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Contrasting effects of microbial partners in the rhizosphere: interactions between Norway Spruce seedlings (*Picea abies* Karst.), mycorrhiza (*Paxillus involutus* (Batsch) Fr.) and naked amoebae (protozoa)

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Abstract

The importance of the soil microbial community for plant mineral nutrition and nutrient cycling has long been recognized. One of the most important interactions is the symbiosis of plants with mycorrhizas. In contrast, the effects of soil microfauna on plant performance have so far received little attention, although soil protozoa in particular, have been shown to beneficially affect plant growth. We investigated in a laboratory experiment the impact of mycorrhiza and protozoa and their interaction on plant performance. Spruce seedlings with or without the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr. were grown in microcosm chambers with defaunated forest soil with naked amoebae (*Acanthamoeba* sp.) or without protozoa for 10 months. The presence of protozoa resulted in the development of a more complex root system by increasing root length (51%), length of fine roots (64%) and number of root tips (43%). The effects of protozoa were more pronounced in the absence of mycorrhiza. In contrast to protozoa, the presence of mycorrhiza resulted in a less complex root system, i.e. root length, length of fine roots and number of root tips were reduced by 47, 47 and 40%, respectively. Shoot height, and stem, shoot and needle mass were at a maximum in the combined treatment with both mycorrhiza and protozoa. The presence of mycorrhiza and protozoa also affected plant nutrient concentrations. In treatments with protozoa shoots of spruce seedlings contained less nitrogen, leading, e.g. to an increased C/N ratio in needles. Conversely, in treatments with mycorrhiza concentrations of phosphorus in needles were increased by a factor of almost two. Mycorrhiza and protozoa also affected rhizosphere microorganisms. Microbial biomass was reduced in the presence of mycorrhiza, mainly due to a reduction in bacterial numbers. Conversely, in the presence of protozoa the length of hyphae in the rhizosphere was reduced. It is concluded that the plant–mycorrhiza mutualism and the bacteria-mediated mutualism between plants and protozoa (microbial loop) complement each other; plant resources presumably are allocated to optimize simultaneous exploitation of both mutualistic relationships. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Plants spend a high portion of their net primary production to fuel microbial interactions below ground. Fogel and Hunt (1983) estimated that trees translocate

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between 40 and 70% of the fixed carbon to the roots and that 30–90% of this carbon is lost by the plant due to rhizodeposition. Plants are often connected with more than one microbial partner, and therefore, should be ideal organisms to compare the mechanisms associated with infecting and non-infecting microbes in the rhizosphere. We, therefore, investigated two contrasting microbial belowground relationships: (a) between tree roots and the ectomycorrhizal fungus *Paxillus involutus* and (b) between roots, bacteria and protozoa. Both are driven by plant carbon allocated below ground, but are based on very different mechanisms.

The mycorrhizal symbiosis is considered as one of the most important mutualisms on earth since most terrestrial plant species are associated with either arbuscular or ectomycorrhizal fungi (Allen, 1991). In the symbiosis with mycorrhiza, the carbon allocated to roots is directly transferred to the fungal symbiont in exchange for nutrients, particularly phosphorus (Allen, 1991; Smith and Read, 1997; Deacon, 1997). Plant investment in this mutualistic relationship is high. The biomass of ectomycorrhiza in a coniferous forest has been estimated to be about 6% of that of the trees (Fogel, 1985) and the carbon cost of maintaining mycorrhizas has been estimated to be at least 10–15% of the total production of a tree (Söderström, 1992; Deacon, 1997).

In addition to the transfer of carbon to mycorrhiza, plants may release up to 40% of their photosynthetically fixed carbon as root exudates (Lynch and Whipps, 1990; Cheng et al., 1994, 1996), fueling a diverse spectrum of non-infecting rhizosphere microorganisms, mainly bacteria (Bååth et al., 1978; Norton and Firestone, 1991). It has been shown that protozoa feeding on rhizosphere bacteria enhance nitrogen mineralization and subsequently stimulate plant growth (Clarholm, 1985; Kuikman et al., 1990; Alphei et al., 1996; Bonkowski et al., 2000a, 2001). Theory suggests that due to the low C/N ratio of the microflora, bacterial-feeding protozoa excrete much of the ingested nitrogen, predominantly as ammonium (Stout, 1973), resulting in a high nutrient flow through protozoa (Griffiths, 1994).

Previously, we have shown that the growth of spruce seedlings with or without mycorrhiza was significantly enhanced in the presence of protozoa in sand culture systems, even when nutrients were added continuously in excess (Jentschke et al., 1995). Recent experiments on watercress also demonstrated that the presence of amoebae led to a greater and more branched root system. The changes resembled hormonal effects and were accompanied by an increase in the proportion of auxin-producing rhizosphere bacteria (Bonkowski and Brandt, unpublished). This indicates that there is another mechanism apart from grazing-associated nutrient release that adds to the indirect relationship between plants and rhizosphere protozoa. Both microbial interactions with plants, that with mycorrhizal fungi and that with protozoa, appear to complement each other in that the mycorrhizal symbiosis may facilitate uptake of relatively immobile nutrients such as P, while the interaction between plants and protozoa predominantly affects plant N acquisition. As both microbial relationships depend on the same energy source — photosynthetically fixed carbon — they may compete for carbon resources from the shared host plant.

This study investigates the response of plants to the presence of mycorrhiza, protozoa or both. By investigating plants with mycorrhiza and plants with protozoa separately and in combination we evaluated whether these microbial relationships in fact complement each other, i.e. whether there are synergistic effects. The laboratory system employed closely resembled field conditions; spruce seedlings were grown in soil from a spruce stand, i.e. under conditions where nutrients are in limited supply.

2. Materials and methods

F- and H-layer material were collected from the uppermost 5 cm of a spruce forest on sandstone in the Solling (Lower Saxony, Germany). The substrates were mixed, defaunated with chloroform (Alphei and Scheu, 1993) and then reinoculated with bacteria from a filtered (3 μm) soil suspension in order to reestablish microbial populations together with microbes originating from bacterial and fungal spores that survived chloroform fumigation.

Laboratory microcosms (cf. Bonkowski et al., 2000a) consisting of Perspex tubes (150 mm length, 60 mm diameter) fixed on ceramic plates were filled with sterilized quarz sand (0.1 mm particle size; 350 g) as drainage layer and defaunated F/H-layer substrate (15 g dry wt.) as organic layer (5 cm). Subsequently, the microcosms were kept in darkness at 15°C and

irrigated for 2 weeks with 30 ml sterile deionized tap water per day to leach excess nutrients mineralized as a result of the fumigation process.

Seeds of *Picea abies* Karst. were surface sterilized by incubation in 3% (w/w) H_2O_2 solution for 12 h followed by 45 min in 20% (w/w) H_2O_2. Then they were rinsed with sterile distilled water and germinated on water agar. The fungus *P. involutus* Batsch 533, isolated from a fruitbody collected at an acidified Norway spruce stand in northern Germany (Schlechte, 1986), was maintained on modified Melin–Norkrans medium (Marx, 1969). When seedlings were 3 weeks old, they were transferred into a sterile perlite culture system (modified after Bigg, 1981). The plants were inoculated with *P. involutus* or remained uninoculated and were grown for 12 weeks in perlite (for details see Marschner et al., 1996). Thereafter, they were transferred into the microcosms. Both non-mycorrhizal and mycorrhizal seedlings had similar dry weights.

Naked amoebae were isolated from the spruce forest soil and kept in culture on Petri dishes with Neffs Modified Amoebae Salinae (NMAS) and native soil bacteria as food source (Page, 1976). Before inoculation the protozoan cultures were pooled, centrifuged (4000 revolutions min^{-1}, 15 min) and washed twice in NMAS. An amount of 10 ml of the solution, containing about 600×10^3 amoebae (numbers obtained from direct counts of cysts and active amoebae in 5 μl) were added per microcosm. Non-protozoan treatments received 10 ml of the culture medium without protozoa.

2.1. Experimental design

The following treatments were set up in a two factorial design with six replicates each.

1. Control soil planted with spruce seedlings without mycorrhiza and protozoa (Ctrl).
2. Soil with spruce seedlings and mycorrhiza (Myc).
3. Soil with spruce seedlings and protozoa (Prot).
4. Soil with spruce seedlings, mycorrhiza and protozoa (Myc × Prot).

During the experiment the microcosms were placed in a temperature controlled room at 15°C, illuminated with a photosynthetically active photon flux density of 200 $\mu mol\ m^{-2}\ s^{-1}$ (Osram L 58 W 21), 16 h day length, and watered with 15 ml of sterile deonized tap water every 4 days. The microcosms were continuously drained using ceramic cup lysimeters and the leaching water was sampled throughout the experiment for further analyses. The experiment lasted for 10 months.

2.2. Sampling and analytical procedures

Leaching water samples of weeks 1–15, 16–30 and 31–44 were pooled and analyzed for NO^{3-}, $NH_4{}^+$, N_{org} and $PO_4{}^{3-}$. The other elements were analyzed from samples pooled for weeks 1–44. Concentrations of P, Ca, Mg, and Fe were determined by inductively coupled plasma atomic emission spectroscopy. Nitrogen ($NH_4{}^+$, $NO_3{}^-$, total N) were photometrically determined using standard auto analyzer procedures (Cenco Instrumenten B.V., Breda, The Netherlands). Organic N was calculated as total N − $NH_4{}^+$–N and $NH_3{}^+$–N. At the end of the experiment, plants were harvested and separated into needles, stems and roots. After washing from adhering soil, root length, root radius, the length of fine roots (diameter <0.5 mm) and the number of root tips were determined using a scanner (HP Scan Jet 4C) and the WinRhizo^{TM} image analysis software (Regent Instruments, Que., Canada). Subsequently, the plant material was dried to constant weight at 60°C. Both C and N contents of plant materials were determined using an elemental analyzer (Carlo-Erba, Na1500, Milan, Italy). For determination of other elements, plant material was wet ashed using 65% (w/w) HNO_3 in closed Teflon vessels under high pressure at 180°C. Concentrations of mineral elements were determined by inductively coupled plasma atomic emission spectroscopy.

Protozoan density was determined from 3 g of fresh soil by a most probable number (MPN) method (Darbyshire et al., 1974). Two-fold dilutions were made from the soil suspensions with eight replicates of each dilution. Protozoan density was calculated from the MPN data according to Rowe et al. (1977).

Microbial biomass was determined by substrate induced respiration (Anderson and Domsch, 1978) using an automated respirometer measuring O_2 consumption rates (Scheu, 1992). Glucose was added as aqueous solution to increase the water content to 2 ml g^{-1}. Samples were supplemented with

20 mg glucose g^{-1} dry wt. which induced maximum initial respiration rates. Readings were made every hour and from the lowest three measurements within the first 10 h the maximum initial respiratory response (MIRR) was calculated. Microbial biomass C (C_{mic}, $\mu g\, g^{-1}$) was calculated as $38 \times$ MIRR ($\mu l\, O_2\, g^{-1}\, h^{-1}$; Beck et al., 1997). Microbial basal respiration was also measured as O_2 consumption using the automated respirometer system. The average of the respiration rates of hours 10–20 after attachment of samples to the system was used.

Numbers of bacteria and length of fungal hyphae were measured by epifluorescence microscopy as outlined by Scheu and Parkinson (1994). Briefly, about 1 g (fresh weight) of F/H material was blended for 60 s in 100 ml sterile 1/4 strength Ringer solution. Aliquots from the solutions were then diluted and filtered through polycarbonate membranes to obtain ca. 1.0 and 0.5 mg of F/H material per membrane (201 mm^2) for measurement of bacterial numbers and hyphal lengths, respectively.

Suspensions diluted for bacterial counts were fixed in 2% formaldehyde and passed through 0.2 μm nucleopore membranes. Bacteria on membranes were stained by acridine orange. Membranes were inspected at 1000× magnification under a fluorescent microscope with appropriate filter combinations. Twenty fields per membrane were counted. Bacterial cell volume was estimated using a videocamera attached to the microscope and the Optimas image-analysis software. The mean cell volume of the treatments ranged between 0.241 and 0.323 μm^3 and did not differ between treatments. We, therefore, did not convert bacterial numbers to volume or biomass since bacterial numbers also represent these parameters.

Suspensions diluted for hyphal volume measurement were stained prior to filtering by adding (1:1) an aqueous solution of calcofluour white M2R (2.3 $g\, l^{-1}$). After 4 h of staining, 2 ml of the suspension was passed through 0.8 μm Millipore polycarbonate membranes. Hyphal length was measured at 400× magnification using the grid intersection method (Olson, 1950); 20 fields were inspected per membrane. Hyphal diameters were measured using the Optimas software system; means of treatments ranged between 2.61 and 2.80 μm and were not significantly different. We, therefore, did not convert hyphal length to volume or biomass since it also represents these parameters.

2.3. Statistical analyses

Data on leaching of N and P from the microcosms were analyzed by a multivariate repeated-measures analysis using Roy's greatest root followed by profile analysis (von Ende, 1993) with factors mycorrhiza (without, with) and protozoa (without, with) as independent variables and time (weeks 0–17, 17–22, and 22–41) as repeated-measures variable. Significance levels of contrast were corrected for the number of contrasts calculated using the Bonferroni method (von Ende, 1993). Other parameters were analyzed by univariate ANOVAs. The statistical analyses were performed using the ANOVA and GLM procedures in SAS 6.12 (SAS Institute, Cary, NC).

3. Results

3.1. Protozoa and microflora

At the end of the experiment 7×10^3 to 10×10^3 naked amoebae g^{-1} dry wt. were present which is low compared to the inoculum density of 40×10^3 ind. g^{-1} dry wt. During the experiment the microcosms were contaminated with flagellates, probably by cysts in the soil filtrate or from the air. Flagellate numbers in protozoan treatments (368×10^3 ind. g^{-1} dry wt.) were 30% lower compared to non-protozoan treatments (532×10^3 ind. g^{-1} dry wt.). However, no amoebae or ciliates were detected in control treatments, indicating that differences between protozoan and non-protozoan treatments were caused by amoebae. Interestingly, flagellate numbers were positively correlated with plant height in non-mycorrhizal treatments ($F = 12.2$, $P = 0.008$, $r^2 = 0.60$), but not in those with mycorrhiza ($F = 0.02$, $P = 0.91$, $r^2 = 0.002$).

Protozoa and mycorrhiza affected the composition of the microflora in opposite ways. The microbial biomass was significantly reduced in the presence of mycorrhiza (−10%) but only in the treatment without protozoa (Table 1). Direct counts indicated that this decline was mainly due to bacteria since bacterial numbers were significantly decreased by 38% in the presence of mycorrhiza. In contrast, the length of fungal hyphae decreased by 18% in the presence of protozoa. Basal respiration was unaffected by mycorrhiza and protozoa. There is a discrepancy between

Table 1
Effects of protozoa and mycorrhiza on microbial biomass, basal respiration, bacterial numbers and hyphal lengths in the rhizosphere of spruce seedlings[a]

	Microbial biomass ($\mu g\ g^{-1}$ dry wt.)	Basal respiration ($\mu g\ O_2\ h^{-1}\ g^{-1}$ dry wt.)	Numbers of bacteria ($10^9\ g^{-1}$ dry wt.)	Length of hyphae ($m\ g^{-1}$ dry wt.)
Means				
Ctrl	928	7.05	27.9	5700
Myc	836	6.81	16.1	5123
Prot	900	7.81	23.9	4624
Myc + Prot	931	7.40	16.2	4271
F-values				
Myc	1.5	1.2	6.5*	1.3
Prot	1.2	0.3	0.3	5.8*
Myc × Prot	4.9*	<0.1	0.3	<0.1

[a] Ctrl, control; Myc, with mycorrhiza; Prot, with protozoa; Myc + Prot, with both mycorrhiza and protozoa.
* $P < 0.05$.

measurements of microbial biomass and direct counts of microorganisms (Table 1). This difference likely results from measuring microbial biomass by a physiological method (microbial respiration) in comparison to direct counts of stained bacteria and fungi, since it is difficult to discriminate between active and inactive microorganisms by staining procedures.

3.2. Nutrients leached

Leaching of NH_4^+ and NO_3^- was high early in the experiment and fell to low levels in all treatments by the end of the experiment (Fig. 1). However, chambers with non-mycorrhizal seedlings inoculated with protozoa showed higher leaching rates than all other treatments, especially in the middle of the experiment (weeks 17–22) where NH_4^+ and NO_3^- leaching was seven- or five-fold that of controls without protozoa, respectively. Repeated-measures analyses revealed significant mycorrhizal effects and significant protozoa × mycorrhiza × time interactions (Table 2). Profile analysis showed that changes within the first 22 weeks of the experiment were responsible for the protozoa × mycorrhiza × time interaction. Leaching of organic nitrogen slightly but significantly decreased during the experiment but was not affected by treatments. Leaching of phosphate strongly decreased after week 17 in all treatments, reaching a minimum between week 17 and 22. Thereafter, it increased on average over all treatments by a factor of 2.5. However, mycorrhiza decreased leaching of P on average by ca. 40%, but the effect was statistically not significant (Table 2).

3.3. Nutrients in needles, stems and roots

Both mycorrhiza and protozoa significantly affected element concentrations in spruce seedlings (Table 3). The presence of mycorrhiza increased the concentration of phosphorus in roots, stems and needles on average by 40, 33 and 55%, respectively. However, the increase in P concentration in stems was less pronounced in the presence of protozoa (+27 and +57% in the presence and absence of protozoa, respectively). Overall, the content of P in seedlings with mycorrhiza (1.20 mg) exceeded that in seedlings without mycorrhiza (0.76 mg) by 58% ($F = 11.6$, $P < 0.01$). In contrast, the P content in seedlings was not significantly affected by protozoa.

Mycorrhiza increased concentrations of carbon in needles by 2.9%, but decreased C concentrations in roots by 3.1%. Concentrations of N were generally not affected by mycorrhiza. In contrast, the presence of protozoa significantly affected nitrogen concentrations in spruce seedlings. Nitrogen concentrations in needles were reduced by 23% resulting in an increase in the C/N ratio by 28%. Overall, however, the content of N (8.5 mg) and P (1.3 mg) in plants was at a maximum in the combined treatment with mycorrhiza and protozoa (+17 and +55% in comparison to the control without protozoa and mycorrhiza, respectively).

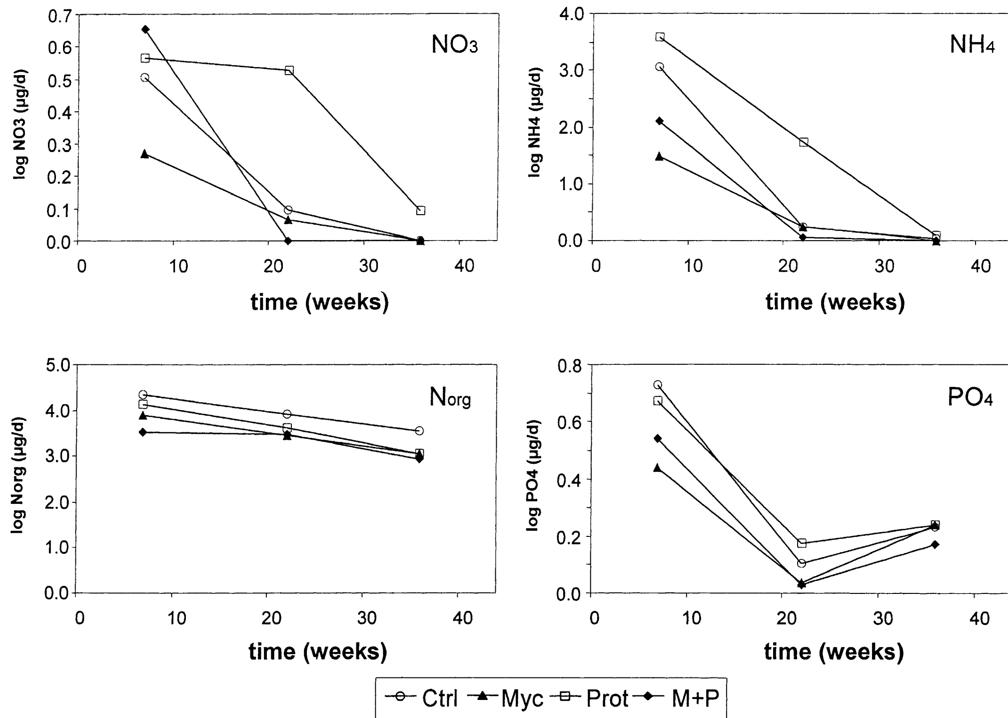

Fig. 1. Leaching rates of nitrate, ammonium, organic N and phosphate from experimental chambers. Ctrl, control; Myc, with mycorrhiza; Prot, with protozoa; M + P, with both mycorrhiza and protozoa. For statistical analyses, see Table 2, note log-scale.

Table 2
Effects of mycorrhiza, protozoa and incubation time on leaching rates of NH_4^+, NO_3^-, organic N and P from experimental chambers with Norway spruce seedlings[a,b]

	NH_4^+	NO_3^-	N_{org}	P
Myc	6.2*	6.0*	2.1	3.1
Prot	0.9	3.0	0.9	<0.1
Time	131***	35***	25***	65***
Myc × Prot	1.9	1.9	0.1	0.1
Myc × time	0.9	2.7	0.3	1.2
Prot × time	0.2	<0.1	0.4	0.4
Myc × Prot × time	4.1*	4.6*	0.8	1.2
Profile analysis for the Myc × Prot × time interaction				
Weeks 0–17 vs. weeks 17–22	8.4*	9.7*	0.8	1.0
Weeks 17–22 vs. weeks 22–41	3.0	1.9	<0.1	<0.1

[a] *F*-values of multivariate repeated-measures analysis of variance and contrasts from profile analysis. Data were transformed into common logarithms before statistical analysis. Probability levels for the profile analysis were adjusted for the number of contrasts.

[b] Myc, with mycorrhiza; Prot, with protozoa; Myc + Prot, with both mycorrhiza and protozoa.

* $P < 0.05$.

*** $P < 0.001$.

Table 3
Effects of mycorrhiza and protozoa on concentrations of C, N, P, Ca, Mg, Mn, Fe and on the C/N ratio in roots, stem and needles of spruce seedlings[a]

	C ($g\,kg^{-1}$)	N ($g\,kg^{-1}$)	C/N	P ($mg\,kg^{-1}$)	Ca ($mg\,kg^{-1}$)	Mg ($mg\,kg^{-1}$)	Mn ($mg\,kg^{-1}$)	Fe ($mg\,kg^{-1}$)
Means								
Roots								
Ctrl	447	10.0	51	909	4106	949	360	1730
Myc	432	13.9	32	1517	3841	877	288	3320
Prot	446	10.2	46	991	4370	1011	238	1789
Myc + Prot	433	10.5	45	1196	3815	906	213	2778
Stem								
Ctrl	483	10.5	52	1070	4567	1458	926	1390
Myc	489	10.7	46	1677	3200	1144	615	1189
Prot	484	9.0	57	1203	4935	1873	821	1449
Myc + Prot	488	8.4	63	1354	4567	1268	636	999
Needles								
Ctrl	453	9.2	53	861	12561	1313	4519	1288
Myc	468	10.5	47	1508	10194	1095	3272	1958
Prot	452	7.9	59	996	15249	1545	4986	1343
Myc + Prot	463	7.2	69	1377	8787	906	2438	963
F-values								
Roots								
Myc	15.4***	2.2	2.8	10.5**	3.6	2.2	0.7	7.6*
Prot	<0.1	1.4	0.4	0.9	0.3	0.6	3.1	0.3
Myc × Prot	<0.1	1.6	2.2	2.6	0.4	0.1	0.2	0.4
Stem								
Myc	3.8	<0.1	<0.1	21.4***	4.3*	1.8	9.6***	3.8*
Prot	<0.1	1.8	2.0	1.4	4.3*	0.6	0.3	0.2
Myc × Prot	0.1	0.1	0.1	7.8*	1.4	1.2	0.6	0.6
Needles								
Myc	6.1*	0.1	0.1	11.8**	6.2*	6.4*	17.0***	0.1
Prot	0.3	4.6*	4.2*	0.1	0.1	<0.1	0.2	0.8
Myc × Prot	0.1	0.9	1.5	0.8	1.3	1.6	2.0	1.0

[a] For treatments, see Table 1.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Both mycorrhiza and protozoa also affected the concentrations of other elements in plant tissue. In the presence of mycorrhiza the concentration of Mg in needles was decreased by 32% and the concentrations of Ca in needles and stems were reduced by 32 and 18%, respectively. Concentrations of Fe in stems were reduced in the presence of mycorrhiza by 23%, but those in roots were increased by 73%. Effects of protozoa were generally less pronounced but, e.g. in the presence of protozoa concentrations of Ca in stems were increased by 20% and those of Mn in roots tended to be reduced by 30%.

3.4. Plant growth and root development

Stem and needle mass, and shoot height and mass were at a maximum in the combined treatment with protozoa and mycorrhiza, however, the interaction between mycorrhiza and protozoa was only significant for stem mass (Table 4). The presence of mycorrhiza significantly increased the plant shoot/root ratio.

The morphology of roots was significantly affected by both mycorrhiza and protozoa (Table 5). The presence of mycorrhiza caused a decrease in root length (on average by −47%), length of fine roots (−47%)

Table 4
Effects of mycorrhiza and protozoa on shoot height, mass of shoot, needles and stem and the shoot/root ratio of spruce seedlings[a]

	Shoot height (cm)	Stem mass (mg)	Needle mass (mg)	Shoot mass (mg)	Shoot/root ratio
Means					
Ctrl	7.5	146	250	396	1.04
Myc	8.1	126	299	424	1.90
Prot	7.7	126	234	360	1.03
Myc + Prot	10.3	174	341	515	1.50
F-values					
Myc	3.9	0.5	4.4*	3.0	4.9*
Prot	2.2	0.5	0.1	0.3	0.5
Myc × Prot	1.5	4.6*	0.6	1.5	0.4

[a] For treatments, see Table 1.
* $P < 0.05$.

Table 5
Effects of mycorrhiza and protozoa on root biomass, root length, root radius, length of fine roots, number of root tips and specific root length of spruce seedlings[a]

	Root biomass (mg)[b]	Root length (cm)[b]	Root radius (mm)	Length of fine roots (<0.5 mm) (cm)	Number of root tips	Specific root length (cm/g)
Mean						
Ctrl	369	642	0.028	464	1586	1820
Myc	192	384	0.028	284	1024	2036
Prot	368	1040	0.021	831	2381	2842
Myc + Prot	385	507	0.030	397	1356	1387
F-values						
Prot	2.6	3.0	3.1	4.8*	2.9	0.1
Myc	2.0	7.8**	8.7**	6.7*	5.4*	6.1*
Prot × Myc	2.6	0.2	8.7**	0.6	0.2	12.7**

[a] For treatments, see Table 1.
[b] Backtransformed means of log-transformed data.
* $P < 0.05$.
** $P < 0.01$.

and number of root tips (−40%). Root biomass of mycorrhizal seedlings was only decreased in treatments without protozoa (−48%), however, the interaction between protozoa and mycorrhiza was not significant.

In comparison to mycorrhiza, protozoa had the opposite effect on root morphology. The presence of protozoa strongly increased length of fine roots (+64%) and also tended to increase root length (+51%) and number of root tips (+43%). However, due to high variation of plant growth in flagellate contaminated controls the latter two effects could not be validated at the 5% significance level (Table 5). Also, in the absence of mycorrhiza protozoa significantly reduced root diameter (−30% in comparison to the control without protozoa and mycorrhiza). Counteracting effects of protozoa and mycorrhiza on root development are most evident when considering ratios between parameters of root dimension and root mass such as the specific root length, i.e. the ratio between root length and root mass. The specific root length was strongly increased when protozoa but not mycorrhiza were present (+56% in comparison to the control without protozoa and mycorrhiza) whereas it was decreased when both protozoa and mycorrhiza were present (−24%); 35% of the variation in this parameter was explained by the interaction between protozoa and mycorrhiza. In general, mycorrhizal colonization reduced the root systems in size and

branching intensity, whereas protozoa increased root elongation and branching.

4. Discussion

Most terrestrial plant species are associated with mycorrhizas (Allen, 1991). In much the same way protozoa occur everywhere in soils (Finlay et al., 1999). In rhizospheres, protozoa are able to release nutrients by grazing on microorganisms (Clarholm, 1985; Kuikman et al., 1991) and to modify the functional and taxonomic diversity of microorganisms in the rhizosphere (Griffiths et al., 1999). The present experiment confirmed our previous results (Jentschke et al., 1995) that both ectomycorrhiza and protozoa significantly affect growth and nutrient uptake of spruce seedlings. In contrast to the present study, our previous experiment was set up under more controlled conditions in a sand culture system and it was the aim of the present experiment to investigate whether our previous findings are also valid under more natural conditions in a soil system. A drawback of the present experiment was that due to a failure in the light source illumination was low during the experiment. In addition, the systems were contaminated by flagellates, thereby increasing variability in control treatments. Nevertheless, consistent effects of protozoa occurred indicating that either flagellates only slightly affect growth of spruce seedlings or that the contamination occurred late in the experiment.

4.1. Changes in the microbial community structure

Nutrient acquisition by plant roots is associated with complex interactions between roots, microorganisms and animals (Bonkowski et al., 2000b). Results of the present experiment indicate that both mycorrhiza and the interaction between bacteria and their protozoan grazers positively affect nutrition and growth of plants. However, very different and in part interacting mechanisms appear to be responsible for these effects.

In comparison to when only a single microbial partner is present in the rhizosphere, the investment of plants in two microbial relationships should result in measurable trade–deficits to each partner, i.e. reduced mycorrhizal and bacterial/protozoan growth. Results of the present experiment indicate that the spruce seedlings indeed modified their carbon allocation in order to optimize nutrient acquisition from both microbial systems. Reduced microbial biomass in mycorrhiza treatments in the absence of protozoa and the strong reduction in bacterial numbers support the hypothesis that less carbon was released by exudation in the presence of mycorrhiza. In contrast, in treatments with protozoa the length of fungal hyphae decreased by almost 20%, suggesting that in the presence of protozoa less carbon has been allocated to mycorrhiza. In accordance with our findings, Olsson et al. (1996) found bacterial production in the rhizosphere of pine to be strongly reduced by the presence of ectomycorrhiza. The conclusion that root exudation was reduced in the presence of mycorrhiza is further supported by data on protozoan numbers. The MPN data on protozoa represent both active protozoa and protozoan cysts which accumulated in soil during the experiment. Therefore, protozoan numbers may be taken as an integrative parameter for the bacterial turnover in the rhizosphere during the experiment. The presence of mycorrhiza caused a decrease in the number of amoebae by 34% indicating that in fact less carbon was available for rhizosphere bacterial production. In agreement with these findings similar or lower rates of root exudation compared to non-mycorrhizal plants were found in ectomycorrhizal seedlings of spruce (Eltrop, 1993), pine (Laheurte et al., 1990; Leyval and Berthelin, 1993), and beech (Laheurte et al., 1990). However, so far this has only been discussed considering investment in mycorrhiza and neglecting other microbial rhizosphere interactions, i.e. the bacteria-mediated plant–protozoan interactions. Even subtle differences in the rate and the composition of root exudates may have large effects on the relative growth rates of even closely related bacterial strains (Bowen, 1991). Arbuscular mycorrhizal infection has previously been shown to alter microbial populations in the rhizosphere (Ames et al., 1984; Meyer and Linderman, 1986) and this is likely also to affect root morphology and plant growth.

4.2. Effects of mycorrhiza and protozoa on plant nutrient acquisition and growth

Mycorrhizal colonization enhances uptake from soil of relatively immobile nutrients such as P or Zn (Marschner and Dell, 1994). Increasing evidence

suggests that one of the main mechanisms responsible for this is the increase in the absorbing surface area by the fungal mycelium foraging into the surrounding soil. In fact, in our study, the presence of mycorrhiza strongly reduced leaching of phosphorus (−40%) and significantly increased the P content of spruce seedlings (+58%). This occurred although the roots of mycorrhizal seedlings were shorter and less branched. As up to 76% of P (and N) may be acquired by the external mycelium of the mycorrhizal fungal strain used (Brandes et al., 1998), it is obvious that the low P uptake at the root surface was overcompensated for by hyphal P acquisition.

In contrast to mycorrhiza, protozoa strongly increased the length of fine roots (+64%) and on average also increased root length (+51%) and the number of root tips (+43%). In our previous study (Jentschke et al., 1995), protozoa caused a similar, but stronger response of spruce seedlings, i.e. root length and number of root tips were increased by factors of 2.6 and 2.7, respectively. Similar findings for grasses and crop plants indicate that the presence of protozoa in general leads to a more branched root system consisting of more fine roots (Bonkowski et al., 2000a, 2001, unpublished).

The changes in root morphology in the presence of protozoa are in accordance with results of our previous study (Jentschke et al., 1995) and strongly suggest that in addition to the stimulation of nitrogen mineralization due to protozoan grazing other, non-nutritional, possibly hormonal effects are responsible for the protozoa-induced changes in plant performance. The production of plant hormones is a common feature of rhizosphere microorganisms, e.g. in the rhizosphere of pine, most culturable microorganisms, including mycorrhizal fungi, actinomycetes and bacteria were capable of auxin production (Strzelczyck and Pokojska-Burdziej, 1984). Other phytohormones, for example cytokinins, are also found in the rhizosphere (von Schwartzenberg et al., 1994; Arshad and Frankenberger, 1998) and may influence plant physiological processes. Protozoan grazing has been found to increase the proportion of auxin producing bacteria in the rhizosphere of watercress significantly and this was accompanied by a four-fold increase in the number and length of lateral roots (Bonkowski and Brandt, unpublished). Therefore, it is likely that microbial phytohormone production is modified by the presence of protozoa.

Even though the root system in protozoan treatments in the present experiment was larger and more intensively branched, it was less effective in nutrient uptake than the smaller less complex root system in the presence of mycorrhiza. As indicated by high leaching rates of NH_4^+ and NO_3^- protozoa increased the turnover and availability of nitrogen but non-mycorrhizal spruce seedlings apparently were unable to use these additional nutrients, even though their root system was enlarged.

In the present experiment, protozoa and mycorrhiza synergistically affected plant performance, i.e. stem mass was at a maximum and specific root length at a minimum in the combined treatment with protozoa and mycorrhiza. These interactions are in accordance with other multiple symbioses of plants. In mycorrhizal plants with *Rhizobium* or *Frankia* nodules plant growth was higher than in plants with mycorrhiza or N-fixing bacteria only (Gardner et al., 1984; Barea and Azcon-Aguilar, 1985; Ames and Bethlenfalvay, 1987). We speculate that both microbial relationships, that between plants and mycorrhizal fungi and that between plants and protozoa, are necessary to maximize plant growth, and therefore, both can be regarded as mutualistic. This hypothesis is supported by the fact that both microbial relationships complemented each other in respect of which of the major nutrient elements, N or P, they facilitated to acquire by the plant. The presence of mycorrhiza strongly reduced protozoa-induced nitrogen leaching from the microcosms indicating that only mycorrhizal plants were able to take advantage of additional N provided by protozoan grazing. In fact, the amount of N and P in spruce seedlings was at a maximum in the combined treatment with protozoa and mycorrhiza. Therefore, in contrast to aquatic systems, the microbial loop in soil, i.e. the fast turnover of carbon and nutrients via bacteria and their microfaunal grazers (Clarholm, 1994), appears to be complemented by a second mutualistic relationship, that between plants and mycorrhiza.

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