



Interactions of Mycorrhiza and Protists in the Rhizosphere Systemically Alter Microbial Community Composition, Plant Shoot-to-Root Ratio and Within-Root System Nitrogen Allocation

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Arbuscular mycorrhizal fungi (AMF) are important symbionts for plant nutrient uptake, but their exact role in plant nitrogen (N) nutrition is unclear. Protists on the other hand play an acknowledged role in plant N acquisition, and there is increasing evidence for a close interaction with AMF. In a split root set up, we investigated the distinct roles of mycorrhiza (*Rhizophagus irregularis*), protists (*Acanthamoeba castellanii*), and their interaction on plant N uptake, within-root system allocation patterns, and shoot-to-root ratio of winter wheat. In addition, we applied a quantitative metabolomics approach to characterize associated changes in soil microbial communities by microbial phospholipid fatty acid (PLFA) analysis from rhizosphere soil. AMF markedly altered plant shoot-to-root allometry by reducing root biomass of wheat, and mycorrhiza partly took over root system functioning. Protists promoted shoot and root growth, and improved plant N uptake by the release of N from consumed bacterial biomass, a mechanism known as microbial loop. The shoot system however responded little to these alterations of the root system and of the rhizosphere community composition, indicating that the plants optimized shoot growth despite varying investment into roots. Mycorrhiza reduced root biomass and plant N, especially in the combined treatments with protists by changing within root system allocation of N and root biomass. These systemic effects on root allocation pattern suggest that mycorrhiza also gained control over N provided by protist grazers. Protists and mycorrhiza altered rhizosphere bacterial communities in contrasting but consistent ways as shown by quantitative shifts in microbial PLFA profiles. Remarkably, the changes in bacterial community composition were systemically conveyed within the root system to the split-root chamber where the symbionts were lacking. Accordingly the synergistic effects of protists and mycorrhiza indicated systemic effects on nutrient- and on root-allocation within root systems as an emergent property

that could not be predicted from single treatments with mycorrhiza or protists alone. The tight plant and microbial feed backs uncovered in this study have far reaching implications for understanding the assembly of plant microbiomes, and testify central roles of both protists and mycorrhizas in the assembly process.

Keywords: arbuscular mycorrhiza, wheat, nitrogen, amoebae, protists, microbial loop, shoot-to-root allocation, split-root experiment

INTRODUCTION

Frameworks on shoot-to-root allometry of plants are based on models of competition between shoots and roots for plant carbohydrates and nutrients (Bloom et al., 1985; Van Noordwijk and De Willigen, 1987; Ericsson, 1995). Recently, models on resource stoichiometry (Johnson, 2010) and microbial markets (Werner et al., 2014; Revillini et al., 2016) have been applied to plant-symbiont interactions, but these approaches have not been rigorously tested on the individual plant level. In particular root traits have been considered to be determined by plant physiology and genetic disposition (Hoad et al., 2001; Wasson et al., 2012) or by external factors, such as different forms of nutrients (López-Bucio et al., 2003; Tian et al., 2014). On the other hand an abundant literature exists on influence of soil microorganisms affecting the root architecture of plants.

In the past decade much has been learned about how free-living rhizosphere microorganisms shape plant traits (Friesen et al., 2011; Lau and Lennon, 2012). Arbuscular mycorrhizal fungi (AMF) are among the most important mutualists improving nutrient acquisition of plants (Smith and Read, 2008; Bonfante and Genre, 2010). This is well confirmed for plant phosphorus uptake (Sanders and Tinker, 1971; Olsson et al., 2010; Smith F. A. and Smith, 2011), but the role of AMF in plant nitrogen (N) uptake is controversial (Smith S. E. and Smith, 2011; Veresoglou et al., 2012a). Although AMF hyphae have been shown to preferentially colonize N-containing organic patches in soil (Bukovská et al., 2016) and can aid in plant N-uptake (Hodge et al., 2001), they also function as N sink (Hodge and Fitter, 2010). Moreover, AMF lack the enzymatic machinery for N mineralization (Smith S. E. and Smith, 2011) and hence rely on other microorganisms for the supply of mineral N (Herman et al., 2012; Koller et al., 2013a).

Protists are ubiquitous predators of soil bacteria in the plant rhizosphere and release considerable amounts of N from consumed bacterial prey (Kuikman et al., 1991), a mechanism described as “microbial loop” (Clarholm, 1985; Bonkowski, 2004). While it has been shown that bacteria suppress AMF colonization of roots when competing for N (Leigh et al., 2011; Hodge and Storer, 2014), protist predation shifts nutrient competition in favor of the mycorrhizal symbiont since AMF hyphae effectively take up the N released by protist predators (Herdler et al., 2008; Koller et al., 2013b; Bukovská et al., 2018).

There is also potential conflict between AMF and protists in respect to the allocation of plant C in the rhizosphere. AMF act as strong sink for host plant C (Gange and Ayres, 1999; Klironomos, 2003; Lerat et al., 2003) and generally lead to reduced root growth

(Veresoglou et al., 2012b). AMF also reduce the concentration of carbohydrates in root exudates (Graham et al., 1981; Ryan et al., 2012), resulting in reduced substrate supply to free-living rhizosphere bacteria and their consumers. Acanthamoebae have been found to stimulate lateral root branching and root growth, thereby likely enhancing root exudation and triggering a positive feedback in the rhizosphere microbial food web (Bonkowski and Brandt, 2002; Kreuzer et al., 2006; Krome et al., 2009, 2010; Bonkowski and Clarholm, 2012). These effects are reminiscent of the stimulation of root growth by plant growth promoting rhizobacteria (Bonkowski and Brandt, 2002; Bonkowski, 2004). In accordance with this view, changes in the rhizosphere bacterial community due to protist predation are non-random (Kreuzer et al., 2006; Jousset et al., 2008, 2009; Rosenberg et al., 2009), but also AMF have been reported to affect rhizosphere bacterial communities in specific ways (Marschner and Baumann, 2003; Rillig et al., 2006; Scheublin et al., 2010; Nuccio et al., 2013; Bukovská et al., 2016), suggesting additive effects on bacterial community assembly.

Here we investigated the specific roles of AMF and protists in determining nutrient fluxes in the rhizosphere. Further, we explore the interaction of AMF and protists as major rhizosphere symbionts and their effects on rhizosphere bacterial communities. A split root experiment was established to investigate systemic effects, and the role of the plant in mediating AMF and protist interactions. Rhizosphere nutrient fluxes were quantified by measuring plant biomass and nutrient concentrations in roots and shoots. Changes in the microbial community composition and activity were measured using phospholipid fatty acid (PLFA) profiles and microbial respiration.

The following hypotheses were tested:

- 1) From the perspective of the plant mycorrhiza and protists appear complementary. Mycorrhizal hyphae take over root system functions by capturing and transporting nutrients to the plant, while protists increase nutrient availability by mineralizing nutrients locked in bacterial biomass.
- 2) Both, mycorrhiza and protists alter the structure of the bacterial community in the rhizosphere with mycorrhiza increasing and protists decreasing bacterial biomass.

MATERIALS AND METHODS

Soil

A clay loess soil was taken from a meadow (“Molino-Arrhenatheretea”) on a basalt outcropping near Darmstadt, Germany. The soil was sampled from 5 to 20 cm depth and sieved

(5 mm) to remove roots and stones. The soil was autoclaved (121°C, 110 kPa, 30 min). The soil pH_(KCl) was 6.9 and the C:N ratio 18 (16 mg g⁻¹ C and 0.9 mg g⁻¹ N).

Plants

Winter wheat (*Triticum aestivum* L. var. Naxos) seeds were sterilized according to Hensel et al. (1990), by subsequent treatment with 96% ethanol (2 min), and 5% NaOCl (5 min) under vacuum in a desiccator. After rinsing with distilled water the seeds were germinated in 100 µl Neff's Modified Amoeba Saline (NMAS; Page, 1976) in individual wells of 96 well microtitre plates (Greiner, Solingen Germany) in darkness at 20°C. After 4 days, sterile seedlings were transferred on sterile 1% Agar containing nutrient broth (NB; Merck, Darmstadt, Germany) and NMAS (at 1:9 v:v; NB-NMAS) in a Petri dish and stored in a climate chamber (20°C, 70% relative humidity, 14/10 h light/ dark cycle). A total of 25 sterile wheat seedlings of 8 cm shoot length and multiple secondary roots of over 5 cm length were selected for the experiment.

Microbial Inocula

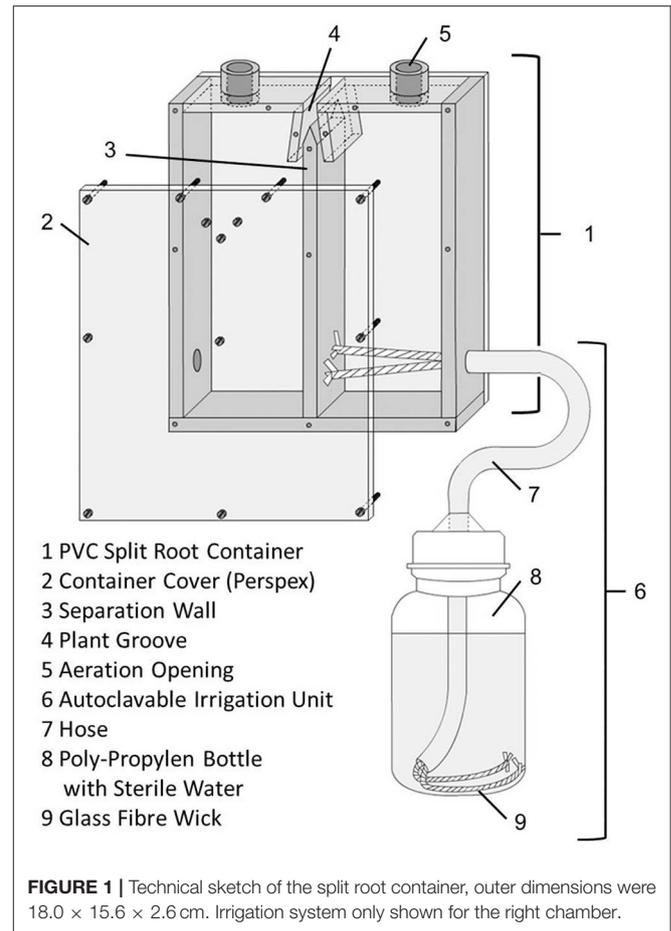
The autoclaved soil was immediately inoculated with a bacterial filtrate from meadow rhizosphere soil to re-establish a diverse bacterial community. The protist-free bacterial filtrate was obtained by suspending 50 g fresh meadow soil (Biology Campus, TU Darmstadt) in 200 ml sterile tap water and filtering it through paper filters (Schleicher & Schuell, Dassel, Germany) and subsequent 5.0 and 1.2 µm Isopore filters (Millipore, Schwalbach, Germany). The filtrate was incubated at 15°C for 7 days and controlled daily for protist contaminations.

Acanthamoeba castellanii Neff, a ubiquitous naked amoeba in soils was used as model protist (Fiore-Donno et al., 2016). *Acanthamoeba castellanii* was isolated from a beechforest soil and kept in NB-NMAS at 5°C on native soil bacteria as food (Bonkowski and Brandt, 2002). The amoebae were separated from the bacteria using repeated centrifugation (1 min, 1,000 rpm) and washing with NB-NMAS (Herdler et al., 2008). The resulting protists suspension was used to inoculate the protist treatments (0.5 ml per chamber). The supernatant containing bacteria from the amoeba culture was filtered (3 µm, Millipore Corporation, Bedford MA, USA) to obtain a protist-free mock community and to inoculate all non-protist treatments.

An AMF *in vitro* culture of *Rhizophagus irregularis* (previously *Glomus intraradices*; Stockinger et al., 2009) was sourced from the Mycothèque de l'Université Catholique de Louvain (MUCL, Belgium) and used as a mycorrhizal inoculum (Bonkowski, 2018).

Split Root Containers

Split root containers were constructed out of PVC with inner dimensions of 8 × 7.5 × 2 cm of each root compartment (Figure 1). The front and back walls were transparent, allowing for monitoring root growth and soil moisture. The containers were divided into equally dimensioned right and left chambers. The only connection point between the two chambers was a groove introduced at the top of the split root container to house the plant's root crown. The plant groove and the mating faces



of the chamber walls were coated with high viscosity silicone paste (Baysilone, Bayer, Leverkusen, Germany) to completely isolate each soil chamber, and the walls were secured at multiple points with screws. The top of each chamber contained a 10 mm diameter aeration opening. Each chamber was watered in an automatic way using a glass fiber capillary wick (Ortmann, Hilden, Germany) housed in a PE hose fed into through the bottom of the chamber, leading into a 250 ml PP wide neck bottle. This enabled the individual plant roots to adjust their water uptake according to demand. This is crucial in the early growth phase, as water demand of individual root systems in a split root chamber cannot be exactly determined by weighing and external watering.

The containers were sterilized in 70% ethanol and the irrigation systems were autoclaved at 121°C and 110 kPa for 30 min prior to construction. Each chamber was filled with soil corresponding to 200 g dry weight. A seedling was set into the plant groove and its roots were equally separated into both root chambers. A 1 cm² piece of agar containing spores and hyphae of *R. irregularis* was applied to the roots in chambers designated for mycorrhiza. Each chamber was inoculated with 1 ml bacterial suspension. Chambers designated for protists were inoculated with 1 ml amoeba suspension, all other chambers received 1 ml NB-NMAS containing the mock community from

amoeba cultures. The aeration openings were closed by sterile cotton plugs to protect the soil from airborne contamination. After inoculation, the chambers were wrapped in aluminum foil to prevent light exposure of soil and roots.

Experimental Design

The experiment was set up in a two-factorial design with the factors protists (*A. castellanii*) and mycorrhiza (*R. irregularis*). Five treatments and treatment combinations were set up, each with five replicates: (1) control (0-0) with only bacterial inoculum in both chambers of the split-root container, (2) protists (0-P) as 0-0, but inoculated additionally with *A. castellanii* in one chamber, (3) mycorrhiza (0-M) as 0-0, but inoculated additionally with *R. irregularis* in one chamber, (4) as 0-0, but protists and mycorrhiza (0-PM) inoculated together in one chamber, (5) protists and mycorrhiza inoculated on separate chambers (P-M), i.e., one chamber containing protists and the other mycorrhiza. The split root containers were arranged in a randomized complete block design with five containers of each treatment in each of five blocks, which were clockwise rotated every 3 days. During these rotations the poly-propylen water supply bottles (Figure 1) were weighed to check for water loss. These measurements allowed calculating the water use of each plant.

The experiment was run for 65 days in a climate chamber at $20 \pm 2^\circ\text{C}$ and a relative humidity of 70%. The climate chamber was illuminated with HI-R lights (Osram, Munich Germany) with $350 \mu\text{mol m}^{-2} \text{s}^{-1}$ and a 14/10 h day/night cycle. After 4 weeks, plant water requirements exceeded the capacity of the capillary wicks, and root chambers were irrigated through the aeration openings with equal volumes of supplementary sterile distilled water every 3 days to adjust for weight lost.

Measurements After Destructive Sampling of the Experiment

Plant roots were carefully picked and sieved from the soil, and the collected roots from both chambers were washed separately and dried, along with the shoots, at 60°C for 72 h, then weighed to obtain dry weight biomass. The dried shoots were blender homogenized (Krupps; Solingen, Germany). One gram of shoot material and 1 g of root material taken from the center of the root sample of each root halve was pulverized in a ball mill (MM 200 Retsch, Haan, Germany), and its C and N concentration measured using an elemental analyser (Na1500; Carlo Erba; Milan, Italy). In addition, 200 mg of the milled shoots and roots were dissolved in 2 ml 65% nitric acid and digested using a closed vessel microwave digester (Agazzi and Pirola, 2000). The concentrations of P, Mn, Mg, Ca, K, Al, Na, Fe, and S were determined using Inductive Coupled Plasma—Atom Emission Spectral analysis (ICP-AES, Thermo Scientific iCAP6500).

Microbial Parameters

Soil remaining after the collection of roots was sieved (2 mm), and a sample corresponding to 2 g dry weight was brought to a moisture content of 30% with distilled water. The O_2 consumption rate of this soil was measured every hour over 48 h using a respirometer (Scheu, 1992). Microbial basal respiration

($\mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$) was measured over 24 h. Microbial biomass (C_{mic} ; $\mu\text{g C g}^{-1} \text{ dw}$) was measured by substrate-induced respiration (SIR; Anderson and Domsch, 1978) after adding glucose ($8 \text{ mg g}^{-1} \text{ dry wt soil}$), and calculated according to Beck et al. (1997). Microbial specific respiration was calculated as the quotient of basal respiration and microbial biomass.

Nutrient limitation of the microbial community was investigated by adding N [as $\text{NH}_4\text{NO}_3(\text{aq})$] and P [as $\text{KH}_2\text{PO}_4(\text{aq})$] along with C [as glucose_(aq); $8 \text{ mg g}^{-1} \text{ dry wt}$] at a C:N:P mass ratio of 10:2:1 (Anderson and Domsch, 1980) and measuring the slope of the corresponding microbial growth curve (log transformed) using the O_2 microcompensation system (Griffiths et al., 2001).

Protist density (Figure S2) was determined by the Most Probable Number technique (MPN; Darbyshire et al., 1974). Five g fresh weight soil from different locations of each chamber were suspended in 20 ml NMAS by shaking (100 rpm, 20 min). Four replicated 100 μl aliquots suspension per sample were 3-fold diluted in NB-NMAS in 96-well microtiter plates and incubated in darkness at 20°C . Wells were checked daily for amoebae, flagellates and ciliates over a period of 10 days using an inverse microscope (100–400x; Fluovort FU, Leitz; Marburg, Germany).

Soil PLFA Analysis

A quantitative metabolomics approach was used to characterize shifts in microbial communities. The phospholipid fatty acids (PLFAs) composing the membrane lipids of the microorganisms in soil were quantitatively extracted from 1 g soil following Frostegård et al. (1991) with Bligh/Dyer solution [chloroform, methanol, citrate buffer (pH = 4; 1:2:0.8; v/v/v)] and the solution passed through silica acid columns (0.5 g silicic acid, 3 ml; Varian Medical Systems, Palo Alto, California).

PLFAs were transformed into fatty acid methyl esters (FAMES) by mild alkaline methanolysis as described in Frostegård et al. (1991). The FAMES were measured using an AutoSystem XL gas chromatograph (Perkin-Elmer Corporation, Norwalk, CT, USA). GC settings were as in Kramer et al. (2013). Identification of FAMES was based on their retention time assessed with a FAME and a bacterial acid methyl ester mix standard (Sigma-Aldrich, St. Louis, USA). An internal FAME standard (Sigma-Aldrich, St. Louis, USA) added to the samples before methanolysis was used for quantification.

FAMES were identified by GC-MS using a HP 5890 Series II Plus coupled with a 5972 mass selective detector (Hewlett Packard/Agilent, Waldbronn, Germany) equipped with a DB-5MS column. GC-MS settings were as in Kramer et al. (2013). A mass range of m/z 50–450 was monitored. The branched fatty acids i15:0, a15:0, i16:0, and i17:0 were considered as Gram positive and the cy17:0 and cy19:0 as Gram negative bacterial markers (Zelles, 1999). The following PLFAs were used to infer microbial community structure: 14:0, i15:0, a15:0, 15:0, i16:0, 16:1 ω 7, 16:0, i17:0, cy17:0, 17:0, 18:2 ω 6, 18:2 ω 6t/18:1 ω 9/18:3 ω 3, 18:1 ω 7/18:1 ω 9t, 18:0, cy19:0 (Frostegård and Bååth, 1996; Zelles, 1999). Since the PLFA 16:1 ω 5, often being used as mycorrhizal marker in environmental studies, can be found in both bacteria and mycorrhiza (see Ngosong et al., 2012) it was discarded from

the analysis. Likewise, unsaturated C20 PLFAs occur in a wide range of eukaryotes (Ruess and Chamberlain, 2010) and were discarded from the analyses.

Statistical Analysis

The data were analyzed by a two factorial (protists and mycorrhiza) general linear model (GLM) with *a priori* singular contrasts. Shoot and root data were analyzed separately. In the first step, treatments with protists (0-P) and mycorrhiza (0-M) alone were compared to the control (0-0). Subsequently, the shoot and root data of the combined treatments (0-PM, P-M) were compared to those of treatments with mycorrhiza alone (0-M) and protists alone (0-P). Roots in the split-root containers had a “treated” and a “non-treated” chamber, except in P-M where both root chambers were differently treated. The split-root set up therefore allowed to compare the “treated” chambers *between* treatments; and further to analyze systemic effects of the “treated” root section on the “non-treated” root section by comparing root chambers *within* treatments. In the 0-0 treatment one root chamber of each replicate was *a priori* randomly assigned as control. For between-treatment comparisons, the root chambers treated with protists (0-P) or mycorrhiza (0-M) were compared to the control chamber of the 0-0 treatment, and to the chamber treated with protists and mycorrhiza (0-PM). In the P-M treatment, where protists and mycorrhiza were spatially separated, the protist-inoculated chamber was compared to the protist treated chamber of 0-P, while the mycorrhiza-inoculated chamber was compared to the mycorrhiza treated chamber of 0-M. Statistically significant contrasts between “treated” root chambers are indicated by horizontal bars in figures. Subsequently, systemic effects of the “treated” root section vs. the “non-treated” root section *within* treatments, and between both treated chambers of the P-M treatment were analyzed by one-way analysis of variance (ANOVA). Data were analyzed using SAS V8.2 software (SAS Institute; Cary, North Carolina, USA). Discriminant function analysis (DFA) was used to inspect the overall structure of the PLFA profiles in order to differentiate the effects of different treatments on soil bacteria. Through subsequent correlation of single fatty acids with both major axes of the DFA, we investigated which fatty acids were responsible for the discrimination of samples. DFA was carried out in R version 3.3.3, using the packages MASS (Venables and Ripley, 2002), and ggplot2 (Wickham, 2016) for plotting.

RESULTS

Plant Biomass

Plant shoot-to-root allometry and within-root system allometry between both split-root chambers provides information on potential competition between shoots and roots and between roots and microbes for carbohydrates and nutrients. Average total biomass of shoots and roots in the control treatment (0-0) was 4.93 ± 0.7 and 6.14 ± 2.3 g dry wt, respectively. Presence of protists (0-P) caused a significant increase in total plant biomass by 24% as compared to the control (**Figure 2A; Table 1**). Protists also tended to increase shoot biomass (+22%). Mycorrhiza in contrast had no effect on shoot biomass but significantly

increased the shoot-to-root ratio (+74%), due to (in trend) reduced total root biomass [-37% , 0-0 vs. 0-M, $F_{(1,23)} = 3.15$; $p = 0.089$; **Figure 2A**]. Root biomass of the mycorrhiza chamber in the 0-M treatment appeared particularly low, but the reduction was not statistically significant due to high variation in the non-inoculated root compartment [$F_{(1,6)} = 3.60$; $p = 0.107$]. Total root biomass was also reduced when both mycorrhiza and protists were combined (0-PM, P-M; **Table 1**). Total root biomass decreased as compared to 0-P by -34 and -31% in the 0-PM and P-M treatments, and resulted in an increased shoot-to-root ratio by 38 and 40% in the 0-PM and P-M treatments, respectively (**Figure 2A; Table 1**).

Protists mitigated some of the mycorrhiza induced reductions of root systems, resulting in a 2.57-fold increase of root biomass in the mycorrhiza chamber of the P-M treatment as compared to the mycorrhiza chamber of the 0-M treatment [$F_{(1,23)} = 5.69$; $p = 0.026$]. This was because root biomass in the mycorrhiza chamber of the P-M treatment was 2-fold higher than that in the neighboring protist chamber, albeit with marginal significance [$F_{(1,8)} = 4.42$; $p = 0.069$]. The complementarity of protists in combination with mycorrhiza was further supported by measurements of plant water uptake, which was significantly higher in 0-PM ($p < 0.001$) and P-M ($p = 0.018$) treatments compared to the 0-M treatment (**Figure S2, Table S2**).

Nutrients

Differences in plant nutrient uptake between control (0-0) and microbial treatments reveal either mutualistic, neutral or parasitic relationships in symbioses. Comparing differently inoculated root chambers can reveal competition or synergism between different microorganism groups. Mycorrhiza and protists altered root N mass in opposite directions, with protists increasing and mycorrhiza reducing it; and the negative mycorrhiza effect continued to dominate in 0-PM and P-M treatments. In the 0-P treatment, root N mass of the protist chamber increased by 52% as compared to the control [0-0; $F_{(1,23)} = 4.32$; $p = 0.049$], but this effect was local and increased total root N mass (+30%) and total plant N mass (+22%) only with marginal significance (**Figure 2B; Table 2**).

In the mycorrhiza chamber of the 0-M treatment, the root N mass decreased by 66% as compared to the control [0-0; $F_{(1,23)} = 6.05$; $p = 0.022$]. This led to a 58% reduction in total root N mass, while total plant N mass was only reduced by 32% (**Figure 2B; Table 2**). The nitrogen reduction in the mycorrhiza chamber of the 0-M treatment led to a 2.72-fold increase in the shoot-to-root N mass ratio as compared to the control (0-0) as shoot N was not affected (**Table 2**). The negative effect of mycorrhiza on plant N mass prevailed in the presence of protists (0-PM, P-M), and was also reflected in root N concentrations (**Figure S3, Table S2**). Root N concentration in the mycorrhiza + protist chamber of the 0-PM treatment (0.60 mg g^{-1}) was significantly reduced as compared to the protist chamber of the 0-P treatment [1.02 mg g^{-1} ; $F_{(1,23)} = 41.3$; $p < 0.001$; **Figure S3**]. This reduced both, total root N mass and total plant N mass by 56% in 0-P vs. 0-PM treatments (**Table 2**). In a similar way total root N mass was reduced in the P-M as compared to the 0-P treatment by 77%. These nitrogen reductions in roots led to

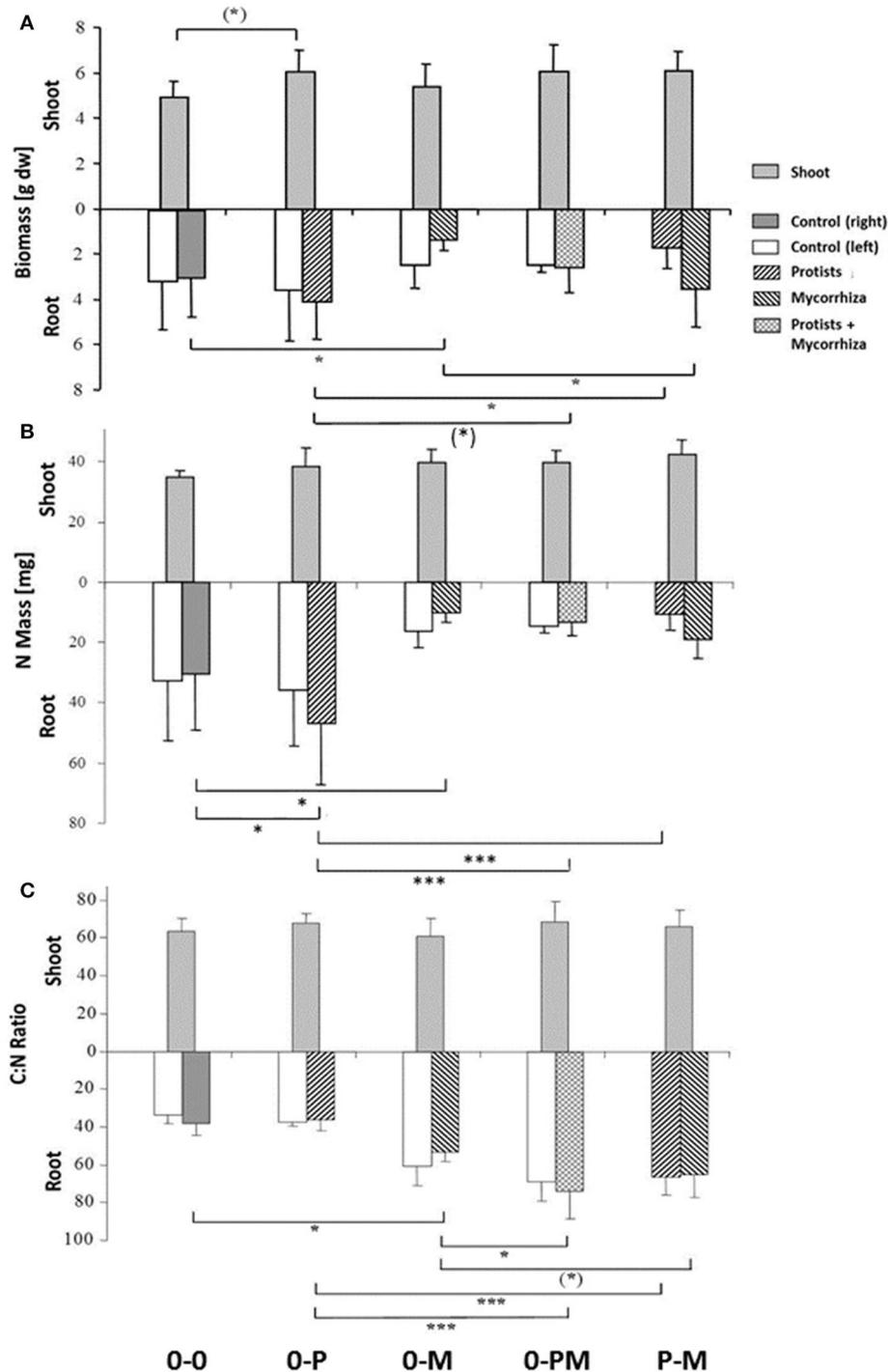


FIGURE 2 | Biomass **(A)** total N mass **(B)** and C:N mass ratio **(C)** of shoots and of roots of *Triticum aestivum* in both root chambers of control plants (0-0), plants treated with protists in the right root chamber (0-P), or treated with mycorrhiza in the right root chamber (0-M), or treated with protists and mycorrhiza together in the right root chamber (0-PM), or where the left and right root chambers were separately treated with protists and mycorrhiza (P-M). Means and standard deviation; horizontal lines denote significant contrasts with (*) $p < 0.1$, * $p < 0.05$, *** $p < 0.001$.

2.57 and 2.58-fold enhanced shoot-to-root N mass ratios in 0-PM and P-M treatments as compared to the control, respectively (Figure 2B, Table 2). Interestingly, the root N concentration of the protist + mycorrhiza chamber in the 0-PM treatment

was also significantly reduced as compared to the mycorrhiza chamber of the 0-M treatment [from 0.75 to 0.60 mg g⁻¹; $F_{(1,23)} = 4.57$; $p = 0.043$], suggesting N uptake by mycorrhiza in PM (Figure S3). Unexpectedly, total mass of root N in the

TABLE 1 | *F*- and *p*-values of GLM contrasts showing treatment effects on total plant biomass, shoot biomass, biomass of the total root system, and shoot-to-root ratio of *Triticum aestivum*.

Comparison	Total biomass		Shoot biomass		Total root biomass		Shoot-to-root ratio	
	<i>F</i> _(1,19)	<i>P</i>	<i>F</i> _(1,19)	<i>P</i>	<i>F</i> _(1,19)	<i>P</i>	<i>F</i> _(4,19)	<i>P</i>
0-0 vs. 0-P	4.46	0.049	3.53	0.077	1.99	0.174	0.67	0.424
0-0 vs. 0-M	1.76	0.201	0.77	0.391	3.69	0.070	11.65	0.003
0-M vs. 0-PM	2.00	0.175	1.05	0.320	1.06	0.316	0.82	0.378
0-M vs. P-M	2.46	0.135	0.96	0.340	1.50	0.235	2.08	0.167
0-P vs. 0-PM	4.08	0.058	0.02	0.836	5.56	0.029	29.35	<0.001
0-P vs. P-M	3.45	0.080	0.01	0.919	4.62	0.045	35.89	<0.001

Treatments: control plants (0-0), root chambers treated with protists (0-P), with mycorrhiza (0-M), with protists and mycorrhiza together (0-PM), or where the left and right root chambers were separately treated with protists and mycorrhiza (P-M). Nominator and denominator degrees of freedom in brackets. Significant effects $p < 0.05$ in bold; $p < 0.1$ in italics.

TABLE 2 | *F*- and *P*-values of GLM contrasts showing treatment effects on total shoot N mass, total root N mass, total plant N mass, and shoot-to-root N mass ratio of *Triticum aestivum*.

Comparison	Shoot N mass		Total root system N mass		Total plant N mass		Shoot-to-root N mass	
	<i>F</i> _(1,19)	<i>P</i>	<i>F</i> _(1,19)	<i>P</i>	<i>F</i> _(1,19)	<i>P</i>	<i>F</i> _(4,19)	<i>P</i>
0-0 vs. 0-P	1.29	0.270	3.20	0.090	4.22	0.055	0.15	0.705
0-0 vs. 0-M	2.65	0.119	10.27	<0.005	7.70	0.012	16.41	<0.001
0-M vs. 0-PM	0.01	0.926	0.01	0.915	0.01	0.909	0.01	0.907
0-M vs. P-M	0.82	0.377	0.08	0.785	0.29	0.599	0.01	0.928
0-P vs. 0-PM	0.24	0.628	25.72	<0.001	23.87	<0.001	20.76	<0.001
0-P vs. P-M	2.41	0.137	23.94	<0.001	19.72	<0.001	21.03	<0.001

Treatments: control plants (0-0), root halves treated with protists (0-P), with mycorrhiza (0-M), with protists and mycorrhiza together (0-PM), or where the left and right root compartments were separately treated with protists and mycorrhiza (P-M). Nominator and denominator degrees of freedom in brackets. Significant effects $p < 0.05$ in bold; $p < 0.1$ in italics.

P-M treatment was almost 2-fold reduced in the protist chamber (10.6 mg N) as compared to the mycorrhiza chamber [19.2 mg N; $F_{(1,8)} = 5.57$; $p = 0.046$; **Figure 2B**].

Root P mass in the 0-M treatment was reduced by 64% as compared to the control [0-0; $F_{(1,23)} = 6.96$; $p = 0.015$], resulting in a 55% reduction in total root P mass (**Figure 3, Table 3**), but a 3.7-fold increase in the shoot-to-root P mass ratio. Although root P mass was not affected in the 0-P treatment (**Table 3**), root P levels were diluted by an increased root biomass, with root P concentration in the protist chamber of the 0-P treatment being only 0.10 mg g⁻¹ as compared to the control (0-0) with 0.14 mg g⁻¹ [$F_{(1,23)} = 4.73$; $p = 0.041$; **Figure S4**].

Root P mass in the protist chamber of the P-M treatment was reduced by 54% as compared to the 0-P treatment [**Figure 3**; $F_{(1,23)} = 5.27$; $p = 0.031$], but in the mycorrhiza chamber of the P-M treatment root P mass was more than 2.5-fold higher than in the 0-M treatment [$F_{(1,23)} = 5.04$; $p = 0.035$]. Comparing both split-root chambers in the P-M treatment, root P mass almost doubled (x 1.91) in the mycorrhiza vs. the protist chamber [$F_{(1,8)} = 5.61$, $p = 0.045$], indicating compensation of P deficiency by mycorrhiza since total root P and total plant P mass were not significantly affected (**Figure 3; Table 3; Table S3**). Only in the 0-PM treatment total root P and whole plant P tended to be reduced by 37 and 16% in 0-PM vs. 0-P, respectively, although with marginal significance (**Figure 3; Table 3**). This was because root P mass in the mycorrhiza + protist chamber of

the 0-PM treatment tended to be 42% lower as compared to the protist chamber in the 0-P treatment [**Figure 3**; $F_{(1,23)} = 3.17$; $p = 0.088$]. Thus, mycorrhiza appeared to reduce the P gain of plants in direct contact with protists (0-PM), but significantly shifted within-root system allocation of P to the mycorrhiza chamber in P-M.

Plant C:N Ratio

The C:N ratio reflects a critical balance of carbohydrate gain (by photosynthesis) and/or investment (e.g., in root symbionts) relative to plant nutrient uptake. Mycorrhiza increased the root C:N ratio across treatments (**Figure 2C**). The root C:N ratio in the mycorrhiza chamber of the 0-M treatment (53) was significantly higher as compared to the control (0-0) at 38 [$F_{(1,23)} = 5.16$; $p = 0.033$]. Interestingly, the effect of mycorrhiza on root nutrient status was systemic, reflected in comparably high root C:N ratios of 61 and 53 in the bacteria only vs. mycorrhiza chambers of the 0-M treatment, respectively [$F_{(1,6)} = 1.74$, $p = 0.24$]. Accordingly, total plant C:N ratio was higher in the 0-M treatment (60) than in the control (46; **Table 4**). Thus, mycorrhiza strongly reduced root N levels relative to C, but the shoot C:N ratio remained constant.

Mycorrhiza effects also dominated the root C:N ratio in the presence of protists, reflected by root C:N ratios of 74 and 66 in the protist chambers of 0-PM and P-M as compared to the 0-P (37) treatment [**Figure 2C**; 0-P vs. 0-PM: $F_{(1,23)} = 37.14$; $p <$

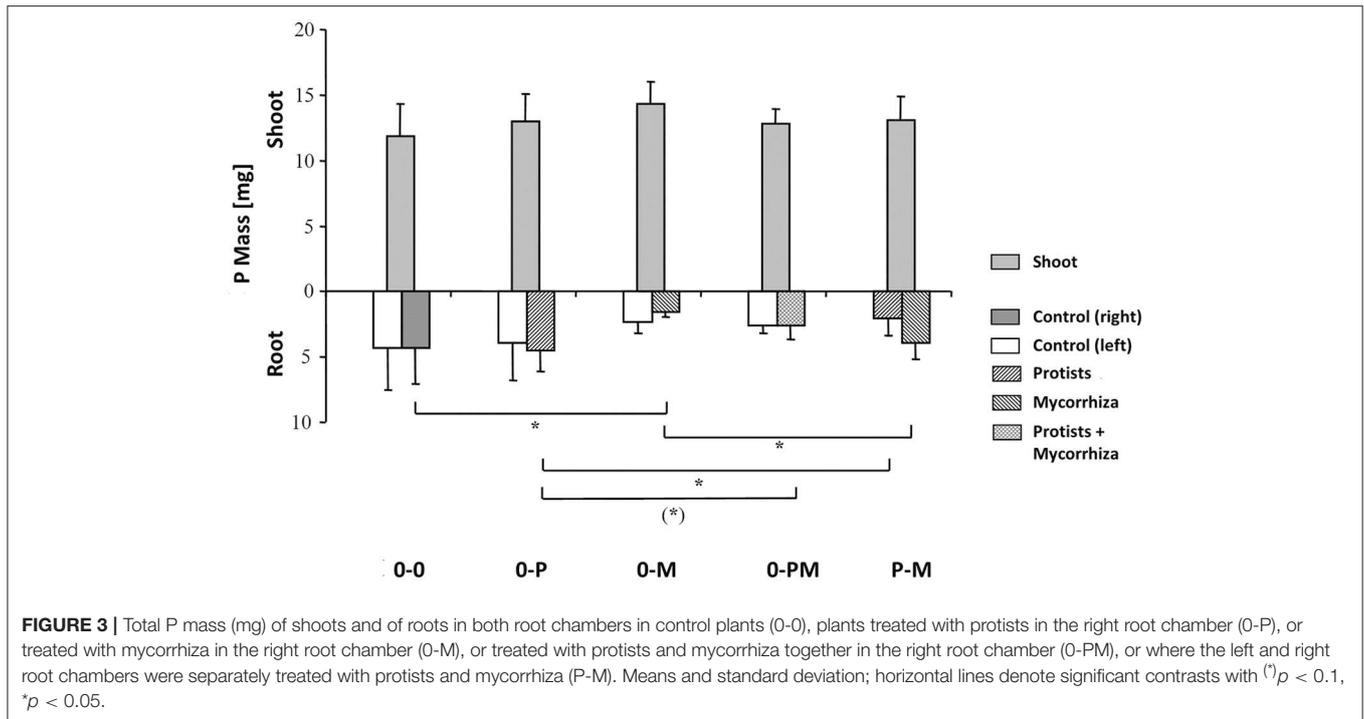


TABLE 3 | F - and P -values of GLM contrasts showing treatment effects on the total shoot P mass, total root P mass, total plant P mass, and shoot-to-root P mass of *Triticum aestivum*.

Comparison	Shoot P mass		Total root system P mass		Total plant P mass		Shoot-to-root P mass	
	$F_{(1,19)}$	P	$F_{(1,19)}$	P	$F_{(1,19)}$	P	$F_{(4,19)}$	P
0-0 vs. 0-P	0.71	0.410	0.02	0.878	0.20	0.663	0.01	0.937
0-0 vs. 0-M	0.30	0.587	9.21	0.007	1.90	0.186	5.50	0.031
0-M vs. 0-PM	1.61	0.219	0.79	0.385	0.00	0.950	1.84	0.192
0-M vs. P-M	1.73	0.204	1.84	0.192	0.26	6.150	3.68	0.072
0-P vs. 0-PM	0.01	0.935	3.96	0.062	3.56	0.076	1.14	0.299
0-P vs. P-M	0.00	0.975	2.32	0.145	1.72	0.207	0.26	0.618

Treatments: control plants (0-0), root halves treated with protists (0-P), with mycorrhiza (0-M), with protists and mycorrhiza together (0-PM), or where the left and right root compartments were separately treated with protists and mycorrhiza (P-M). Nominator and denominator degrees of freedom in brackets. Significant effects $p < 0.05$ in bold; $p < 0.1$ in italics.

0.001; 0-P vs. P-M: $F_{(1,23)} = 23.40$; $p < 0.001$]. At the whole plant level the C:N ratio increased from 46 in the 0-P treatment to 70 and 66 in the 0-PM and P-M treatments, respectively (Table 4).

The N sink effect of the combined treatments with mycorrhiza and protists was also enhanced in comparison to the 0-M treatment. The root C:N ratio was particularly enhanced in the protist + mycorrhiza chamber of the 0-PM treatment (74) as compared to the mycorrhiza chamber of the 0-M treatment [53; $F_{(1,23)} = 10.41$; $p = 0.004$], and also tended to be higher in the mycorrhiza chamber of the P-M treatment [65; $F_{(1,23)} = 3.49$; $p = 0.075$].

Microbial Biomass and Nutrient Limitation of Microbial Growth

Microbial biomass in this experimental set up was almost exclusively bacterial biomass. Its measurements may allow to

detect differences in biomass pools (e.g., by protist grazing), C release (basal respiration), and microbial C turnover (specific respiration). Microbial biomass in the protist chamber of the 0-P treatment tended to be reduced by 9% as compared to the control (0-0), although the effect was only marginally significant [$F_{(1,21)} = 3.07$; $p = 0.09$]. There were no treatment effects on microbial basal respiration and specific respiration (data not shown).

Steeper slopes of microbial growth after nutrient addition reflect limitation of microbes and of plant growth by this particular nutrient. Slopes of microbial growth after CN addition were 30-fold higher than slopes of microbial growth after CP addition [$F_{(1,91)} = 692.6$, $p < 0.001$], showing a strong N limitation of microbial growth in our experimental soil. Compared to the 0-P treatment, microbial growth increased by 34% after CN addition in the protist chamber of the 0-PM

TABLE 4 | *F*- and *P*-values of GLM contrasts showing treatment effects on the C:N ratio of *Triticum aestivum* shoots, the total root system, and of whole plants.

Comparison	Shoot C:N ratio		Total root system C:N ratio		Total plant C:N ratio	
	<i>F</i> _(1,19)	<i>P</i>	<i>F</i> _(1,19)	<i>P</i>	<i>F</i> _(1,19)	<i>P</i>
0-0 vs. 0-P	0.63	0.4380	0.05	<i>0.8260</i>	0.06	0.8160
0-0 vs. 0-M	0.21	0.6550	25.35	0.0001	8.37	0.0097
0-M vs. 0-PM	1.92	0.1820	9.05	0.0070	4.76	0.0426
0-M vs. P-M	0.81	0.3810	2.82	0.1090	2.05	0.1690
0-P vs. 0-PM	0.04	0.8470	69.03	0.0001	26.74	0.0001
0-P vs. P-M	0.10	0.7510	47.59	0.0001	19.12	0.0004

Treatments: control plants (0-0), root halves treated with protists (0-P), with mycorrhiza (0-M), with protists and mycorrhiza together (0-PM), or where the left and right root compartments were separately treated with protists and mycorrhiza, respectively (P-M). Nominator and denominator degrees of freedom in brackets. Significant effects $p < 0.05$ in bold; $p < 0.1$ in italics.

treatment [$F_{(6,22)} = 5.16$, $p = 0.034$], indicating a much stronger N-limitation in PM as compared to P. Also compared to the 0-M treatment, there was a tendency of increased growth after CN addition (+26%) in the protist + mycorrhiza chamber of the 0-PM treatment [$F_{(6,22)} = 3.88$, $p = 0.062$], suggesting an even stronger N-limitation in PM as compared to M. There were no treatment effects on microbial growth after CP addition (data not shown).

PLFA Profiles

Since PLFA profiles reflect the composition of microbial membrane lipids they are highly sensitive to detect changes in microbial community composition. DFA clearly separated the PLFA profiles of the 0-0, 0-M, and 0-P treatments (Figure 4); mycorrhiza and in particular protists led to strong and contrasting shifts in microbial PLFA patterns as compared to the control. The first axis of the DFA represented 77.1% of the variation and correlated closely with short chain branched fatty acids typical for Gram positive bacteria (i15:0, a15:0, 15:0, i16:0; Table S1). It clearly separated the 0-P treatment from the 0-M and 0-0 treatments, with the PLFA patterns of the combined treatments with mycorrhiza and protists (0-PM and P-M) being more similar to the 0-P treatment. The second axis represented 12.2% of the variation and clearly separated 0-P and 0-M treatments from the control (0-0) and the combined treatments (0-PM, P-M). The second axis correlated with fatty acids typical for Gram negative bacteria (cy19:0; Table S1).

Notably, as indicated by DFA, bacterial communities between different root chambers within the same treatment did not differ significantly except for the protist and mycorrhiza chambers of the P-M treatment ($p < 0.05$; Figure 4). The protist chamber contained fewer fatty acids typical for Gram positive bacteria than the chamber without protists. Specifically, these fatty acids were significantly reduced in the 0-P as compared to the control (0-0) treatment, and both combined treatments had significantly lower levels of these fatty acids than the MYC treatment (Figure 5).

Treatment Performance

In the 15 root chambers treated with *A. castelanii*, the amoebae reached densities of 16,700 (0-P, protist treated chamber),

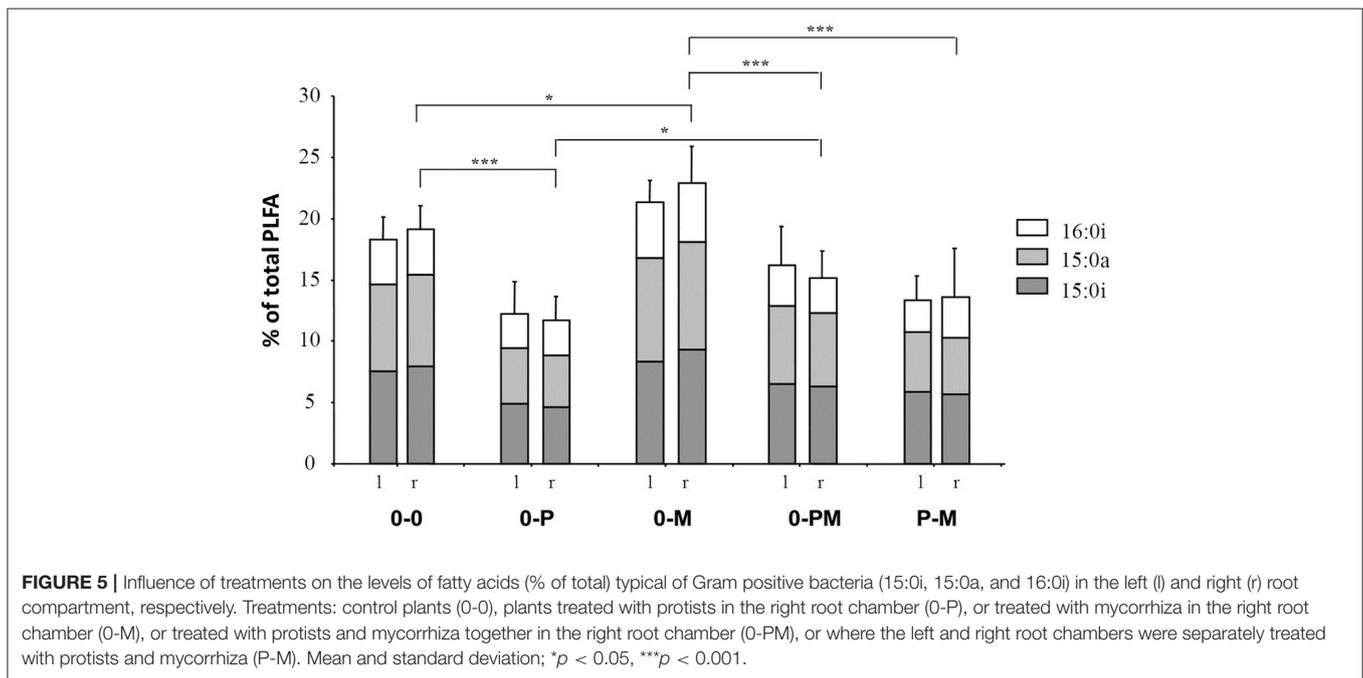
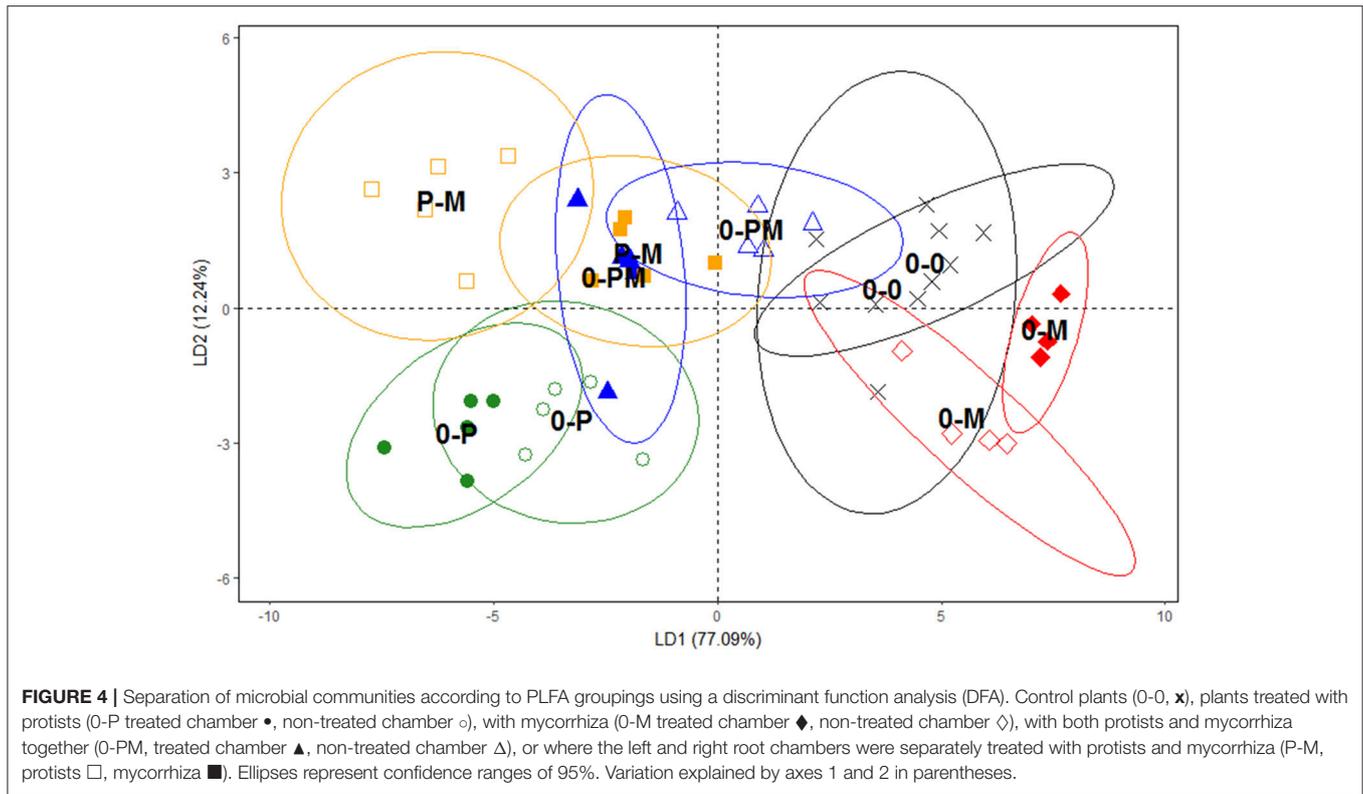
14,400 (0-PM, protist+mycorrhiza treated chamber) and 10,200 ind. g^{-1} dw soil (P-M, protist treated chamber; Figure S1). One replicate of the 0-M treatment was discarded from the statistical analyses as it was contaminated by protists.

Unfortunately, the determination of root colonization by mycorrhiza failed because the roots disintegrated after boiling in KOH. However, mycorrhiza in this experiment is a treatment factor and not treated as a continuous variable. The clear treatment effects by mycorrhiza on plant performance leave no doubt that mycorrhization was successful.

DISCUSSION

We hypothesized that both, mycorrhiza and protists, would show complementary functions for plant growth, comparable to findings from tripartite symbioses between rhizobia, mycorrhiza and their host plants (Kafle et al., 2018). As expected, mycorrhiza and protists, when inoculated separately, had opposite effects on plant N limitation with fundamental consequences for plant stoichiometry and shoot-to-root allometry. High shoot biomass relative to roots is an important plant trait in modern wheat cultivars (Siddique et al., 1990; Slafer and Araus, 2007). Conform to earlier studies (Veresoglou et al., 2012b) and our hypothesis, mycorrhiza increased plant shoot-to-root biomass ratio and shoot-to-root N mass ratio almost 3-fold, and shoot-to-root P mass ratio almost 4-fold. In part this was due to reducing root biomass (−38%), root N mass (−58%), and P mass (−55%), however, shoot biomass was not significantly affected by mycorrhiza. Split root experiments with barley already described *R. irregularis* as a strong C-sink for its plant hosts (Lerat et al., 2003). The strong reduction of root biomass, despite unchanged shoot biomass of wheat indicates that mycorrhiza indeed partly replaced root system function as hypothesized. It seems that plants preferentially invested in the shoot system even though root biomass and root nutrient levels were strongly reduced by mycorrhiza.

We further hypothesized that mycorrhiza would increase the P content of their plant hosts, but complemented by protist grazers of bacteria (Trap et al., 2016), we expected mycorrhiza to capture and transport N released by protist predation to the plant (Koller et al., 2013b; Trap et al., 2016). Mycorrhiza did



not increase plant P uptake, but despite reduced root biomass, mycorrhiza infection kept whole plant P uptake constant. Interestingly, mycorrhiza caused a translocation of P from roots to shoots. This was confirmed by the lower N:P ratio of shoots, the strongly reduced mass of P in roots, but unaltered total mass of plant P in the 0-M treatment. It has been argued

that replacement of root system function by mycorrhiza might be more economic to plants than investment into own roots (Fitter, 1991; Hodge, 2004). The reduction of root biomass indeed indicates a replacement of root system function by mycorrhiza, but at the same time mycorrhiza caused a severe reduction of total plant N (−32%). The reduced plant N levels show that

a replacement of roots by mycorrhiza was not beneficial to the plants at N-limiting conditions. Rather, the results suggest that the plants partly lost control over root system function—a symbiont strategy to make itself indispensable according to microbial market theory (Werner et al., 2014). Likely the fungus diverted N for its own investment, e.g., into chitin polymers, for the establishment of an extraradical hyphal network (Herdler et al., 2008; Hodge and Fitter, 2010).

In contrast to AMF, protists are known to increase N availability to plants by releasing significant amounts of NH_4^+ from consumed microbial biomass, a mechanism known as microbial loop (Clarholm, 1985; Bonkowski and Clarholm, 2012). Such changes in nitrogen form can feed back on mycorrhizal development (Giovannetti et al., 2017; Bukovská et al., 2018; Mollavali et al., 2018). Corresponding to the N-limiting experimental conditions, the increase in total plant N uptake (+22%) in the 0-P treatment corresponded to a 24% increased plant biomass as compared to the control (0-0). However, the protist effect on plant N nutrition was mainly due to a local increase in N contents in the protist chamber of the 0-P treatment (+52%). Further, contrasting the effect of mycorrhiza, protists did not change the shoot-to-root biomass ratio, suggesting that they stimulated above- and belowground growth in a balanced way.

In soils mycorrhiza and protists always co-occur and therefore their individual effect on plants is difficult to study. By manipulating them independently from each other, our split root design not only allowed deeper insight into their individual effect but also their interactions. The positive influence of AMF on plant growth primarily manifests as an improvement of P status against a background of P limitation (Nagy et al., 2009; Smith F. A. and Smith, 2011). Thus, from the plant's perspective, mycorrhiza, and protists seem complementary by respectively improving its P and N status. According to the functional equilibrium model (Mooney, 1972; Johnson et al., 2003), plants should allocate more biomass to those specific structures and rhizosphere symbionts that best garner the limiting resource (Revillini et al., 2016). Assuming that plants are under full control of the mycorrhiza symbiosis (Smith S. E. and Smith, 2011), plants would gain most benefit by investing in the protist rather than the mycorrhizal partner under N limiting conditions, as shown by increased plant biomass in the 0-P treatment. Recent studies indicated that *R. irregularis* is unable to compete for N mineralized by bacteria, but effectively captures N released from consumed microbial biomass by protist grazers (Koller et al., 2013a,b). Here we showed that AMF, although specialized for efficient P foraging, also gain control over N. It has been widely speculated why AMF hyphae proliferate in organic patches, despite being unable of a direct uptake of organic N (Hodge and Storer, 2014). The significantly stronger microbial N limitation in the mycorrhiza + protist chamber of the 0-PM treatment as compared to the protist chamber of the 0-P treatment indicates that mycorrhiza efficiently captured N mobilized by protists (Herdler et al., 2008; Koller et al., 2013b). This finding positions mycorrhiza as a critical player in the microbial loop (Bukovská et al., 2018).

However, plants did not benefit from the interaction between protists and mycorrhiza as indicated by the absence of plant

growth promotion as compared to the 0-P treatment, and the widest C:N ratio of shoots and roots in the 0-PM as compared to all other treatments. Evidently the N released by protists was not delivered to the plant host, since plant N contents in the 0-PM and P-M treatments were as low as in the 0-M treatment. The mycorrhiza effects on plants clearly dominated in the combined treatments with mycorrhiza and protists (0-PM, P-M), suggesting that mycorrhiza, at least in part, took over control of the interactions—a crucial prerequisite for the equitable exchange of resources in mutualisms (Werner et al., 2014).

Remarkably, allocation of resources into roots considerably changed in the P-M treatment where protists and mycorrhiza were spatially separated. According to the 0-P and 0-M treatments one would expect a narrow C:N ratio and increased root growth in presence of protists, and a wide C:N ratio and reduced root growth in presence of mycorrhiza. Strikingly, the opposite pattern was found. Protists generally had a positive effect on root proliferation as observed here in the 0-P treatment and earlier (Bonkowski, 2004; Krome et al., 2010), but the AM fungus is a strong C sink and likely thereby root allocation was translocated to the mycorrhiza chamber in the P-M treatment. In addition, mycorrhiza altered N allocation within roots in a systemic way. Root system architecture is highly sensitive to different forms of nutrients, particularly N (Tian et al., 2014). Mycorrhiza generally reduced root N mass and root concentration of N, but in the 0-M treatment root N mass was also reduced in the non-mycorrhiza chamber of the split-root containers. This was reversed in the P-M treatment, where the doubling of N uptake in the mycorrhiza chamber as compared to the protist compartment suggests that N taken up by roots in the protist chamber was subsequently translocated to the mycorrhiza chamber. Taken together, the provisioning of roots by N through the microbial loop in the protist chamber was coupled with a systemic shift of N allocation to mycorrhiza in P-M. At the same time, also root proliferation shifted to the mycorrhiza chamber, potentially resulting from of a systemic protist effect on root growth. The combination of these effects caused the observed wide C:N ratio of roots in the protist chamber and the shift of root allocation within root systems in the P-M treatment. Importantly, the synergistic effects of protists and mycorrhiza in the P-M treatment could not have been predicted from the individual effects of protists and mycorrhiza in the 0-P and 0-M treatments. Rather, the interactions suggest that mycorrhiza as the root-infecting symbiont at least in part took over control of the tripartite interaction for diverting resources to its own benefit.

Mycorrhiza and Protists as Drivers of Rhizosphere Microbial Community Composition

In recent years much has been learned how plants shape their root-associated soil and its inhabiting microorganisms (Berg and Smalla, 2009; Hartmann et al., 2009). It is assumed that soil type and rhizodeposits act as environmental filters for the enrichment of specific microorganisms (Bais et al., 2006; Bulgarelli et al., 2012; Schreiter et al., 2014), while host genotype-dependent

fine tuning at the rhizoplane further selects for specific root-associated microbial consortia (Edwards et al., 2015; van der Heijden and Schlaeppi, 2015). Our results partly challenge this plant-centered view of community assembly, because the unique PLFA groupings in the 0-P and 0-M treatments as compared to the control (0-0) suggest fundamentally different effects of protist grazers and mycorrhiza on bacterial community assembly in the wheat rhizosphere. Also previous studies support this rhizosphere symbiont view of structuring rhizosphere microbial communities by documenting that both protists through top-down control (Kreuzer et al., 2006; Rosenberg et al., 2009) and AMF through bottom-up modifications of resources alter bacterial communities in the rhizosphere (Marschner and Baumann, 2003; Rillig et al., 2006; Bukovská et al., 2016; Rodríguez-Caballero et al., 2017). Since protist grazing is highly selective, preferential grazing together with competitive shifts in bacterial community composition are likely responsible for changes in root-associated bacterial communities (Jousset et al., 2008; Jousset, 2012; Flues et al., 2017). The shifts in PLFA pattern by mycorrhiza and protists support our general hypothesis on differences in bacterial community composition. However, the fact that the non-treated chambers of the split root containers displayed the same PLFA patterns as the treated chambers indicates that the effects of protists and mycorrhiza were communicated by the roots to the chamber not colonized by these symbionts. This supports the view that root symbionts exert systemic effects on rhizosphere community assembly (Marschner and Baumann, 2003; Marschner and Timonen, 2005; Veresoglou et al., 2012a) and that this also applies to the studied protists. Interestingly, microbial communities in the mycorrhiza chamber of the P-M and 0-M treatments were most different, indicating that in addition to mycorrhiza, protists may have altered microbial community assembly in the myco-rhizosphere in a systemic way (Bukovská et al., 2018). Implications of systemic root-responses to protists and mycorrhiza would be far reaching, indicating an adjustment of the root response via changing the quantity and quality of root exudates (Benizri et al., 2002, 2007; Jones et al., 2004). Such responses however, need to be confirmed in subsequent studies by gene expression studies of roots and their metabolites.

The strong effect of protists on the rhizosphere microbial community was unexpected considering the dominance of mycorrhiza on shoot-to-root allometry and plant nutrient uptake. The discovery that the genome of *A. castellanii* harbors genes for auxin biosynthesis as well as for free-auxin deactivation via formation of IAA conjugates led Clarke et al. (2013) to suggest that *A. castellanii* directly manipulates the root architecture of plants. Certainly, more research on how protist-root and protist-mycorrhiza interactions alter root allometry, and the assembly and functioning of the plant microbiome is needed. Split-root experiments as used in the present study provide an essential tool to uncover systemic root responses and their modulation by rhizosphere symbionts (Bonkowski, 2018).

CONCLUSIONS

Results of the present study provide compelling evidence for a tight functional coupling of the microbial loop and nutrient

foraging by AMF. Mycorrhiza markedly altered plant shoot-to-root allometry by reducing root biomass of wheat, and it partly took over root system functioning. Notably, the shoot system responded little to alterations in the root system and rhizosphere community composition, indicating that the plants optimized shoot growth despite varying investment into roots. Protists increased plant growth and N uptake via the microbial loop, but this effect vanished in combined treatments with mycorrhiza, suggesting that mycorrhiza gained control over N provided by protist grazers. Combined effects of protists and mycorrhiza revealed systemic effects on within root system biomass allocation and root N allocation of winter wheat, and on rhizosphere microorganisms. Structuring rhizosphere communities by plants (roots) and root allocation thereby represent emergent properties that could not be predicted from individual treatments with mycorrhiza or protists alone. Notably, protists and mycorrhiza altered rhizosphere microbial communities in contrasting ways and these changes were systemically conveyed to the root side where the symbionts were lacking. The tight plant and microbial feed backs have far reaching implications for understanding the assembly of plant microbiomes, and testify central roles of both protists and mycorrhizas in the assembly process.

AUTHOR CONTRIBUTIONS

MB and SS conceived and designed the experiment. GH and MB designed the split root containers. GH performed the experiment and measurements after harvest, except the PLFA analyses that were carried out by SM and EK. Statistical analyses were performed by GH and MB drafted the manuscript. All co-authors contributed to the interpretation of the data and significantly improved the manuscript by critical revisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2018.00117/full#supplementary-material>

Figure S1 | Numbers of amoebae in soil (ind. g^{-1} soil dry wt) of protist-inoculated root-chambers at the end of the experiment. No protists were detected in non-inoculated chambers, except in one replicate of the 0-M treatment, which was excluded from all analyses. Differences between treatments were not statistically significant.

Figure S2 | Cumulative average requirements of water (g) of plants in split-root microcosms until 32 days after the set up of the experiment, measured by weighing the water supply bottles attached to each microcosm. Treatments: control plants (0-0), plants treated with protists in one root chamber (0-P), or treated with mycorrhiza in one root chamber (0-M), or treated with protists and mycorrhiza together in one root chamber (0-PM), or where the root chambers were separately treated with protists and mycorrhiza (P-M).

Figure S3 | Concentration of N (%) in shoots and in roots of both root chambers in control plants (0-0), plants treated with protists in the right root chamber (0-P), or treated with mycorrhiza in the right root chamber (0-M), or treated with protists and mycorrhiza together in the right root chamber (0-PM), or where the left and right root chambers were separately treated with protists and mycorrhiza (P-M). Means and standard deviation; horizontal lines denote significant contrasts with (*) $p < 0.1$, * $p < 0.05$, *** $p < 0.001$.

Figure S4 | Concentration of P (%) in shoots and in roots of both root chambers in control plants (0-0), plants treated with protists in the right root chamber (0-P), or treated with mycorrhiza in the right root chamber (0-M), or treated with protists and mycorrhiza together in the right root chamber (0-PM), or where the left and right root chambers were separately treated with protists and mycorrhiza (P-M). Means and standard deviation; horizontal lines denote significant contrasts with (*) $p < 0.1$, * $p < 0.05$.

Table S1 | Correlation of single PLFAs with the major axes of the DFA (**Figure 4**). Significant correlations ($p < 0.05$) are shown in bold.

Table S2 | *F*- and *P*-values of GLM contrasts showing treatment effects on N concentration (%) of shoots, N (%) of the treated root chambers inoculated with protists or/and mycorrhiza, N (%) of the whole root system, N (%) of the whole plants, and treatment effects on the total use of water of *Triticum aestivum*. Treatments: control plants (0-0), root halves treated with protists (0-P), with mycorrhiza (0-M), with protists and mycorrhiza together (0-PM), or where the left and right root compartments were separately treated with protists and mycorrhiza (P-M). Nominator and denominator degrees of freedom in brackets. Significant effects $p < 0.05$ in bold; $p < 0.1$ in italics.

Table S3 | *F*- and *P*-values of GLM contrasts showing treatment effects on P concentration (%) of shoots, P (%) of the treated root chambers inoculated with protists or/and mycorrhiza, P (%) of the whole root system, P (%) of the whole *Triticum aestivum* plants. Treatments: control plants (0-0), root halves treated with protists (0-P), with mycorrhiza (0-M), with protists and mycorrhiza together (0-PM), or where the left and right root compartments were separately treated with protists and mycorrhiza (P-M). Nominator and denominator degrees of freedom in brackets. Significant effects $p < 0.05$ in bold; $p < 0.1$ in italics.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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