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# Protozoa stimulate N uptake and growth of arbuscular mycorrhizal plants

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ABSTRACT

Roots of most terrestrial plants interact with arbuscular mycorrhizal (AM) fungi and soil protozoa with each individually increasing nitrogen (N) supply to plants and plant growth. We investigated if AM fungi and protozoa interactively affect plant N acquisition and growth. We used stable isotopes ($^{15}$N, $^{13}$C) to assess plant N uptake, C fixation and C partitioning. Shifts in the microbial community structure were evaluated by analysing phospholipid fatty acids. Protozoa and AM fungi enhanced plant N uptake. Protozoa mobilized N from bacterial biomass and AM fungi increased the translocation of the mobilized N to the host plant; if both were present, plant biomass, C fixation and C allocation to roots increased twofold. Protozoa alone and in the presence of AM fungi altered microbial activity and community structure in the rhizosphere. The results indicate that plant N uptake *via* mycorrhizal symbiosis strongly benefits from the presence of protozoa.



## 1. Introduction

Arbuscular mycorrhizal (AM) fungi increase the fitness of plants by improving access to growth limiting soil nutrients such as phosphorus (Smith and Read, 2008). In exchange AM fungi receive plant carbon (C) and in turn benefit from enhanced plant growth (Bago et al., 2000; Jakobsen and Rosendahl, 1990). In addition to phosphorus, AM fungi transport nitrogen (N) to their host plants which they in part acquire from decomposing litter material (Hodge et al., 2001; Hodge and Fitter, 2010; Leigh et al., 2009; Tu et al., 2006). However, the mechanisms by which AM fungi capture N from organic matter are little understood as they are unlikely to decompose litter themselves (Herman et al., 2012; Smith and Read, 2008). Rather, AM fungi presumably rely on saprotrophic microorganisms mobilizing N from litter for hyphal uptake (Herman et al., 2012). Indeed, AM fungi affect microbial community composition and thereby may increase litter decomposition (Andrade et al., 1997; Barea et al., 2002; Marschner and Baumann, 2003; Scheublin et al., 2010).

Protozoa are ubiquitous in soil (Finlay, 2002). By feeding on bacteria they mobilize mineral N from bacterial tissue thereby making it available to plants (Kuikman and Van Veen, 1989). The 'microbial loop in soil' concept assumes that by fostering plant growth protozoa benefit from increased allocation of plant C belowground (Bonkowski and Clarholm, 2012; Krome et al., 2009). In addition to increasing plant nutrient supply, protozoa alter plant growth by selective feeding on microorganisms thereby altering the community composition of rhizosphere microorganisms (Bonkowski, 2004; Rosenberg et al., 2009).

Despite the ubiquity of AM fungi and protozoa and their importance for plant nutrition and growth, only few studies investigated their interactions. Most studies which included both AM fungi and protozoa focused on AM fungi-mediated changes in the density of protozoa (Rønn et al., 2002a; Vestergard et al., 2008; Wamberg et al., 2003). Using axenic AM fungal and protozoan cultures Herdler et al. (2008) showed that protozoa modify the functioning of the AM fungus – plant symbiosis. However, it remains elusive if alterations in AM fungi–plant interactions in presence of protozoa are based on protozoa-mediated changes in nutrient mobilization from OM in the root zone of plants.

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We tested the following hypotheses: (1) AM fungi and protozoa enhance plant N nutrition from litter *via* protozoa mobilizing N from consumed bacterial biomass and AM fungi transferring the mobilized N to plant roots. (2) Being an obligate biotroph, AM fungi promote the allocation of plant C to roots; this is fostered by protozoa-mediated N mobilization from litter and subsequent transfer of the mobilized N *via* AM fungi to plants. (3) The interaction between AM fungi, protozoa and plant roots changes the size, activity and structure of the rhizosphere microbial community.

We established a model laboratory system combining natural bacterial communities with axenic cultures of protozoa and AM fungi in order to dissect the interaction between plant roots, AM fungi and protozoa. We focused on the initial phase of litter decomposition because at this phase bacteria predominate (as compared to saprotrophic fungi) and strongly interact with AM fungi (Leigh et al., 2011) and protozoa (Bonkowski and Clarholm, 2012; Bonkowski et al., 2000; Rønn et al., 2002a). Using $^{15}N$ labelled litter we followed the flux of N into plants and its modification by AM fungi and protozoa. To follow plant C allocation to roots and its modification by AM fungi and protozoa plants were labelled with $^{13}CO_2$. Shifts in the microbial community structure due to grazing of protozoa and presence of AM fungi were assessed by phospholipid fatty acid analysis.

## 2. Materials and methods

### 2.1. Soil, microcosms and microorganisms

The experimental soil was taken from the uppermost 20 cm of grassland (Hedlund et al., 2003). The soil was stored in plastic bags at 4 °C for four weeks until use. The soil was mixed with sand at a ratio of 1:1 to reduce nutrient concentrations, autoclaved (20 min, 121 °C) and washed with a threefold volume of tap water in order to deplete nutrients and toxic compounds mobilized by autoclaving. The soil-sand mixture contained 5.02 g $kg^{-1}$ organic carbon, 0.33 g $kg^{-1}$ total N, 0.07 g $kg^{-1}$ phosphorus, and 0.037 g $kg^{-1}$ potassium, and had a pH of 6.66.

$^{15}N$ labelled *Lolium perenne* L. shoots (45.2 atom% $^{15}N$, C-to-N ratio 15.1) were milled and mixed with milled non-labelled *L. perenne* shoots (C-to-N ratio 16.5) to obtain litter containing about 10 atom% $^{15}N$. From the litter 0.47 g was mixed homogeneously with 780 g dry weight of autoclaved soil-sand mixture and the material dried to approx. 50% of the water holding capacity. Then, it was transferred into microcosms consisting of transparent twist off glass jars with a volume of 660 ml (Parisienne de Verreries, Orly, France). The soil in the jars was compacted leaving about 2 cm space underneath the lid. The lid contained an opening allowing plants to grow through and ensuring gas exchange. The opening was closed with hydrophobic cotton wool to avoid contaminations from airborne cysts of protozoa (supplementary information Figs S1–3). Subsequently, the jars were autoclaved and the soil moisture content was adjusted to 75% of the water holding capacity.

The experiment was set up in a full factorial design with protozoa and AM fungi as treatments with nine replicates each. Prior to establishment of the treatments the jars were inoculated with bacteria. The inoculum was prepared by suspending 200 g fresh weight of rhizosphere soil taken from grassland on the campus of the Faculty of Biology, Darmstadt University of Technology, Germany, in 300 ml Neff's Modified Amoebae Saline (NMAS; Page 1976). The slurry was filtered though paper filters (595½ Schleicher and Schuell, Dassel, Germany) to remove organic and mineral particles. Then, the filtrate was passed successively through 5 and 1.2 μm filters to exclude protozoa and fungi (Bonkowski and Brandt, 2002). The filtrate was stored in a culture flask (Nunc, Roskilde, Denmark) at 15 °C and checked for contamination with protozoa and fungi using an inverted microscope for seven days prior to use (×200 magnification; Leitz, Wetzlar, Germany). Each jar received 6 ml of this filtrate. Protozoa treatments further received 2 ml of axenic *Acanthamoeba castellanii* (Douglas) containing ca. 95,000 individuals, equivalent to ca. 100 cells $g^{-1}$ soil in sterile filtered mineral water. The amoebae were taken from cultures established with specimens isolated from woodland soil (Bonkowski and Brandt, 2002) kept axenically in sterile Peptone Glucose Yeast medium (1% peptone, 1% glucose, 0.5% yeast; VWR, Darmstadt, Germany; cf. Rosenberg et al., 2009). Prior to inoculation, *A. castellanii* cells were washed twice with mineral water and sedimented at 800 rpm for 5 min. Non-protozoan treatments received 2 ml of sterile filtered mineral water.

### 2.2. Plants and incubation procedure

Seeds of *Plantago lanceolata* L. (Appels Wilde Samen GmbH, Darmstadt, Germany) were surface sterilized (Hensel et al., 1990) and germinated individually in 96-well microtitre plates filled with 100 μl sterile nutrient broth (NB, Merck, Darmstadt, Germany) in NMAS at 1:9 v:v (NB-NMAS). The microtitre plates were incubated in darkness at 20 °C and checked for microbial contamination. Eight days after germination plants were transferred into sterile polypropylene tubes (diameter 14 mm, length 20 mm; see Fig. S1) filled with quartz sand under sterile conditions. Plants of the mycorrhiza treatments were inoculated with agar pieces of approximately 27 $mm^3$ containing spores and mycelium of the AM fungus *Glomus intraradices* Schenk cultivated axenically (Bago et al., 1996; Mycovitro S.L. Biotechnología Ecológica, Granada, Spain). After five days the tubes were placed on the surface of the soil in the jars (see Fig. S1) and the microcosms incubated in a plant growth chamber at 18/22 °C night/day temperature, 70% humidity, 16 h light at $460 \pm 80$ μmol $m^{-2}$ $s^{-1}$ light photon flux density in the PAR range at plant level. Soil moisture was checked gravimetrically and kept at 75% of the water holding capacity by adding sterile distilled water every second day through a sterile filter (pore size 0.2 μm; Sartorius, Göttingen, Germany; see Figs S1–3).

### 2.3. $^{13}CO_2$ pulse labelling and stable isotope analyses

Thirty days after transplantation of the seedlings the microcosms were transferred into an assimilation chamber for pulse labelling with $^{13}CO_2$ (Robin, 2006). Temperature and light conditions during the labelling period were similar to those in the plant growth chamber (see above). The $CO_2$ concentration in the chamber was reduced by 50% to 180 μl $l^{-1}$ and re-adjusted to 360 μl $l^{-1}$ by a pulse of $^{13}CO_2$ by adding 1 M lactic acid to $NaH^{13}CO_3$ (99 atom %). During the 5 h labelling period, the $CO_2$ concentration in the chamber was kept constant at 360 μl $l^{-1}$ by adding $NaH^{13}CO_3$ (50 atom%) to 1 M lactic acid. Belowground respiration was measured during the first 48 h after labelling by passing $CO_2$ free air through the microcosms and into a trap with 60 ml 1 M NaOH.

Total C concentration in NaOH was measured using a TOC analyser (TOC-VCSH CSH/CNS, Shimadzu, Champs-sur-Marne, France). $^{13}C$ isotopic excess of the trap was determined after precipitation of carbonates in saturated $SrCl_2$ (Harris et al., 1997) and centrifugation. The supernatant was decanted and the pellet was freeze-dried before $^{13}C$ analysis by an elemental analyser coupled with an isotope mass spectrometer (see below).

Plants were sampled four days after labelling at the end of the vegetative growth stage ensuring that plants had taken up most of the nutrients in the system but did not start investing resources into reproductive structures. Subsamples of shoots and roots were freeze dried for biomass determination. Soil samples were dried at 80 °C for 48 h, milled and analyzed for total C and N, as well as isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) by an elemental analyzer