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# **Protozoa: small organisms in soil but strong effects on plant functioning**

## **Les Protozoaires : petits organismes dans le sol mais grands effets sur le fonctionnement des plantes**

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### **Summary**

Plants produce organic resources of different quantity and quality for soil organisms. In turn, the plant receives nutrients from soil organisms which mediate the nutrient remobilization by their decomposing activities. Thus, the plant links above and belowground ecosystem processes.

This work aimed to demonstrate how rhizosphere microbial communities of different complexity can mediate plant foraging for light (Carbon, C) and nutrients (Nitrogen, N). Nitrogen mineralization and fluxes of C and N within the plant and between soil and plant compartments were quantified with <sup>13</sup>C and <sup>15</sup>N stable isotopes. We established model systems of different trophic level in planted microcosms. Our results

- (a) confirm the plant growth promoting effect of protozoa in showing that protozoan grazing on the bacterial community in the rhizosphere increased root and shoot surface area;
- (b) show that morphological effects and N uptake from soil vary among plant species;
- (c) demonstrate that plant N uptake from decomposing organic patches and net CO<sub>2</sub> assimilation were stimulated when arbuscular mycorrhiza fungi and protozoa interact.

Thus, we conclude that protozoa play a key role in plant nutrition through their interactions with microbial communities, highlighting the importance of biotic interactions in the rhizosphere for the productivity of both natural- and agro-ecosystems.

### **INTRODUCTION**

The plant is the basis of existence for several groups of organisms above- and belowground [1]. In most terrestrial ecosystems nitrogen (N) is the primary limiting nutrient for plant growth [2]. All nutrients that are taken up by the plant pass the 'rhizosphere' which is the volume of soil in the immediate vicinity of roots. This zone is predominantly affected by the activity of plants and contains a complex network of microorganisms [3]. In contrast to plants, the activity and growth of heterotrophic soil organisms are generally not nutrient-limited but they are limited by the availability of carbon (C). Hence, the microbial and faunal activities in the rhizosphere are much higher as compared to bulk soil, since this zone is fuelled by plant-derived easily available C. The C transfer toward root-colonizing and free-living microbes accounts for up to 50 % of the net photosynthetically fixed C [3,4]. From a plant perspective, investing about half of its C budget must be fundamental, because it could otherwise be spent in the construction of light-capturing or defensive structural tissues above ground [3, 4].

The answer why plants invest such high amounts of C lies partly in the loop structure of the bacterial energy channel in soil: fast growing bacteria are typically colonizers of the

rhizosphere and are strongly top-down controlled by protozoa. Consequently, numbers of bacterial feeding protozoa increase up to 30-fold as compared to bulk soil [5,6]. Beneficial effects of protozoa in the rhizosphere on plant growth are well documented [3] and are mostly due to a mechanism which is known as “microbial-loop” [7]. In the microbial-loop concept, protozoa contribute significant to the remobilization of nutrients – especially N - for the uptake for the plant, by feeding on bacterial biomass. Protozoa may excrete one third of the N locked up in bacterial biomass as  $\text{NH}_4$  thereby making it available for the plant uptake [3]. The most important group of protozoa in soil are naked amoebae because of their high biomass, turnover and specific grazing [3]. Additional to the positive effect due to N mobilization, selective grazing induces a fundamental microbial community shift toward a hormonal (auxin-like) plant growth promoting response [3]. Thus, even if soil protozoa are relatively small single cell organisms (diameter = 10-100 $\mu\text{m}$ ) they are fundamental for nutrient cycling in soil. Root colonizing arbuscular mycorrhizal (AM) associations are the most widespread root-microbe relation, occurring in about 80% of all terrestrial plants and thus are a normal part of root function and ecology [4,8]. Like protozoa, AM fungi provide their host plant with mineral nutrients [4,9] but in contrast they e.g. reduce plant root growth [3]. However, there is little information on how mycorrhiza-protozoa interactions affect the mobilization of N for plants. Consequently, it is a good model system to study multitrophic interactions in the rhizosphere.

## OBJECTIVES

The objectives of the conducted experiments were to

- (1) prove positive protozoan effects in our specific model system, e.g. the increase in plant biomass and root surface area (uptake of nutrients) / shoot surface area (C assimilation).
- (2) test whether these positive effects on plant growth and increased mobilization by protozoa are plant species dependent
- (3) examine whether organisms of different trophic levels (bacteria – AM fungi-protozoa) in the rhizosphere interact to increase plant N uptake from fresh organic matter.

## MATERIAL AND METHODS

Controlled soil microbial communities of contrasted trophic level were set up after sterilisation (autoclaving 20 min, 121°C) of a soil-sand mixture (50:50). A natural protozoa-free bacterial inoculum was used to re-establish the functional diversity of a representative bacterial community filtrated from a soil slurry. However, for setting up experiments on the effect of protozoa on soil processes, gnotobiotic cultures are needed [10]. Consequently, *Acanthamoeba castellanii* (free living naked Amoebae, Protozoa) was selected because it is (a) an abundant and common bacterial grazer in soil and (b) available in gnotobiotic culture. *Glomus intraradices* is an arbuscular mycorrhiza and was selected since it as an opportunistic root colonizer and available in gnotobiotic culture. *Holcus lanatus*, *Lotus corniculatus*, *Plantago lanceolata* were chosen because they were the common EU-project model plants and *Zea mays* was tested because it is a laboratory model plant in Nancy. Stable isotope labelling techniques were used in our model systems to quantify mineralization and fluxes of C ( $^{13}\text{C}$ ) and N ( $^{15}\text{N}$ ).

### Experiment 1

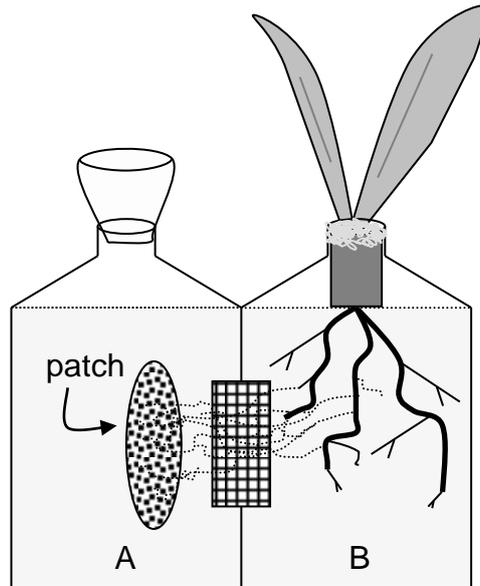
After surface sterilization, seeds of *P. lanceolata* were individualized and transferred aseptically in a nutrient solution (NB-NMAS, 100µl) in 96 well micro-titer-plates (Grainer). After 7 days of growth (18°C / 22°C night day temperature 70% humidity, 16 h of photoperiod and  $460 \pm 80 \mu\text{mol m}^{-2} \text{s}^{-1}$  light photon flux density in the PAR range) seedlings were transferred into the microcosms. Plant shoots were wrapped at their basis with sterile cotton wool to avoid protozoan contaminations from air. After 31 days of growth, plants were harvested. Aboveground parts were cut, scanned for surface determination and lyophilised for biomass determination. Roots were separated from soil by handpicking, washed and treated as aboveground parts of plants. The soil was checked for contaminations with protozoa by using the Most Probable Number technique.

### Experiment 2

Seeds of *H. lanatus*, *Z. mays* and *P. lanceolata* were surface sterilized as described in exp.1 (except of *Z. mays* which was directly transferred on nutrient agar). After germination the seedlings were transferred on 10% nutrient agar in Petri dishes and cultured at 24°C until the transfer in the microcosms. After 3 weeks of growth *Z. mays* and after 5 weeks of growth *H. lanatus* and *P. lanceolata* plants were harvested. The plant and soil compartment were treated as in exp.1. Furthermore, aboveground and belowground parts were analyzed for atom%  $^{15}\text{N}$  from labelled and ground *Lolium perenne* litter added to soil prior to soil autoclaving.

### Experiment 3

*Plantago lanceolata* seeds were surface sterilized as described in exp.1. Seven days after growth the plants were inoculated with a gnotobiotic culture of the AM fungus *G. intraradices* and transferred into the 'plant-site' of a two compartmentalized microcosm (Fig. 1). Foraging of the plants for N in a nutrient patch was tested by fixing a mesh between the 'plant' and the 'patch' compartment. Due to the pore size of the mesh AM fungi hyphae only - not plant roots - could grow from the 'plant' into the 'patch' compartment of the microcosms. Transfer of N from the patch to the plant via hyphae was analysed by adding  $^{15}\text{N}$  labelled litter (10 atom%) to the patch. Net  $\text{CO}_2$  assimilation rate and leaf area was measured at harvest. Two days before harvest, shoots were pulse labelled with  $^{13}\text{CO}_2$  to quantify the uptake, partitioning and to follow the transfer of recently fixed C via hyphae into the patch. Data shown here are from treatments where *G. intraradices* could grow from the plant into the patch compartment and *A. castellanii* were (a) not added (M-treatment) and (b) added into the soil of both compartments (AA-treatment).

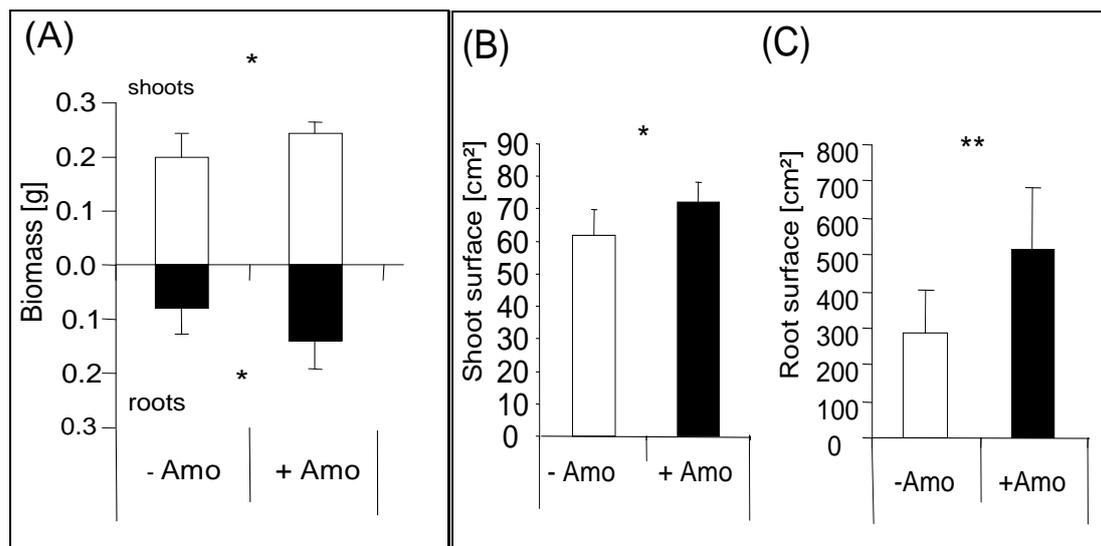


**Figure 1.** Schematic representation of the microcosm set-up in experiment 3. Each microcosm consisted of two compartments: a ‘patch’ compartment without plant (A) and a ‘root’ compartment with plant (B). *Plantago lanceolata* roots growing in the B compartment were colonized by the arbuscular mycorrhizal fungus *Glomus intraradices*. Both compartments were separated by a double layer mesh with 0.20  $\mu\text{m}$  pore size that allowed hyphae but not roots to grow into the ‘patch’ compartment. Naked amoebae, *Acanthamoeba castellanii*, were either not added or added to both compartments

## RESULTS

### Experiment 1

In presence of amoebae the biomass of *P. lanceolata* increased 1.2 times for shoots from  $0.2 \pm 0.04$  to  $0.24 \pm 0.02$  and 1.8 times from  $0.08 \pm 0.05$  to  $0.14 \pm 0.05$  for roots (Fig. 2a).



**Figure 2:** Effect of *Acanthamoeba castellanii* in the rhizosphere of *Plantago lanceolata* on (a) shoot and root biomass [g], (b) shoot and (c) root surface area [cm<sup>2</sup>]; error bars are one standard deviation from the mean. Significant differences are indicated by \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (GLM); - AMO, without; +AMO, with *A. castellanii*

Thirty days after germination, the presence of amoebae significantly increased the assimilatory surface area of shoots of *P. lanceolata* by a factor of 1.2 and that of roots by a factor of 1.8 (Fig. 2b,c).

### Experiment 2

In contrast to *Z. mays* and *H. lanatus*, the presence of amoebae increased the specific leaf area (leaf area per mass unit) in *P. lanceolata* from  $266 \pm 34.1$  to  $354 \pm 31.7$   $\text{cm}^2$  (Fig. 3). Thus, *P. lanceolata* leaves were significantly thinner when amoebae were present.

Depending on the plant species, the presence of *A. castellanii* did not change or increase  $^{15}\text{N}$  atom % in shoots and roots. The effect of amoebae was most pronounced in *Z. mays*; in the presence of *A. castellanii*  $^{15}\text{N}$  atom% was increased by a factor of 0.8 and 1.5 in shoots and roots, respectively (Fig. 4). For *P. lanceolata*, respective factors were 1.3 and 1.4, but these effects were only marginally significant.

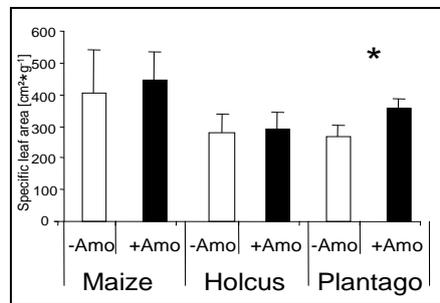


Figure 3. Specific leaf area ( $\text{cm}^2 \text{g}^{-1}$ ) of *Zea mays* (Maize), *Holcus lanatus* (Holcus) and *Plantago lanceolata* (Plantago) for plant without (-Amo) and with (+Amo) *Acanthamoeba castellanii* (Significant differences are indicated by: \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ )

The presence of *A. castellanii* in the rhizosphere of *H. lanatus* did neither affect specific leaf area nor  $^{15}\text{N}$  uptake.

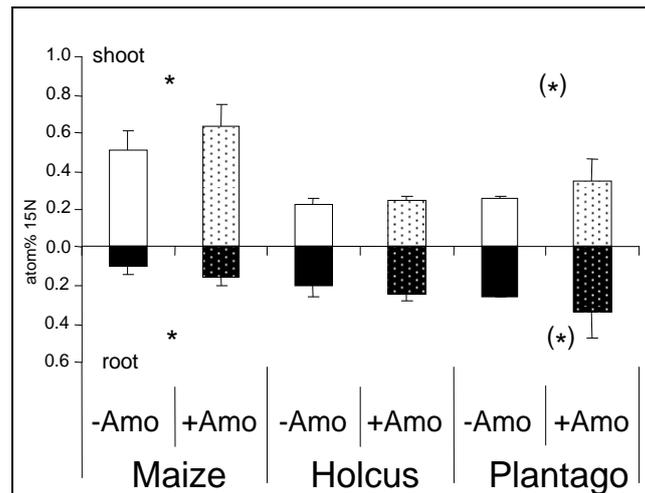


Figure 4.  $^{15}\text{N}$  atom% in root and shoots of *Zea mays* (Maize), *Holcus lanatus* (Holcus) and *Plantago lanceolata* (Plantago) for plant without (-Amo) and with (+Amo) *Acanthamoeba castellanii* (Significant differences are indicated by: \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ )

### Experiment 3

In presence of amoebae in the patch and root compartment, total net CO<sub>2</sub> assimilation ( $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ) increased by a factor of 2.1 from 2.7 to 5.8 (Table 1). Subsequently, the amount of recently fixed C recovered at harvest (<sup>13</sup>C) increased by a factor of 2.5 from 0.25 to 0.78 mg (Table 1).

The amount of <sup>15</sup>N transferred from the patch via fungal hyphae into the plant increased by a factor of 5.1 (Table 1).

Table 1: Total net CO<sub>2</sub> assimilation of *Plantago lanceolata*, total amount of fixed <sup>13</sup>C and total amount of <sup>15</sup>N in *P. lanceolata*. For detailed information see text.

| Parameter   | M          | AA        | p-value (M vs. AA) |
|---|------------|-----------|--------------------|
| total net CO <sub>2</sub> assimilation ( $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ) | 2.7±1.69   | 5.8±0.28  | 0.0005             |
| total amount of recently fixed C ( <sup>13</sup> C) [mg]                          | 0.3±0.08   | 0.8±0.25  | 0.0059             |
| total amount of <sup>15</sup> N [mg]  | 0.02±0.011 | 0.11±0.03 | 0.00001            |

## DISCUSSION AND CONCLUSIONS

Complex multitrophic microbial interactions occur in the rhizosphere [3]. We currently have a limited understanding of the interactions of plants with free living and root infecting microbes [3]. To shed light on how microbial interactions affect morphology, C assimilation, nutrient uptake of host plants and its feed backs, we conducted three experiments differing in complexity. It is known that *A. castellanii* increases the total biomass of plants by the ‘microbial-loop’, i.e. by mobilization of microbial fixed nutrient pools [7]. This was proven in our model system for *P. lanceolata*, since the presence of *A. castellanii* increased plant biomass (exp.1) and <sup>15</sup>N mobilization from labelled *L. perenne* litter added to soil (exp.2). Morphological and nutritional changes of the host plant species depend on the capacities to exploit N pools in soil, to store nutrients for later use, to mobilise stored pools at different developmental stages and to respond to environmental constraints [12]. The specific leaf area is an important parameter reflecting favourable and unfavourable conditions for plants: high specific leaf area reflects large supply of resources and high quantities of assimilated CO<sub>2</sub> [12]. The relation between specific leaf area (SLA) and nutrient (N) concentration in the leaf is linear [12]. In consequence, we conclude that the N mobilized by grazing of amoebae on microorganisms was insufficient to increase the SLA in *Z. mays* and *H. lanatus*, but sufficient for *P. lanceolata*. Since in our experiments plants were only harvested once, effects on morphology and N uptake might have appeared at later plant developmental stages. Consequently, multiple harvesting points during plant growth are needed in future experiments.

Generally, nutrients are patchily distributed in soil. Bonkowski et al. [13] showed the importance of protozoa in the degradation of organic matter patches and nutrient acquisition from patches by plants. Although the mechanism remained unknown, Hodge et al. [14] showed that arbuscular mycorrhiza play an important role in plant N uptake from nutrient patches. In our experiments protozoa strongly increased the hyphal pathway of N acquisition by the plant. This positive interaction of free living and root associated symbionts underlines the complexity of microbial interactions in mineral nutrition of plants. Furthermore, similar to SLA, leaf N concentrations are correlated with net photosynthesis (NPP) [12]. Subsequently, NPP and the amount of recently fixed C by plants in presence of amoebae further improved plant growth (exp.3). Root

infesting (AM fungi) and free living microorganisms (naked amoebae) interact to improve the host plants foraging for light (C, aboveground) and nutrients (N, belowground) resulting in increased plant biomass. However, we showed that N uptake, morphological changes in root and shoot surface, as well as stimulation of plant growth are plant species-dependent. In the past, studies have been devoted to investigate the physiochemical components of soil quality [15]. In consequence, modern agriculture is based mainly on the use of mineral fertilizers to increase plant growth (e.g. ‘Liebig’s law’). Soil biological properties have received less attention and only few studies considered trophic level interactions of the microbial soil food web [3, 16, 17]. Results of the present work suggests that the maintenance of diverse and active soil microbial communities is fundamental to soil quality and consequently to the stability and productivity of both agro-ecosystems and natural ecosystems. It is time to appreciate the key role of biotic interactions in plant nutrition.

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