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Review paper

Stimulation of Plant Growth through Interactions of Bacteria and Protozoa: Testing the Auxiliary Microbial Loop Hypothesis

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Abstract. By feeding on bacterial biomass protozoa play an acknowledged role in the liberation of nutrients in the plant rhizosphere. In addition there are suggestions that plants have mechanisms working through changes in root architecture and initiation of active release from soil organic matter, which are used to improve uptake and recirculation of nutrients in the ecosystem. All processes are carried out on a local scale in soil with roots, bacteria and protozoa interacting. The many actors and the small scale of interactions make experimentation difficult. We discuss mistakes, pitfalls and misinterpretations and provide suggestions for improvement. Recent methodological progress has opened new exciting avenues for protozoan research. New techniques have already helped to reveal protozoan regulation of cooperation as well as conflict in bacterial communities. These mechanisms in turn affect bacterial functioning and target molecular control points in rhizosphere food webs in relation to plants. Integrating nutritional and regulatory aspects into new concepts of protozoan functioning in soil is a challenging frontier in protozoology.

Key words: Protozoa, bacteria, microbial loop, plant growth, priming effect, rhizosphere ecology.

INTRODUCTION

The term "microbial loop" was coined when Azam *et al.* (1983) described a new pathway forming a loop at the base of the classical food chain in aquatic systems. They discovered that a substantial fraction of dissolved organic carbon (DOC) was utilized by bacteria, which led to sequestration of growth-limiting nutrients in bacterial biomass; and that protozoa were later responsible for the remobilization of nutrients from consumed bacterial biomass. This new concept radically modified the traditional views at that time regarding bacteria as (re) mobilizers of nutrients and primary producers only as nutrient sinks in aquatic systems (Caron 1994, Fenchel 2008).

At the same time strong effects of protozoan grazers on nutrient mineralization and plant growth were observed also in soil systems (Clarholm 1981). These results were in general accordance with effects of protozoa in aquatic systems (Azam *et al.* 1983) causing high turnover and nutrient release from bacterial biomass. However, in addition Clarholm (1985a) included a step that transferred N from soil organic matter (SOM) to primary producers via bacterial and protozoan activi-

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ties. She suggested that the carbon (C) released by roots provided means for bacteria to mineralize N from soil organic matter for their own use, and that this N would be subsequently liberated by protozoan grazers in the form of ammonium, creating a feed back to enhance plant growth and further release of C, the much cited hypothesis on the "microbial loop in soil" (Clarholm 1985a, 2005).

Plant growth in soil is strongly limited by the availability of mineral nutrients, in particular nitrogen (N) (Mokhele *et al.* 2012). As compared to the aquatic environment, there is also an uneven distribution of plant-growth-limiting supplies of water caused by intricate spatial conditions separating processes within the soil pore network at a fine scale of resolution. There is also a large organic N pool in soil. Plant roots constantly release large amounts of their photosynthates below ground, partly by active mechanisms to lubricate the growing root tip, and partly passively since root tips are inherently leaky for low-molecular weight C compounds (Farrar *et al.* 2003). These plant exudates stimulate rapid growth and activity of microorganisms, since C-availability strongly limits microbial growth in soil (Paterson 2003, Jones *et al.* 2009).

In a series of experiments, Bonkowski and co-workers confirmed the plant growth promoting effects of protozoa. However, the increase of plant biomass was not always accompanied by enhanced plant N uptake, as assumed by the original microbial loop hypothesis (see Bonkowski 2004). Instead, a recurrent pattern in their experiments was a dramatic change in the root architecture of plants, characterized by a strong stimulation of lateral root growth in presence of protozoa (Jentschke *et al.* 1995, Bonkowski and Brandt 2002, Kreuzer *et al.* 2006). Lateral root growth in plants is regulated by complex internal hormonal control, with auxins, most notably indole-acetic acid (IAA), being the master regulators of the initiation of lateral root primordia and root elongation (Aloni *et al.* 2006). Any positive microbial effect on lateral root growth must target the IAA pathway in plants (Shi *et al.* 2009, Contesto *et al.* 2010). This has also been confirmed when investigating protozoan effects on root growth (Krome *et al.* 2010).

The changed root growth pattern caused by addition of protozoa was in good agreement with effects reported for "plant growth promoting rhizobacteria" (PGPRs) (Glick *et al.* 2007, Lugtenberg and Kamilova 2009). Among plant growth promoting rhizobacteria the ability to synthesize the plant hormone IAA seems a common mechanism for bacterial manipulation of root architecture (Spaepen *et al.* 2007, Lugtenberg and Kamilova 2009). Bonkowski and Brandt (2002) suggested that specific PGPR likely increased during protozoan predation in the rhizosphere. The results suggest that besides the nutrient release from bacterial biomass there are auxillary indirect effects of protozoa on plant growth, most likely caused by changes in the bacterial flora (Bonkowski 2004). According to current understanding (Phillips *et al.* 2003), this explanation requires that regulatory roles of rhizosphere signal molecules and corresponding plant genes are taken into account.

Lately, doubt was expressed about the existence of mechanisms responsible for the stimulation of plant growth, apart from grazing increased nutrient release from bacteria. Ekelund *et al.* (2009) investigated the effects of protozoan presence on growth of a grass species in an experiment with three flagellate species added. They found evidence that the protozoa enhanced N availability to plants, but the authors did not find evidence in support of the auxiliary "microbial loop" hypotheses involving priming of soil organic matter (SOM) (Clarholm 1985a), or bacterial signalling affecting root structure (Bonkowski 2004). Therefore the authors ruled out indirect effects and suggested that increased N availability was the only key factor in explaining protozoan effects on plant growth (Ekelund *et al.* 2009).

Their blunt critique of the auxiliary microbial loop hypothesis may lead to a decreased interest in microbial interactions in the rhizosphere. This could in turn discourage protozoologists from participating in the exciting field of rhizosphere research preventing further development of a modern theoretical framework for rhizosphere interactions. The relevance of the experiment and the conclusions drawn from the results by Ekelund *et al.* (2009) will be discussed below. We will i) address the microbial loop concept and pitfalls in the design of experiments affecting rhizosphere interactions, ii) encourage research on tripartite protozoa-bacteria-plant interactions by pointing out what has been learned about protozoa-bacteria interactions in recent years, and finally iii) emphasize important gaps of knowledge, scientific challenges and avenues for further research.

THE MICROBIAL LOOP CONCEPT

In order to penetrate into soil, plants lubricate their root tips by active release of high-molecular weight carbon compounds, but root tips of plants are also leaky,

leading to significant losses of low-molecular weight carbon molecules (exudates) belowground (Farrar *et al.* 2003, Paterson 2003, Jones *et al.* 2009).

Using ^{15}N-labelled bacteria, Kuikman and colleagues provided compelling evidence that it is not until rhizosphere bacteria are grazed by protozoa that nitrogen held in bacterial biomass is released in plant accessible inorganic form (Kuikman *et al.* 1989, 1990, 1991). Plant-derived C temporarily offsets normal microbial C-limitation in soil (Cheng *et al.* 1996), and plays the same important role in soil as DOC in aquatic systems, namely providing energy to the C-limited microflora. Because of the spatial constrains in soil, the C influence acts on a spatially highly restricted scale near each growing root tip. A growing root system, however, has many root tips and as a result, plant rhizospheres host a rich microbial community with significantly higher rates of metabolism and microbial biomass relative to the bulk soil further apart from plant roots (Griffiths 1990, Rutherford and Juma 1992, Alphei *et al.* 1996, Badalucco *et al.* 1996). This zone has been suggested to be characterized by fierce microbial competition between bacteria and plant roots for available nutrients (Hodge *et al.* 2000, Jones *et al.* 2009). With their normal C limitation lifted, bacteria should win the N because of a higher substrate affinity. Still plants do take up N and transport it out from soil to aboveground parts. An alternative to fierce competition is a sequential use of N along the root. Using root tip exuded C, bacteria first take up N from the soil solution and concentrate it in their growing biomass. In a second step protozoa release bacterial N through grazing. Two days later, the apically growing root tip has moved forward. The N is released in the original area of C exudation now situated further behind the tip. Here bacteria have again become C-limited and N is now taken up by the root (Clarholm 1985b). This process has been described as apparent priming (Kuzyakov 2010). For the plant growth to continue without fertilizer addition, it is necessary to enter additional N released from SOM into the original microbial loop.

THE PRIMING EFFECT

A crucial addition as compared to the original microbial loop concept developed for aquatic systems is the assumption that microbes in soil would tap new nutrient sources by using the easily-available C compounds provided by plant exudates for increased mineralization of SOM (Clarholm 1985a). For a long time the large majority of SOM has been considered to be recalcitrant with little accessibility to microorganisms even in soils rich in SOM (Coleman and Jenkinson 1995). However, small amounts of easily available carbon, such as root exudates, can lead to enhanced microbial decomposition of recalcitrant SOM and eventually enhanced availability of growth limiting nutrients to plants, a mechanism well known as "priming." Lately, C in SOM previously considered as stable has been shown to be as vulnerable to priming as more labile C in soil (Guenet *et al.* 2012). Priming effects in the plant rhizsosphere are reported to be common in natural soils (Parnas 1976; Kuzyakov *et al.* 2000; De Nobili *et al.* 2001; Kuzyakov and Cheng 2001; Kuzyakov 2002, 2011; Dijkstra and Cheng 2007; Jackson *et al.* 2008; Dijkstra *et al.* 2009), thus undoubtedly confirming Clarholm's early hypothesis (Clarholm 1985a).

To study priming of SOM, Ekelund *et al*. (2009) investigated effects of root exudation, which in their experiment was added as a single pulse of glucose-C (corresponding to 10,000 ppm glucose ml^{-1} added water or 0.04 ppm glucose per microcosm) at the soil surface. With this experimental set up Ekelund and co-workers found no stimulating effect of the C addition. It is well known that glucose is rapidly utilized by microorganisms (Rønn *et al.* 2001), being completely metabolized within 1–4 days after addition to soil (Fig. 1). A single pulse of glucose is definitely insufficient to induce priming effects due to root exudation, while a continuous addition of glucose can stimulate the mineralization of SOM (see Macura *et al*. 1963 for a detailed analysis). Besides increased bacterial biomass production, there are also other effects of experimental C inputs. Griffiths *et al*. (1998) and more recently Jenkins *et al*. (2011) have shown that bacterial taxa responding to low inputs of glucose-C are quite distinct from taxa responding to high C-inputs.

In a further attempt to study microbial nutrient transfer from organic matter to plants, Ekelund *et al*. (2009) mixed ^{15}N-labelled grass residues into sterilized soil. Addition of fresh plant remains to an experimental system introduces not only N, but also easily available C. It has been shown that the release of C in the added residues is linearly coupled to the release of N from the same material (Hodge *et al.* 1998, Bonkowski *et al.* 2000). Therefore, potential effects of root-C are strongly confounded by excessive bacterial growth on C originating from the fresh added organic material.

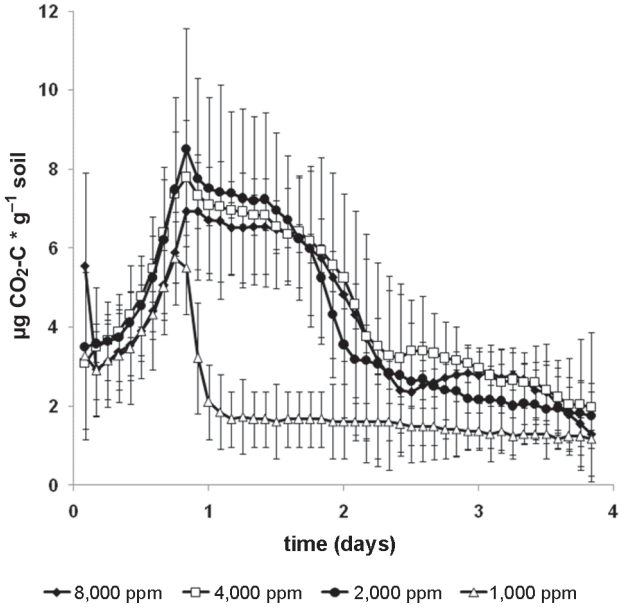

Fig. 1. Respiration of glucose-C (µg CO_2-C * g^{-1} soil) after addition of 1,000, 2,000, 4,000, and 8,000 ppm glucose to soil from the Heteren field site (Scheu 1992). 1,000 ppm glucose are completely respired by soil microorganisms within a single day, but glucose was not lasting longer than 4 days after saturation of the soil with glucose at 2,000–8,000 ppm (mean of 3 replicates ± 1 SD, see Ekelund *et al*. (2009) for a characterization of the soil).

In a comparable experimental set up to that of Ekelund *et al*. (2009), but using grass residues double-labelled with ^{13}C and ^{15}N, Bonkowski *et al.* (2000, 2001a) demonstrated that the protozoa-mediated release and subsequent incorporation of N in plant biomass is purely based on easily available C and N from grass residues, but did not originate from soil organic matter. Applying basic stoichiometric principles, protozoan grazing clearly liberates N from excessive bacterial growth on fresh detritus material (Hodge *et al.* 1998; Bonkowski *et al.* 2000, 2001a). In experiments with additions of fresh plant residues to soil, microbes have been shown to use plant exudates primarily to scavenge the nutrients becoming easily available during decomposition of added material, but no priming of old SOM will occur (Nicolardot *et al.* 1995). The incorporation of fresh plant material therefore has a highly predictable effect on the transfer of nutrients via bacteria and protozoa to plants (Bonkowski *et al.* 2000, 2001a), but the approach allows no conclusions on priming of SOM. Since the driving force is the C originating from grass residues, this mechanism even works in complete absence of plants (Rønn *et al*. (2001), and will overcast any rhizosphere effects (Bonkowski *et al.* 2000, 2001b).

To understand priming, studies of naturally developed situations in the field are more valuable. During photosynthesis, plants fixing C according to the C3 pathway strongly discriminate against the heavy ^{13}C isotope naturally occurring in CO_2 from the air, leaving a clear isotopic imprint in soil organic matter after plant death (Bowling *et al*. 2008). C4 plants discriminate less against ^{13}C and are much less depleted than C3 plants. This shift in ^{13}C content can be used as a natural tracer to follow the fate of plant-derived C in soils with a C3 plant history. Kramer and Gleixner (2006) and later Nottingham *et al*. (2009) used the change in stable-C isotopic signature in natural field soils after a change from C3 to C4 plants to investigate the change in carbon signature in rhizosphere microorganisms. They showed that Gram-negative rhizosphere bacteria were directly linked to priming effects, since they contained both young carbon from the present C4 crop and older C from the preceding C3 crops. These examples show that the duration and magnitude of soil carbon inputs has a profound influence on priming effects via plants and bacteria (Macura *et al.* 1963, Blagodatskaya *et al.* 2011), and both aspects must be considered when performing experiments investigating rhizosphere processes. The additions made by Ekelund *et al*. (2009) failed to adequately mimic root C inputs and consequently they in turn failed both to induce effects on root architecture and mechanisms that release N from SOM. Therefore, the observations on which the authors drew their conclusions that the microbial loop is not needed to explain protozoan effects on plant growth, are invalid for that purpose.

To understand real priming of SOM for N it is valuable to know the age of the N delivering substrate. By recurrent ^{15}N fertilizer additions for 10 consecutive years, thus obtaining SOM with N of different known ages, it was possible to show that N released by priming in the agricultural field of study originated predominantly from young but stable material (de Graaff *et al.* 2009). It has been estimated that protozoa assimilate a third of the N in bacterial biomass and release about a third of ingested bacterial N as water-soluble ammonium (Griffiths 1994), a form highly accessible to plants (Kuikman *et al.* 1990; Zwart *et al.* 1994; Bonkowski *et al.* 2000, 2001a). The N in protozoan biomass will also be quite available once protozoa die through predation or adverse conditions like drying out events.

Field observations indicate that under favorable conditions protozoan turnover rates are counted in days. The remaining third of N in ingested bacteria is egested as bacterial cell walls and organelle remains. These parts are insoluble, but contain organic N of good quality. They contribute to SOM and will be readily decomposed, if accessed by proteolytic enzymes.

The examples above show that designing experiments to elucidate the interactions of bacteria and protozoa in the rhizosphere of plants is highly challenging. Confounding artefacts are easily introduced and may lead to overly simplistic interpretations. It appears highly questionable whether traditional concepts focusing on gross nutrient and C flows will contribute much further to our understanding of plant-microbe interactions considering the rapid progress in rhizosphere research (Friesen *et al.* 2011). This is especially true when research aims to investigate effects of protozoa on root growth (Dubrovsky and Forde 2012).

MOLECULAR CONTROL POINTS IN RHIZOSPHERE FOOD WEBS

Protozoa have been clearly shown to enhance plant biomass without increasing plant N uptake (Kuikman *et al.* 1991, Jentschke *et al.* 1995, Alphei *et al.* 1996, Bonkowski *et al*. 2001b), but with profound positive effects on root architecture (e.g. Jentschke *et al.* 1995, Bonkowski and Brandt 2002, Kreuzer *et al.* 2006, Somasundaram *et al.* 2008). It is crucial to understand that these findings do not refute the microbial loop concept, but expand the traditional view into a new framework of more complex co-evolved plant-microbe interactions (Bonkowski and Brandt 2002, Phillips *et al.* 2003, Friesen *et al.* 2011). Both increased root surface and increased root exudation to initiate priming can be seen as traits evolved by biota to increase reuse of a plant limiting N resource, which has become increasingly locked up in SOM with time.

In a detailed study, Krome *et al.* (2009) have shown that within the first three days after addition of amoebae, *Arabidopsis thaliana* responded with enhanced C-allocation to shoots, while N-uptake increased only six days after addition. At that time plants also had allocated more resources to root growth. In this context increased nutrient availability could be clearly explained by the microbial loop process. However, the increased plant nutrient uptake can also be a secondary result of an increased root surface area (Herdler *et al.* 2008, Somasundaram *et al.* 2008). These effects may have escaped the attention of protozoologists, because the growth of the very finest roots is often stimulated, enhancing root surface area, while total root biomass may not change (Fig. 2).

Increased growth of lateral roots in the presence of naked amoebae has been observed in several experiments. Despite being the most obvious explanation, and despite early evidence of protozoa directly stimulating bacterial IAA production (Bonkowski and Brandt 2002), IAA production was not confirmed to be a general bacterial trait for protozoan prey selection (Bjørnlund *et al.* 2006, Vestergård *et al.* 2007, Ekelund *et al.* 2009). Applying basic evolutionary theory, IAA production does not confer a direct fitness benefit to the bacterial cell confronted with a predator. Therefore, it is in fact unlikely to serve as a selected trait of bacteria under protozoan predation pressure. This reasoning raises the crucial questions whether specific bacterial traits exist that are able to directly enhance individual bacterial fitness in the presence of protozoan grazers, and also if associated microbial signal molecules other than IAA may interfere with root auxin signalling.

There are many reported specific responses of plants to microbial root colonization, spanning from exudation of distinctive antimicrobial compounds to

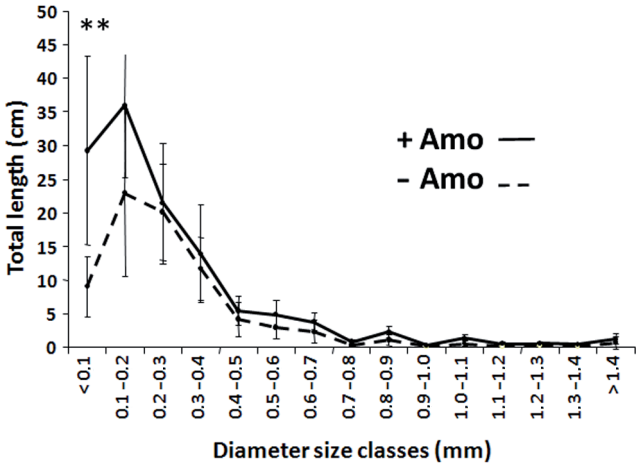

Fig. 2. Effects of *Acanthamoeba castellanii* on root diameter size classes of a grass (*Lolium perenne*). Grass plants were grown for 20 days in presence (+ Amo) and absence (– Amo) of amoebae on 1% agar with ½ Murashigge and Skoog medium in Petri dishes with a diverse soil bacterial community (Kreuzer, unpublished).

exudate-mediated indirect activation of plant defences, or reciprocal signal exchange to manipulate microbial behaviour, all demonstrating genetic control points in plant roots. Roots are extremely perceptive. They will differentiate diverse microbial signals and respond with profound changes in plant physiology, including quality and quantity of exudation (Dunn and Handelsman 2002, Walker *et al.* 2003, Phillips *et al.* 2004, Bais *et al.* 2006, Lanoue *et al.* 2010, Henkes *et al.* 2011). The findings of increased root branching are consistent with the idea of molecular control points in rhizosphere food webs, emphasizing the roles of regulatory signal molecules targeting plant genes in plant-microbe interactions (Phillips and Strong 2003). Applying this modern, evolutionary view on plant microbe interactions, Phillips *et al.* (2003) suggested that "if microbial signals would enhance root elongation, plants would gain greater access to N liberated by …[microbial grazers], while bacteria would benefit from a larger surface area for exudation and colonization."

Plant diversity in the field has lately been strongly positively linked to protozoa and in particular to numbers of soil amoebae (Scherber *et al.* 2010). Amoebae in turn are suggested to regulate the composition, size, and productivity of the rhizosphere bacterial community through selective grazing (Rosenberg *et al.* 2009). Various species of protozoa have been shown to modify bacterial communities in specific and often highly predictable ways (Bjørnlund *et al.* 2006, Rosenberg *et al.* 2009, Glücksman *et al.* 2010). However, we are still far from predicting the outcome of protozoan grazing on bacterial functions in the plant rhizosphere, or possible further effects on plants.

EFFECTS OTHER THAN NUTRIENT RELEASE

When Bonkowski and Brandt (2002) and Phillips and Strong (2003) proposed that protozoan grazing had a decisive influence on the bacterial species composition, it was still an unsolved question how specific bacterial taxa should directly benefit from protozoan grazing. Using *Pseudomonas fluorescens* as model organism, Jousset *et al*. (2006) showed that production of specific toxins confers grazing resistance to pseudomonads. Continuing these studies, Jousset *et al*. (2008, 2009) uncovered that the pseudomonads in fact used the toxins to deflect protozoan grazing pressure to their non-defended neighbours, thus maximizing their individual fitness in presence of protozoa. Today there are strong indications of a highly coevolved chemical warfare between pseudomonads and protozoa (Mazzola *et al.* 2009). Certain amoeba species seemed able to counteract bacterial toxin production (Jousset *et al.* 2010), while flagellates were strongly inhibited (Pedersen *et al.* 2010). Actually, gene expression of functional genes in pseudomonads changed (Rosenberg *et al.* 2009), even when only supernatant of protozoan cultures was added (Mazzola *et al.* 2009, Jousset and Bonkowski 2010, Jousset *et al.* 2010). In other words, the sole presence of products released by protozoa was sufficient to change bacterial "behaviour" and functioning in significant ways. Therefore, interactions of PGPR with plant roots may only be understood if their functional changes are studied in presence of their protozoan grazers. Pseudomonads in the rhizosphere may also directly (De Leij *et al.* 2002) or indirectly (Combes-Meynet *et al.* 2011, Couillerot *et al.* 2011) increase root growth of their host plants and in this way benefit both from increased exudation and nutrients released from consumed microbial competitors.

The example of pseudomonads demonstrates the high level of co-evolution in bacteria-protozoa interactions. However, the study of Rosenberg *et al*. (2009) also demonstrated that a single amoeba species did not only affect pseudomonads, but changed the whole bacterial community composition in the rhizosphere of *A. thaliana* compared to control plants without protozoa. Therefore, it is not possible yet to link the resulting effects on plant performance (Krome *et al.* 2009) to a single bacterial taxon, or a single bacterial or protozoan trait. It is even still largely unclear to which degree grazing by different protozoan taxa is complementary (i.e. selecting for different bacterial traits), or redundant (i.e. selecting for similar bacterial traits). There is an urgent need for more detailed studies on the mechanisms of protozoan predation in the rhizosphere of plants.

EXPERIMENTAL SET UPS FOR RHIZOSPHERE RESEARCH

A shift in focus regarding plant-microbial interactions from nutrient effects of protozoa to investigations on genetic control points makes the design of experimental set ups and experimental analyses increasingly challenging. Some major points need to be considered i) Protozoa occur ubiquitously and cannot be easily

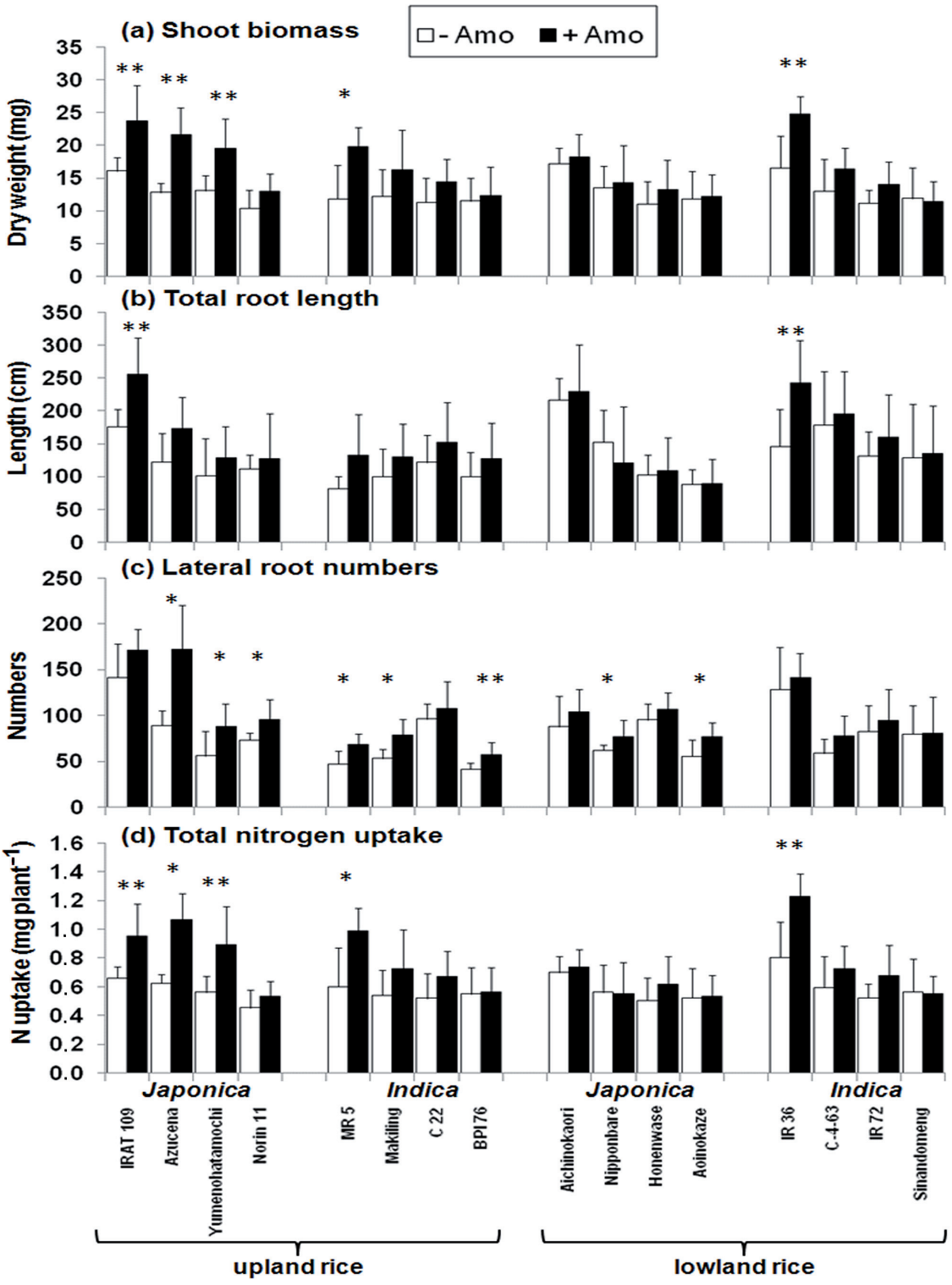

Fig. 3. Difference in growth responses of 16 cultivars of rice (*Oryza sativa* L.) grown in autoclaved soil and with a diverse soil bacterial filtrate reinoculated into the farmland soil in presence (black bars) and absence (white bars) of *Acanthamoeba* sp. Shoot dry weight **(a)**, total root length **(b)**, number of laterals at seminal root **(c)**, and total nitrogen uptake **(d)**. Vertical error bars represent standard deviation ($n = 4–9$). The symbols * and ** indicate a significant difference at $P < 0.05$ and 0.01 by one way ANOVA, respectively. Data from Somasundaram *et al*. (2008).

eliminated from soil to produce a protozoan-free medium. Therefore laborious soil sterilization and inoculation methods are needed to establish a protozoa-free control (see Alphei and Scheu (1993) for a comparison of methods). Re-introduced bacteria do not settle in soil in the same way as indigenous bacteria occurring in biofilms, but more superficially. This could change grazing conditions for protozoa (Clarholm *et al.* 2007). ii) After soil sterilization it is crucial to reduce easily available carbon- and nutrient sources, which are abundant after soil sterilization, either by soil leaching and/or by diluting soil with sand. As discussed earlier, also the addition of fresh organic matter may confound any rhizosphere effects. iii) Competitive protozoan grazers with high consumption rates of bacteria, thus expected to be common under natural conditions, should be used. Because of their ecology, naked amoebae are the naturally dominating grazers in areas like the rhizosphere with a high bacterial production on root surfaces (Clarholm *et al.* 2007). Flagellates will be able to dominate in rhizosphere experiments only if amoebae are excluded. The former never showed any effects on root growth when compared to amoebae (Bonkowski *et al*. 2001b, Herdler *et al.* 2008). Flagellates are known for their high selectivity and often ingest bacteria one by one (Boenigk and Arndt 2002). Due to relatively long prey handling, flagellate grazing strategy may not be efficient enough to keep up with exponential bacterial growth in the plant rhizosphere (Bjørnlund *et al.* 2006, Vestergård *et al.* 2007). A careful selection of bacterial prey is also important in the study of protozoan effects on root growth, as shown by Bonkowski and Brandt (2002). iv) The choice of plant cultivar is likewise crucial, especially when working with domesticated plant species. Recent studies demonstrate that agricultural plants repeatedly lost genes responsible for root-animal interactions (Rasmann *et al.* 2005, Kollner *et al.* 2008). By comparing the growth response of 16 different rice cultivars to presence of soil amoebae, we found a clear distinction between lowland cultivars of *Oryza sativa* cv Japonica, which showed little response, and upland cultivars. The latter, which are grown in aerated soils, generally responded strongly with an increase in root branching and shoot biomass (Somasundaram *et al.* 2008) (Fig. 3). Lowland cultivars which are grown in anoxic wetlands, had apparently encountered different selection pressures during plant breeding. Similarly, root growth responses of maize cultivars differed strongly in response to amoebae (Koller, unpublished). These results indicate that plant breeding can have a profound influence on naturally co-evolved rhizosphere processes. When these interactions are to be studied cultivars responsive to plant-microbial interactions must be selected.

OUTLOOK

The different roles of protozoa observed for rhizosphere bacterial communities (Rosenberg *et al.* 2009) and the complex regulatory network of root responses to external signals are two clearly underrepresented aspects in current protozoological research (Krome *et al.* 2010, Alvarez *et al.* 2012). Recent developments in molecular biology now offer all the tools necessary to identify molecular control points in plants and microorganisms (Heidel *et al.* 2010). High throughput sequencing methods combined with stable isotope probing allow us for the first time to identify important protozoan players in the plant rhizosphere (Lueders *et al.* 2004, 2006; Urich *et al.* 2008). A drawback is that we still have very sparse information on molecular genetic markers for species of amoebae compared to other microbial groups (De Jonckheere *et al*. 2012). Well-characterized bacterial model species, their mutants and reporter strains can be used to uncover specific bacteria-protozoa (Jousset 2012), as well as specific bacteria-plant interactions (Jousset *et al.* 2011); these methods combined with plant gene-expression systems (Kang and Baldwin 2008) offer a great possibility to advance our future understanding of rhizosphere processes, particularly if applied under appropriate conditions. Protozoologists should play an active part in this development.

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