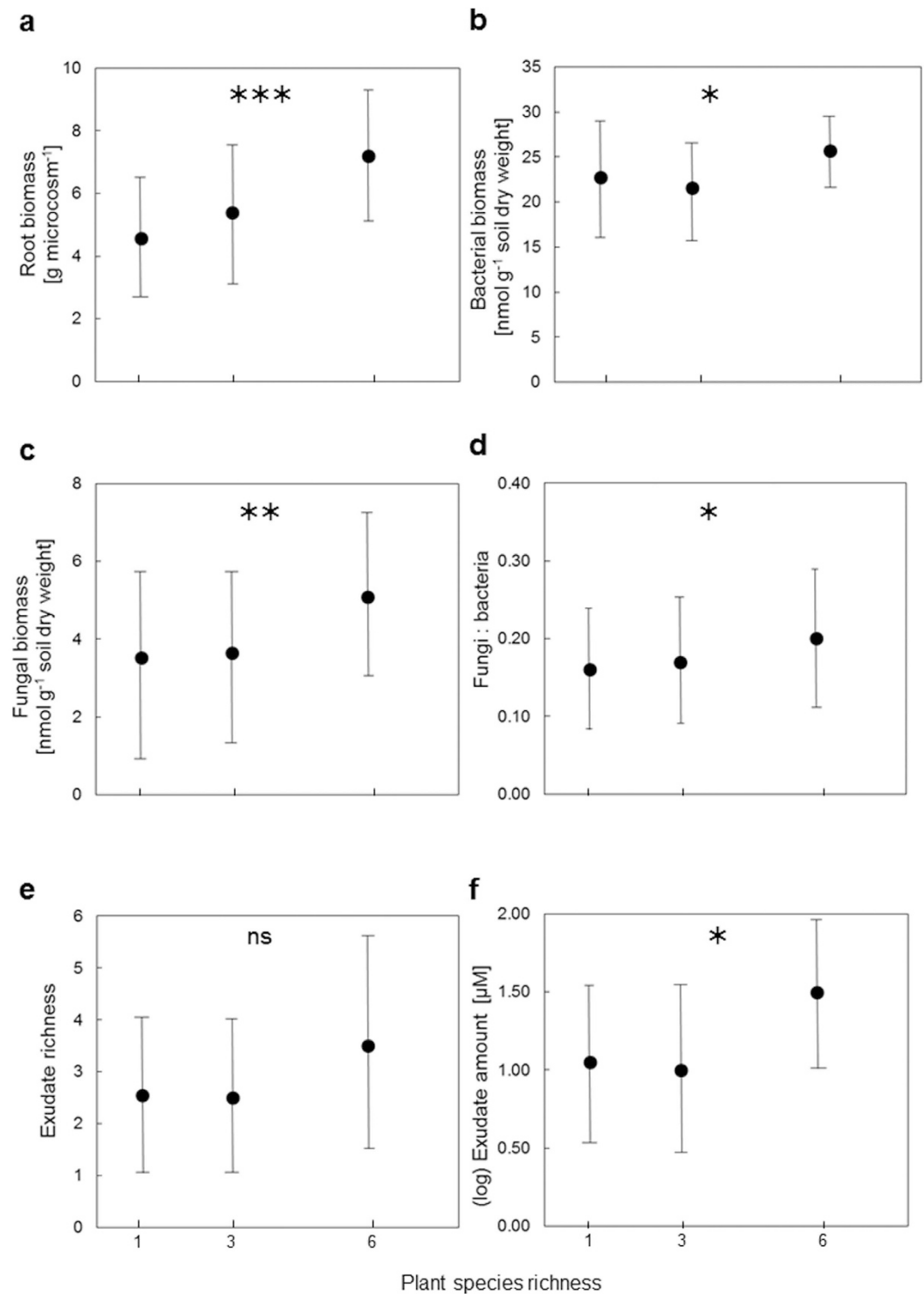


Figure 1. Plant diversity effects on root biomass, soil microbial properties, and root exudates. Root biomass [g microcosm⁻¹] (a), bacterial biomass [nmol g⁻¹ soil dry weight] (b), fungal biomass [nmol g⁻¹ soil dry weight] (c), ratio between fungal and bacterial biomass (d), root exudate diversity [richness of different compounds] (e), and root exudate amount [μM] as the sum of the most abundant compounds fumaric acid, 4-hydroxybenzoic acid, p-coumaric acid, and vanillic acid; see main text for details (f) as affected by plant species richness. Given are means with 95% confidence intervals. *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$ (see Table 1 for details).

Discussion

The present microcosm experiment mirrors field plant diversity experiments by showing increased above-^{1,31} and belowground^{13,14} plant biomass with increasing plant diversity. Consistent with field experiments^{26,32}, soil microbial biomass increased with plant diversity, and soil microbial communities shifted along the plant diversity

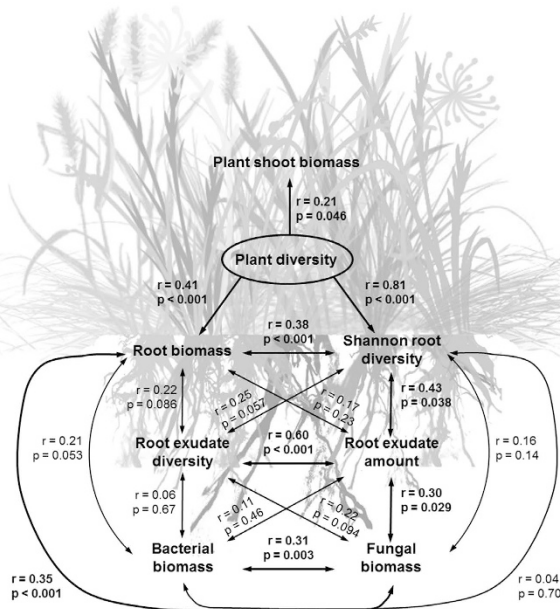


Figure 2. Relationships between plant diversity, root biomass and exudates, and soil microorganisms. Correlations among plant diversity (experimental treatment; 1, 3, or 6 plant species) and plant shoot biomass, root biomass (both [g microcosm⁻¹]), Shannon diversity of roots, root exudate diversity [richness of different compounds], amount of root exudates (log total of the most frequent compounds fumaric acid, 4-hydroxybenzoic acid, p-coumaric acid, and vanillic acid [μM]; see main text for details), soil bacterial biomass, and soil fungal biomass (both [nmol g⁻¹ soil dry weight]). One-headed arrows indicate treatment effects on response variables, while double-headed arrows indicate correlations. Significant relationships ($p < 0.05$) are given in bold; r stands for correlation coefficient. Note that results of structural equation modelling are not shown due to low replication number, but confirmed the significant relationship between the amount of root exudates and fungal biomass ($p = 0.046$).

gradient towards more fungal-dominated communities^{6,33}. While our results confirm previous experiments that reported plant biomass-mediated effects of plant diversity on soil microbial biomass³², the present study provides one of the first empirical evidences that this plant diversity effect could be driven –at least in part– by changes in plant root exudation.

Plants often select their rhizobiomes *via* root exudation²¹. Indeed, an elevated amount, and to a lower extent diversity, of root exudates at high plant diversity shifted fungal-to-bacterial ratio in the present study. A recent laboratory experiment also suggests that variations in the diversity and stoichiometry of root exudates can alter soil microbial communities and functions as well as influence the relationship between plant diversity and microbial communities²⁹. However, it is important to note that soil microbial communities can also influence the amount and composition of root exudates³⁴. The marginally significant positive relationship between the diversity of root exudates and root biomass found in the present study may have been due to larger surface area of the roots^{35,36} and a higher Shannon diversity of roots (with the latter being directly related to the experimental plant diversity gradient).

The investigation of root exudates is challenging, and we had to accept some limitations of our approach. First of all, we were able to identify only a fraction of the compounds detected in the HPLC; nevertheless we used identified plant products only, because organic compounds in the soil will always contain soil microbial products³⁰ that were not in the focus of this study. Thus, the measures of root exudate amount and diversity should be regarded as proxies representing relative differences among experimental treatments rather than absolute measures. Despite those caveats, the present study provides empirical evidence for the significant role of root exudates in linking above- and belowground communities and the diversity of plant communities with the functional composition of soil microbial communities^{12,22} stimulating future work on the mechanisms of rhizosphere interactions^{26,28,33}.

Ecosystem services, such as soil carbon storage⁶ and soil aggregate stability³⁷, are known to be driven by soil microorganisms and contingent upon plant diversity. The fungal-to-bacterial biomass ratio is a powerful predictor of soil ecosystem functioning, varies with biotic and abiotic environmental factors^{3,38}, and thus is likely to be affected by root litter and organic compounds exudated by roots into the rhizosphere⁶. Hooper *et al.*²⁹ proposed that resource heterogeneity determines the composition and diversity of soil microorganisms, although experimental evidence is scarce³⁹. The present study is one of the first indicating that the diversity of roots and root exudates, i.e., the heterogeneity of resources of soil microorganisms, indeed influence the composition and biomass of soil microbial communities, although the quantity of roots and root exudates were the most significant determinants of fungal biomass and the functional composition of soil microbial communities (fungal-to-bacterial ratio). Integrative approaches combining chemical and microbial ecology^{40,41} are needed to improve mechanistic

understanding of aboveground-belowground linkages. Understanding the links between root diversity, root biomass production, root exudates, and soil microorganisms and how they are affected by plant diversity is important for maintaining ecosystem service provision at times of globally declining diversity.

Material and Methods

Experimental set-up. Three different plant diversity levels were established using a pool of six species: monocultures (N = 54), 3- (N = 24), and 6-species mixtures (N = 15). Plant species were selected based on their functional dissimilarity in spatial resource use according to Ebeling *et al.*⁴²: the grasses *Festuca rubra*, *Phleum pratense*, *Anthoxanthum odoratum* and the forbs *Plantago lanceolata*, *Centaurea jacea*, *Rumex acetosa*. This trait-based approach was chosen as our main hypothesis, of plant diversity effects on soil microorganisms being mediated by changes in root biomass and the diversity and quantity of root exudates, required functional dissimilarity among plant species. The initial design of the experiment included an orthogonal manipulation of plant diversity and decomposer diversity (see Supporting Information). However, as the decomposer treatments could not be successfully implemented, and decomposers could not be retrieved at the end of the experiment (probably due to a dry period during the experiment; microcosms were watered manually, see below), we focus on plant species richness effects in the present study and regard the decomposer treatments as potential source for variability in the dataset (e.g., due to potential nutrient effects of decomposing soil animals).

Microcosms consisted of PVC tubes (inner diameter 10 cm, height 20 cm) sealed with PVC lids at the bottom. Each microcosm was filled with 1.6 kg fresh, sieved (4 mm), and defaunated (heating and drying the soil⁴³) soil (pH 8.1, carbon concentration 4.6%, nitrogen concentration 0.3%, C-to-N ratio 15.7; water content 14%) from the grassland field site of the Jena Experiment⁴⁴. The soil was washed to reduce potential effects of nutrients released by the defaunation process. Seedlings (at least 3 cm in height) of six pre-grown plants (also grown in the defaunated and washed soil mentioned above) were transplanted into microcosms in a defined pattern to enhance interspecific interactions; i.e., plant individuals always had neighbours from other plant species in mixtures. During the experiment, microcosms were irrigated with deionized water with the amount increasing from 50 ml/2d to 50–100 ml/day later in the experiment. Microcosms received the same amount of water because variations in soil water content may co-determine plant diversity effects on soil microorganisms²⁴. No nutrients were added to during the experiment. Microcosms were incubated in a climate chamber (day/night = 16/8 h and 22/18 °C ± 3.5 °C; PAR 400 μE) for 104 days.

Harvest and laboratory analyses. Root exudates were sampled directly before the harvest with micro-suction cups (Rhizon Mom 10 cm, Rhizosphere Research Products, Wageningen, Netherlands) and stored until analyses at –20 °C (see Supporting Information for more details). Root exudate solutions (10 ml) were filtered through a 0.22 μm filter (Millipore, Bedford, MA, USA), acidified to pH 2 with 1 N HCl, and partitioned against 5 ml ethyl acetate on an orbital shaker at 290 rpm (Bühler, Hechingen, Germany) for 1 h at room temperature. The organic phase was reduced to dryness under nitrogen and the residue taken up in 100 μl of methanol for high pressure liquid chromatography (HPLC) analyses (see ref. 40; Supporting Information). We only considered compounds that could be ascribed doubtlessly to plant products among many peaks in the HPLC chromatograms, because soluble organic compounds in the soil can contain root exudates as well as soil microbial products³⁰. Thus, our index of root exudate diversity can only be a proxy measure of true diversity of root exudates. Due to the laborious analysis, root exudates could be quantified in 54 microcosms (29, 16, and 9 replicates in monocultures, 3- and 6-species treatments, respectively).

As given above, we used the most frequent compounds for quantification of root exudates: fumaric acid, vanillic acid, p-coumaric acid, and 4-hydroxybenzoic acid. We used this approach as we had to focus on well quantifiable compounds. Every analytical method involves a Limit Of Quantification (LOQ) and a Limit of Detection (LOD). “Not quantified” means that the detected signal does not allow a robust quantification, but not that the compound is absent. Here, the amount of detected root exudates was very low, which still represents a limit for the study of belowground signalling molecules⁴⁰, probably due to the intrinsic limitations of the sampling method (micro-suction cups) and the high turnover of root exudates. Thus, the sum of those four root exudates provides a stoichiometric-based calculation of the total amount of root exudates. While we limited the quantification of root exudates to the four compounds that were above the LOQ, other compounds were only detectable (above LOD) but not quantifiable (below LOQ) and thus were only used for the assessment of root exudate diversity.

We restricted our study to the water-soluble fraction of root exudates as defined in Bais *et al.*⁴⁵ and Baetz & Martinoa⁴⁶. These exudates cover plant secondary metabolites affecting soil microorganisms. Although unidentified peaks observed during HPLC analyses indicate that other compounds, such as phenolic acids, were present in the root exudate solution and could also contribute to the mediation of plant diversity effects on soil microorganisms, the characterization of the root exudates remains a challenge. Novel analytical platforms likely will offer unprecedented opportunities to unravel the complex composition of root exudates in future studies⁴⁰.

The root exudates analyzed in the present study were indubitably identified by the use of pure external standards purchased from Sigma-Aldrich (St Louis, MI, USA). 4-hydroxybenzoic acid, p-coumaric acid, and vanillic acids belong to the class of phenolic compounds. This class of plant secondary metabolites derives from the shikimate pathway and phenylpropanoid metabolism and has been extensively studied as plant allelochemicals. They are commonly found in natural soils^{47,48}, and there are known as plant root exudates playing a role in root inhibition⁴⁹, plant defense^{50,51}, and in the attraction of some soil-borne microorganisms⁵². Fumaric acid is an organic acid that is a key intermediate in the tricarboxylic acid cycle and plays a role in many other plant metabolic processes. Organic acids are commonly detected in the rhizosphere. Their presence in root exudation have been associated with nutrient deficiencies, exposure to toxic cations and anoxia⁵³, and contribute to the stimulation of microbial activity⁵⁴. Previous studies using sterile hydroponic plants have shown that the described molecules (belonging to organic and phenolic acids) are part of plant root exudation; however, often to a lower

extent compared to real soil-root systems. The study of root exudates as signalling molecules of belowground plant interactions necessarily implies to work with microorganisms at the root-soil interface, which may degrade these compounds sooner or later.

Plants shoot material was cut at the soil surface, dried (70 °C, 72 h), and weighed. For soil microbial analyses, three soil cores (1 cm diameter, 10 cm deep) were taken per microcosm, pooled, and frozen at –20 °C until analysis. For molecular species-specific root quantification, one subsample was taken in the centre of the microcosm to include roots of all plant species (0–10 cm: 1 cm diameter, 10–20 cm: 2.5 cm diameter). Roots were washed (1 mm-sieve), thoroughly mixed, and 100 mg of fresh root material was stored at –80 °C until DNA extraction. The remaining soil in the microcosm was washed, and roots were dried and weighed like described above.

For preparation of reference standards for quantitative analysis of species-specific root biomass with real-time PCR, hand-made mixed ratios of all 6 species (1:1:1:1:1:1) were prepared from roots originating from the respective monocultures. The DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) was used for DNA isolation. For qPCR, species-specific primers of *A. odoratum* and *F. rubra* were used as published in refs 12, 55; primers for *P. lanceolata*, *R. acetosa*, *P. pratense*, and *C. jacea* were developed similar to the protocol described in ref. 55. The qPCR plates (96 wells system) were run per species in triplicates, including 4 reference standards. We used the following protocol for the qPCR with HOT FIREPol EvaGreen (Solis BioDyne) with an addition of 0.94 μM MgCl₂, 1 ng of genomic DNA for all species except for *Plantago* for which 7.5 ng of genomic DNA was used, a primer concentration of 120 nM for *Festuca*, *Plantago*, *Rumex*, and *Phleum* and 60 nM for *Anthoxanthum* and *Centaurea* and in a total volume of 20 μl. qPCR reactions were performed on a CFX96 Real-Time system (Biorad). The qPCR reaction conditions were as follows: 15 min at 92 °C, followed by 45 cycles of 20 s at 95 °C, 30 s at 62 °C, and 15 s at 72 °C and finally 42 cycles of 5 s at 75 °C with an increase of 0.5 °C per cycle (melting curve). For the calculation of relative species abundances see Mommer *et al.*⁵⁵. Root DNA analyses could only be performed for 27 plant species mixtures (17 3-species mixtures and 10 6-species treatments) due to logistic constraints as a consequence of the plethora of different laborious analyses.

To determine soil microbial community composition and biomass at the end of the experiment, phospholipid fatty acid (PLFA) analysis was used. PLFA extraction was done following the method of ref. 56, based on the method of ref. 57, and PLFAs were assigned to Gram-positive bacteria, Gram-negative bacteria, fungi, and unidentified microorganisms.

Calculations and statistical analyses. Species-specific root biomass allowed performing additive partitioning⁵⁸ for calculating belowground net biodiversity effect (NE), complementarity effect (CE), and selection effect (SE). Further, the Shannon diversity index of root biomass was calculated to include the relative abundances of different plant species in the belowground plant diversity measure. General Linear Models were used to test plant species richness effects on shoot biomass, root biomass, Shannon diversity of roots, complementarity effect, selection effect, net biodiversity effect, root exudate richness, root exudate amount, bacterial biomass, fungal biomass, and the ratio between fungal and bacterial biomass (Table 1). Further, linear regressions were used to test the relationships between plant species richness and plant shoot biomass, root biomass, and soil microbial variables as well as between different response variables (Supporting Information). Statistical analyses were performed using Statistica 10 (Statsoft). Given the rather low number of replicates for several variables (root exudates and species-specific root biomass), structural equation modeling could not be used to test our hypotheses⁵⁹. We ran SEM and piecewise SEM⁶⁰, but the best model did not meet the criteria based on Fisher's C statistic (Fisher's C = 66.93, p-value = 0.009, AIC = 168.93, AICc = –27.51), which was most likely due to a low number of replicates (n = 25; i.e., in 25 microcosms both species-specific root biomass and root exudates were analyzed). Despite the flexibility of using piecewise SEM for lower number of replicates, our model was still invalid due to several variables and paths. Thus, despite the targeted experimental design, most of the present results are based on correlations, which may not necessarily imply causalities. Notably, however, results of structural equation modeling confirmed the significant relationship between the amount of root exudates and fungal biomass ($p < 0.046$).

Data accessibility. Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.nj3c0>.

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Author Contributions

N.E. conceived the research question; N.E., T.S. and S.S. were involved in the study design; A.L., K.S., T.S., M.P.T. and L.M. carried out data collection and analyses; N.E. and M.P.T. performed the statistical analyses, and N.E. wrote the first draft of the manuscript; all authors were involved in writing and gave final approval for publication.

Additional Information

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